SMAP 2019

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SMAP 2019

September 16-19
Strasbourg

Société Française de Spectrométrie de Masse (SFSM)

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Planning

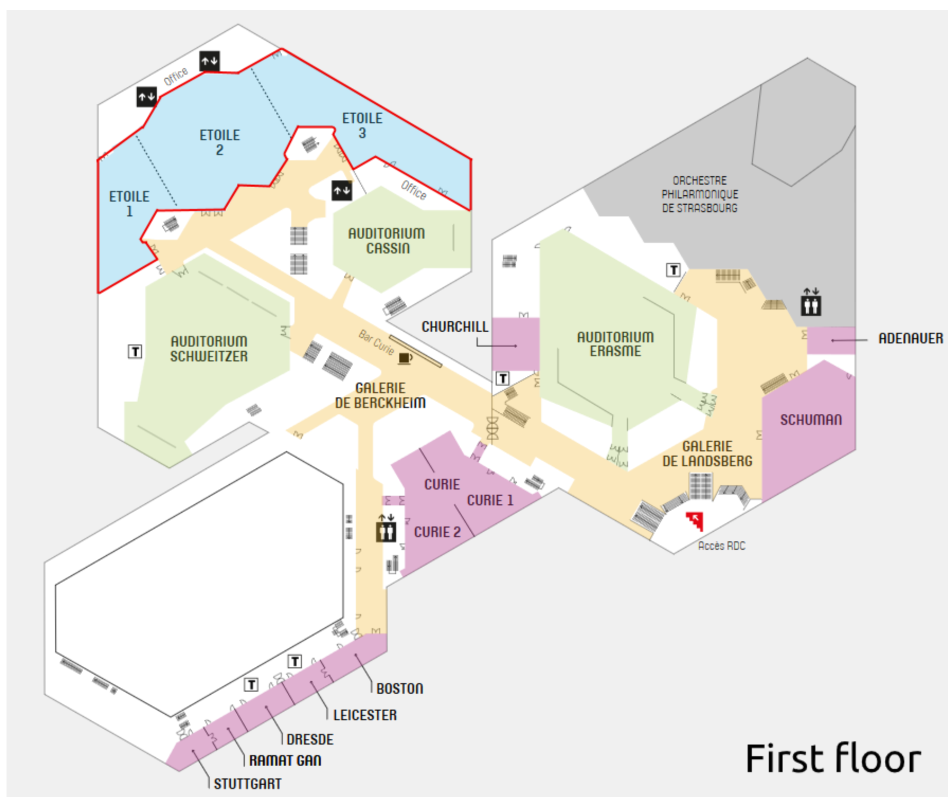
Tuesday, 17 September 2019		
08:00	**Registration**	
09:00 – 09:30	**Opening of SMAP2019 Strasbourg**	**Auditorium CASSIN**
09:30 – 10:30	**Opening Lecture**	**Auditorium CASSIN**
	PL1 - Rainer Bischoff - University of Gröningen, The Netherlands	
	Biomarker discovery and validation – from shotgun proteomics to targeted methods	
10:30 – 11:00	**Coffee Break / Poster Session (even N°)**	**Hall RHIN – Galerie SCHWEITZER**
11:00 – 12:30	**Parallel Session 1: Ion Mobility**	**Auditorium CASSIN**
11:00 – 11:30	**KL1 - Hélène Rogniaux** - BIBS, INRA, Nantes, France	
	Structure determination of large isomeric oligosaccharides of natural origin through multi-pass and multi-stage cyclic traveling wave ion mobility mass spectrometry	
11:30 – 11:50	**OC 1 - Aurélien Le Fèvre** - ISA, Lyon, France	
	Temperature-controlled IMS-IMS measurements for conformational thermodynamics	
11:50 – 12:10	**OC2 - Frédéric Rosu** - IECB, Bordeaux, France	
	Circular dichroism mass spectrometry of nucleic acids ions	
12:10 – 12:30	**OC3 - Oscar Hernandez-Alba** - LSMBO, Strasbourg, France	
	Intact- and middle-level Collision Induced Unfolding experiments to decipher gas-phase unfolding mechanism of hybrid and canonical mAbs	
11:00 – 12:30	**Parallel Session 2: Clinical Mass Spectrometry**	**Room LONDRES 2**
11:00 – 11:30	**KL2 - Nina Ogrinc** - PRISM, Lille, France	
	Towards in-vivo molecular diagnostics of esogastric cancer with SpiderMass real-time, mini invasive	
11:30 – 11:50	**OC4 - Romain Carrière** - ISA, Lyon, France	
	Multiplexed detection and quantification of the protein effectors of antibiotic resistance in clinical strains of Pseudomonas aeruginosa and Enterobacteriaceae by Scout-MRM – Application to positive blood cultures	
11:50 – 12:10	**OC5 - Cynthia Mongongu** - AFLD, Châtenay-Malabry, France	
	Quantitative determination of IGF-I in blood using DBS and VAMS after solid phase extraction microelution and analysis by LC-HRMS for doping control purposes	
12:10 – 12:30	**OC6 - Mathieu Dupré** - Pasteur Institute, Paris, France	
	De novo sequencing of patient-derived monoclonal antibody light chains by top-down and bottom-up proteomics	
12:30 – 14:30	**Lunch Break / Poster Session**	**Hall RHIN – Galerie SCHWEITZER**
12:45 – 13:45	**Shimadzu Lunch Seminar**	**Room LONDRES 2**
14:30 – 16:00	**Plenary Session**	**Auditorium CASSIN**
14:30 – 15:15	**PL2 - Christine Enjalbal** - IBMM, Montpellier, France	
	Mass spectrometry and peptide analysis: our contribution to a long lasting story	
15:15 – 16:00	**PL3 - Lukas Käll** - KTH, Stockholm, Sweden	
	Machine learning methods for the interpretation of label-free proteomics data	
16:00 – 16:20	**Award – Best communication from the Young Scientists Societies**	**Auditorium CASSIN**
16:20 – 16:50	**Coffee Break / Poster Session (odd N°)**	**Hall RHIN – Galerie SCHWEITZER**
16:50 – 18:20	**Parallel Session 3: Instrumentation**	**Auditorium CASSIN**
16:50 – 17:20	**KL3 - Thanh-Loan Lai** - IPNO, Orsay, France	
	Andromede: MeV-Nanoparticle-SIMS for surface analysis	
17:20 – 17:40	**OC7 - Michel Sablier** - MNHN, Paris, France	
	Benefits of Py-GCxGC/MS for the analysis of complex samples from cultural heritage	
17:40 – 18:00	**OC8 - Vincent Jung** - 3P5-Necker, Paris, France	
	BLI-MS: coupling BioLayer Interferometry with Mass Spectrometry	
18:00 – 18:20	**OC9 - Anthony Lechner** - LSMIS, Strasbourg, France	
	Implementation and evaluation of a homemade nanoflow interface for capillary electrophoresis-mass spectrometry coupling	

16:50 – 18:20	**Parallel Session 4: Bio-Chemo-Informatics and Statistics**	**Room LONDRES 2**
16:50 – 17:20	**KL4 - Jean Armengaud** - CEA, Marcoule, France	
	Striking the current metaproteomics dogma for deeper characterization of microbiota	
17:20 – 17:40	**OC10 - Alyssa Imbert** - CEA, Saclay, France	
	Proteomics and metabolomics data integration for deep phenotyping	
17:40 – 18:00	**OC11 - Nicolas Elie** - ICSN, Gif-sur-Yvette, France	
	MetGem software for the generation of molecular networks based on the t-SNE algorithm	
18:00 – 18:20	**OC12 - Thomas Burger** - CEA, Grenoble, France	
	Replacing target-decoy competition to stabilize the FDR control of peptide identification in MS-based discovery proteomics	
18:20 – 18:50	**Sponsor Communications**	**Auditorium CASSIN**
	SC1 - Tanguy Fortin - Anaquant	
	HCP Profiler, a global solution for individual identification and quantification of your Host Cell Proteins	
	SC2 - Nicolas Autret - Covaris	
	Universal and reproducible sample preparation with adaptive focused acoustics	
	SC3 - Nico Wortel - MS Vision	
	ECD: a powerful tool for the fragmentation of intact proteins under denaturing and native conditions	
19:00 – 21:00	**Welcome Cocktail**	**Hall RHIN – Galerie SCHWEITZER**
Wednesday, 18 September 2019		
08:00	**Registration**	
08:30 – 10:00	**Plenary Session**	**Auditorium CASSIN**
08:30 – 09:15	**PL4 - Mickael Glocker** - Proteome Center Rostock, Germany	
	Mass spectrometric epitope mapping	
09:15 – 10:00	**PL5 - Paola Picotti** - ETH Zurich, Switzerland	
	Proteomes in 3D	
10:00 – 10:30	**Sponsor Communications**	
	SC4 - Pierre-Olivier Schmit - Bruker Daltonics	
	PASEF & beyond: developing the potential of the TIMS-Q-TOF architecture for proteomics	
	SC5 - Thierry Legoupil - Shimadzu	
	Innovations and new products introduced by Shimadzu	
	SC6 - Fabien Guérin - Thermo Fisher Scientific	
	Orbitrap IDX : make the most of Orbitrap technology for your structural analysis	
10:30 – 11:00	**Coffee Break / Poster Session (odd N°)**	**Hall RHIN – Galerie SCHWEITZER**
11:00 – 12:30	**Parallel Session 5: Proteomic Sciences**	**Auditorium CASSIN**
11:00 – 11:30	**KL5 - Pascal Cosette** - PISSARO, Rouen, France	
	Proteomics for hunting molecular targets to combat biofilms and proteomics again for deciphering associated molecular mechanisms	
11:30 – 11:50	**OC13 - Chiara Guerrera Ida** - 3P5-Necker, Paris, France	
	Critical role of a phosphorylation in sheath assembly and function of an atypical type VI secretion system	
11:50 – 12:10	**OC14 - Serge Urbach** - IGF, Montpellier, France	
	Functional proteomics of glioblastoma: investigation of tumor initiating cell secretome	
12:10 – 12:30	**OC15 - Anne Gonzalez De Peredo** - IPBS, Toulouse, France	
	Dynamic analysis of the VAV1 interactome in primary T cells reveals a crosstalk between the TCR and CD226	

11:00 – 12:30	**Parallel Session 6: Structural Characterization**	**Room LONDRES 2**
11:00 – 11:30	**KL6 - Friedel Drepper** - University of Freiburg, Germany	
	From molecular recognition to cellular function: Pex5p receptor phosphorylation controls peroxisomal matrix protein import	
11:30 – 11:50	**OC16 - Clothilde Comby-Zebrino** - iLM, Lyon, France	
	Negatively charged liganded gold clusters: where is the charge? Insight from IMS-MS	
11:50 – 12:10	**OC17 - Mira Merdas** - Protim, Rennes, France	
	Distribution study of paracetamol and its metabolites in rat whole body after on-tissue chemical derivatization by MALDI Imaging Mass Spectrometry	
12:10 – 12:30	**OC18 - Marie Yammine** - MSAP, Lille, France	
	Glycoproteomic study of *Saccharomyces cerevisiae* yeast cell wall mannoproteins	
12:30 – 14:30	**Lunch Break / Poster Session**	**Hall RHIN – Galerie SCHWEITZER**
12:45 – 13:45	**Bruker Daltonics Lunch Seminar**	**Room LONDRES 2**
14:30 – 16:00	**Plenary Session**	**Auditorium CASSIN**
14:30 – 15:15	**PL6 - Frank Sobott** - University of Leeds, UK	
	Mass spectrometry approaches to dynamic protein structure: from disorder to membrane pores	
15:15 – 16:00	**PL7 - Jean-Luc Le Quéré** - INRA, Dijon, France	
	Direct-Injection Mass Spectrometry (DIMS) for the analysis of aroma compounds	
16:00 – 16:30	**Sponsor Communications**	**Auditorium CASSIN**
	SC7 - Mickael Hybois - Waters	
	Novel cyclic IMS and new SYNAPT XS lead full portfolio of mass spectrometry innovations	
	SC8 - Serge Desmoulins & Philippe Firmin - Agilent Technologies	
	Be sure in your analysis of Host Cell Protein (HCP) impurities. From sample preparation to HRMS analysis for HCP identification and quantification	
	SC9 - Joerg Dojahn - Sciex	
	Advanced quantitative approaches made easy with TripleTOF(R) 6600+ system	
16:30 – 17:00	**Coffee Break / Poster Session (even N°)**	**Hall RHIN – Galerie SCHWEITZER**
17:00 – 18:30	**Parallel Session 7: Structural Biology**	**Auditorium CASSIN**
17:00 – 17:30	**KL7 - Julien Marcoux** - IPBS, Toulouse, France	
	Study of the largest and most heterogeneous macromolecular complex by HDX-MS, bringing new important mechanistic insights in proteasome regulation	
17:30 – 17:50	**OC19 - Eduard Puig** - IRB, Barcelona, Spain	
	Structure of Aβ42 pore-forming oligomers	
17:50 – 18:10	**OC20 - Leïla Bechtella** - LBM, Paris, France	
	Benzophenone photoreactivity in a lipid bilayer to probe cell-penetrating peptides/lipid membrane interactions by affinity photocrosslinking coupled to mass spectrometry	
18:10 – 18:30	**OC21 - Martial Rey** - Pasteur Institute, Paris, France	
	An innovative in vivo cross-linking mass spectrometry workflow for proteome-wide studies	
17:00 – 18:30	**Parallel Session 8: Geochemistry/Environment/Polymers**	**Room LONDRES 2**
17:00 – 17:30	**KL8 - William Kew** - EMSL, Edinburgh, UK	
	Laser Desorption Ionisation coupled to FTICR for analysis of whole soils	
17:30 – 17:50	**OC22 - Fabrice Bray** - MSAP, Lille, France	
	Robust proteomics workflow for the high throughput identification and classification by MALDI FT-ICR MS of paleontological bones indiscernible by their anatomy	
17:50 – 18:10	**OC23 - Jasmine Hertzog** - Helmoltz Institute, München, Germany	
	Non-targeted analysis applied to archaeological samples for a fast composition overview	
18:10 – 18:30	**OC24 - Oscar Lacroix** - COBRA, Rouen, France	
	Analysis of bitumen by high performance thin layer chromatography coupled to laser desorption ionization Fourier transform ion cyclotron resonance mass spectrometry	
18:30 – 20:00	**General Assemby – SFSM**	**Auditorium CASSIN**
18:30 – 20:00	**General Assemby – SFEAP**	**Room LONDRES 2**
20:00 – 01:00	**Gala Dinner and Party**	**Room ETOILE**

Thursday, 19 September 2019		
08:30	**Registration**	
09:00 – 10:25	**Plenary Session**	**Auditorium CASSIN**
09:00 – 09:45	**PL8 - Glen Jackson** - West Virginia University, Morgantown, WV, USA	
	Hair reveals what people conceal: biometric traits from the chemical analysis of human hair	
09:45 – 10:05	**Award – Thesis Prize SFSM Tingting Fu** - ISA, Lyon, France	
	3D and high sensitivity micrometric mass spectrometry imaging	
10:05 – 10:25	**Award – Thesis Prize SFEAP Charlotte Gaviard** - PISSARO, Rouen, France	
	Post-translational modifications effect: small chemical groups, influential consequences? Characterization of modified proteins in Pseudomonas aeruginosa PA14 by proteomic analysis	
10:25 – 11:00	**Coffee Break / Poster Session**	**Hall RHIN – Galerie SCHWEITZER**
11:00 – 12:30	**Parallel Session 9: Proteomic Sciences**	**Auditorium CASSIN**
11:00 – 11:30	**KL9 - Donatien Lefebvre** - CEA, Gif-sur-Yvette, France	
	Quantitative determination of *Staphylococcus aureus* enterotoxins in complex food matrices by a multiplex immunocapture mass spectrometry	
11:30 – 11:50	**OC25 - Martha Zoumpoulaki** - ENS, Paris, France	
	Quantifying the cellular redoxome : effect of MnSOD mimic, a potential metallodrug against Inflammatory	
11:50 – 12:10	**OC26 - Liz Paola Cantero Mendieta** - LSMBO, Strasbourg, France	
	Development of quantitative targeted proteomics for the diagnosis of Lyme borreliosis	
12:10 – 12:30	**OC27 - Céline Henry** - PAPPSO, Jouy-en-Josas, France	
	Tricks to overcome challenges in metaproteomics of the human intestinal microbiota	
11:00 – 12:30	**Parallel Session 10: Lipidomics/Metabolomics**	**Room LONDRES 2**
11:00 – 11:30	**KL10 Isabelle Schmitz-Afonso** - COBRA, Rouen, France	
	Molecular networking and ion mobility complementarity in metabolites identification of a *Fagara heitzii* extract	
11:30 – 11:50	**OC28 - Kathleen Rousseau** - CEA, Saclay, France	
	Convenient production of deuterium-labelled internal standards for large scale quantitative metabolomics	
11:50 – 12:10	**OC29 - Hoang Thi Phuong Thuy** - ICSN, Gif-sur-Yvette, France	
	Supercritical-Fluid Chromatography coupled to High-Resolution Mass Spectrometry (SFC-HRMS) for qualitative and quantitative analysis of N-acylhomoserine lactones implied in quorum-sensing	
12:10 – 12:30	**OC30 - Laurent Debrauwer** - INRA, Toulouse, France	
	Untargeted profiling of toxicologically relevant reactive metabolites using all ion MS/MS and specific data filtering	
12:30 – 14:30	**Lunch Break / Poster Session**	**Hall RHIN – Galerie SCHWEITZER**
12:45 – 13:45	**Thermo Fisher Scientific Lunch Seminar**	**Room LONDRES 2**
14:30 – 16:00	**Plenary Session**	**Auditorium CASSIN**
14:30 – 15:30	**PL9 - Alain Van Dorsselaer** - LSMBO, Strasbourg, France	
	From analytical proteomics to biology or from biology to analytical proteomics ?	
15:30 – 16:00	**Closing of the Conference**	**Auditorium CASSIN**

PMC Plans



- **Auditorium CASSIN**: main room for plenary sessions
- **Room LONDRES 2**: secondary room for parallel sessions and lunch seminars
- **Hall RHIN**: lunch breaks, posters
- **Galerie SCHWEITZER**: posters
- **Room ETOILE** (upstairs): gala dinner

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Plenary lectures

Biomarker discovery and validation – from shotgun proteomics to targeted methods [PL1]

Rainer BISCHOFF

Mass spectrometry is an indispensable tool in modern protein research. Mass spectrometry of proteins has come a long way, since the introduction of electrospray ionization and matrix-assisted laser desorption ionization allowed the analysis of high-molecular weight proteins for the first time. Mass spectrometry has also contributed significantly to the discovery and validation of biomarkers, which are an indispensable part of the toolbox of modern medicine. Biomarkers are related to the physiological state of an individual and indicate pathophysiological deviations from homeostasis. While many biomarkers are currently in use, further developments are needed to address unmet clinical needs, notably in predicting response to therapy on an individualized level (personalized or precision medicine) [1]

The discovery of biomarkers is one of the greatest challenges of modern molecular medicine. This has many reasons starting from the design of a clinical study to sample handling and storage all the way to data processing and statistical data analysis [2]. Proteomics analyses, generate highly complex data sets that comprise many more data points than the number of measured samples, which results in a fundamental statistical challenge when trying to find changes that are related to the study question (e.g. disease progression or response to therapy) rather than to chance. Reducing the number of data points (often also referred to as 'features' or 'variables') without removing relevant information is a critical step of data analysis and a challenge that cannot be met by a "one size fits all" approach [3].

In this lecture, I will cover fundamental aspects of biomarker discovery by bottom-up (also referred to as "shotgun") proteomics on the example of tissue analysis of biopsies from cervical cancer patients after laser capture microdissection (LCM) [4]. This will be followed by a discussion of biomarker selection and prioritization for Chronic Obstructive Pulmonary Disease (COPD), one of the most prevalent diseases worldwide with a high level of mortality and morbidity [5]. I will provide a comparison of different targeted LC-MS/MS methods to quantify the soluble receptor of advanced glycation end products (sRAGE), a candidate biomarker for emphysema development during COPD, which is currently under consideration for qualification by the US Food and Drug Administration (FDA) [6-9]. This methodological comparison serves to discuss differences between LC-MS/MS and a widely used commercial enzyme-linked immunosorbent assay (ELISA). I will conclude with future perspectives on how multiplexed, targeted LC-MS/MS assays may be used to quantify proteins comprising biochemical pathways using concatenated, stable-isotope-labelled internal standards [10].

Biomarker discovery, validation and qualification has made significant progress in the last decade due to better experimental design, diligent method optimization and validation and thorough testing of the resulting statistical models. However, bringing a candidate biomarker from discovery to clinical practice remains a considerable challenge, since the final test must show added value in the clinic. This is an area that the field is currently only beginning to appreciate.

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Authors

Frank Klont [1], Simon D. Pouwels [2], Marrit Hadderingh [1], Marc Joosten [1], Coşkun Güzel [3], Jos Hermans [1], Natalia I. Govorukhina [1], Christoph Stingl [3], Lennard J. Dekker [3], Bea A. Wisman [4], Ate G.J. van der Zee [4], Harry Hollema [5], Victor Guryev [6], Nico C. van de Merbel [1], [7], Justina C. Wolters [8], Péter Horvatovich [1], Theo M. Luider [3], Nick H.T. ten Hacken [2], Rainer Bischoff [1]

1. Department of Analytical Biochemistry, Groningen Research Institute of Pharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands
2. Department of Pulmonary Diseases, University Medical Center Groningen, University of Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands
3. Laboratory of Neuro-Oncology, Clinical and Cancer Proteomics, Department of Neurology, Erasmus University Medical Center Rotterdam, Rotterdam 3015 CN, The Netherlands
4. Department of Gynecologic Oncology, Cancer Research Center Groningen, University of Groningen, University Medical Center Groningen, Groningen 9713 GZ, The Netherlands

5. Department of Pathology, University Medical Center Groningen, University of Groningen, Groningen 9713 GZ, The Netherlands
6. European Research Institute for the Biology of Ageing, University Medical Center Groningen, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands
7. Bioanalytical Laboratory, PRA Health Sciences, Early Development Services, Amerikaweg 18, 9407 TK Assen, The Netherlands
8. Department of Pediatrics, Center for Liver, Digestive and Metabolic Diseases, University of Groningen, University Medical Center Groningen, 9713 AV, Groningen, The Netherlands

Mass spectrometry and peptide analysis: our contribution to a long lasting story [PL2]

Christine ENJALBAL

Life science research linked to health societal issues requires at one stage the discovery of new biomarkers. Peptides and proteins constitute one of the most important class of such bioactive compounds that have thus been the subject of many researches for their detection, structural characterization and finally quantitation. Mass spectrometry represents the state-of-the-art analytical science to monitor such molecules of interest present at trace level in complex biological matrices as encountered in the context of pharmacology, pharmacy, toxicology and clinic applications. For the last two decades, we were involved in such investigations dealing with various method developments for efficient peptide sequencing, sensitive detection and robust quantitation applied to natural peptides mainly linked to pharmacology and chemical ecology issues.

Gas-phase fragmentations of synthetic peptides were studied to contribute to the in-depth knowledge of fragmentation rules that are specifically required for efficient and reliable MS/MS *De Novo* sequencing. Although a variety of search algorithms has been developed for that purpose, numerous high-signal/noise MS/MS spectra cannot still be matched to predicted protein spectra or are even misinterpreted. A large set of home-made synthetic peptides dissociated upon vibrational activation on different mass analyzer configurations (MALDI-TOF/TOF, ESI-QqTOF) allowed to evidence specific peptide backbone dissociation pathways triggered by the presence of basic residues within the sequences which is predominantly found in peptides issued from natural extracts such as venoms. Besides, the diversity of post-translational modifications and the possibility of sequence folding by multiple disulfide bridges necessitated specific ion mobility-tandem mass spectrometry experiments (IM-MS) to elucidate such complex peptides and achieve the most complete structural characterization.

Furthermore, various strategies to enhance peptide detection and quantitation were also investigated by designing either specific functionalization organic chemistry (labeling tag) or material preparation (inert surface) to a dedicated MS technology. For instance, Surface Assisted Laser Desorption Ionization-Mass Spectrometry (SALDI-MS) performed on silicon nanostructures revealed to be a very attractive alternative to conventional MALDI technology for the analyses of peptides. Both material architecture and physico-chemical properties were probed as crucial parameters for achieving detection sensitivity down to femtomolar concentrations, good reproducibility/repeatability as well as method robustness. High-throughput identification of protein tryptic digests was achieved. Surface functionalization by grafting a MALDI matrix as well as peptide immobilization were achieved with the introduction of an analytical construct bearing a photolinker to covalently anchor the peptide and to trigger its subsequent photorelease upon laser irradiation. Additionally, other peptide chemical labeling strategies aiming to enable sensitive quantitation of natural bioactive peptides were designed, the functionalization tag being adapted to either molecular (MALDI-MS/MS, ESI-MS/MS) or elemental (ICP-MS) mass spectrometry. The proof-of-concept was demonstrated in pharmacology on the Vasopressin V1a receptor (AVP peptide ligand) for both methodologies (HCCA-labeling/MALDI-MS and Se-labeling/ICP-MS). Saturation and competitive binding experiments enabled to recover figures of merit (Ki, Kd, IC50) in accordance to literature data.

Machine learning methods for the interpretation of label-free proteomics data [PL3]

Lukas KALL

Protein quantification by label-free shotgun proteomics experiments is plagued by a multitude of error sources. Typical pipelines for identifying differential proteins use intermediate filters to control the error rate. However, they often ignore certain error sources and, moreover, regard filtered lists as completely correct in subsequent steps. These two indiscretions can easily lead to a loss of control of the false discovery rate (FDR). We propose a probabilistic graphical model, Triqler, that propagates error information through all steps, employing distributions in favor of point estimates, most notably for missing value imputation. The model outputs posterior probabilities for fold changes between treatment groups, highlighting uncertainty rather than hiding it. Further, the model can be combined with unsupervised clustering on both MS1 and MS2 level to summarize all analytes of interest without assigning identities, a method we call quandenser. Not only does this eliminate the need for redoing the quantification for each new set of search parameters and engines, it also reduces search time due to the data reduction by MS2 clustering. Both methods are distributed under an open source licence from https://github.com/statisticalbiotechnology/triqler and https://github.com/statisticalbiotechnology/quandenser

Mass spectrometric epitope mapping [PL4]

Michael GLOCKER

Mass spectrometric epitope mapping has become a versatile method to precisely determine a soluble antigen´s partial structure that directly interacts with an antibody in solution [1]. Typical lengths of investigated antigens have increased up to several hundred amino acids while experimentally determined epitope peptides have decreased in length to on average ten to fifteen amino acids. Since the early 1990s sophisticated methods have been developed more and more and have forwarded a bouquet of suitable approaches for mass spectrometric epitope mapping with permanently immobilized, temporarily immobilized, and free-floating antibodies. While up to now monoclonal antibodies have been mostly used in mass spectrometric epitope mapping experiments, the applicability of polyclonal antibodies has been proven. The antibody´s resistance towards enzymatic proteolysis has been of key importance for the two mostly applied methods: epitope excision and epitope extraction. Sample consumption of both, the antigen and the antibody, has dropped to low picomol amounts.

Epitopes are identified by us using our newly developed mass spectrometry-based method, termed "Intact Transition Epitope Mapping (**ITEM**)" [2]. This method is now renamed to "Intact Transition Epitope Mapping – One-step Non-covalent force Exploitation (**ITEM-ONE**)" since it has been surpassed by our most recently developed epitope mapping method which is entitled "Intact Transition Epitope Mapping – Targeted High-Energy Rupture of Extracted Epitopes (**ITEM-THREE**)" [3]. Intact Transition Epitope Mapping - Thermodynamic Weak-force Order (**ITEM-TWO**) at last, is a mass spectrometry-based method that we have developed to determine an equally important antibody feature, affinity, i.e. binding strength, in a straightforward gas phase experiment [4].

All three ITEM methods make use of ion mobility mass spectrometry for rapid and accurate determination of antigen-derived peptides, the epitopes, that undergo specific in-solution interactions with an antibody of interest. The rapidity by which mass spectrometric epitope mapping and binding strength determination is executed outperforms most if not all alternatives. Thus, it can be asserted that mass spectrometric epitope mapping has reached a state of maturity, which allows it to be used in any mass spectrometry laboratory around the world. After now thirty years of constant and steady improvements, its application to clinical samples, *e.g.* for patient characterization and patient stratification, is anticipated to become true in the near future.

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Proteomes in 3D [PL5]

Paola PICOTTI

Protein structural changes induced by external perturbations or internal cues can profoundly influence protein activity and thus modulate cellular physiology. Mass spectrometry (MS)-based proteomic techniques are routinely used to measure changes in protein abundance, post-translational modification and protein interactors, but much less is known about protein structural changes. In my talk, I will present a recently developed structural proteomics method that enables analysis of protein structural changes on a proteome-wide scale and directly in complex biological extracts. The approach relies on the coupling of limited proteolysis (LiP) tools and MS. LiP-MS can detect subtle alterations in secondary structure content, larger scale movements such as domain motions, and more pronounced transitions such as the switch between folded and unfolded states. I will describe how we are applying this approach to study the molecular bases of protein aggregation diseases and to the identification of protein-small molecule interactions (e.g drug targets). I will also propose that monitoring protein structural states on a proteome-wide scale can serve as a new powerful readout to pinpoint altered protein functional states and the (de)regulation of biochemical pathways. Last, I will discuss the power and limitations of the new approach.

Mass spectrometry approaches to dynamic protein structure: from disorder to membrane pores [PL6]

Frank SOBOTT

Our work focuses on aspects of dynamic and heterogeneous protein conformations and assemblies, using an integrated structural approach based on "native" mass spectrometry, ion mobility, H/D exchange and surface mapping techniques (e.g. Fast Photochemical Oxidation of Proteins) in combination with electron microscopy and other biophysical methods.

Specifically, we are going to show recent results on the detection and characterization of conformational dynamics and intrinsic disorder in proteins, including alpha-synuclein. A range of folding states, from disordered to compact, are characterized and interpreted using conformational footprinting and molecular dynamics approaches. These data link the conformational state of the protein with their association into larger oligomers, which are believed to be able to form membrane pores. We use detergent micelles, lipid bilayers (bicelles) and nanodiscs for both native MS and covalent labelling of exposed parts of the protein, and apply these techniques to various different ion channels including the mechanosensitive channel of large conductance (MscL). Using covalently attached, charged ligands inside the MscL channel, we can mimic the effect of mechanical pressure on the surrounding membrane and characterize various opening states using ion mobility-MS, electron microscopy, EPR spectroscopy and other biochemical and computational methods, in the absence of lipids. We also develop novel MS and EM methods to study membrane proteins in SMALPs, amphipols and other lipid nanoparticles.

Direct-Injection Mass Spectrometry (DIMS) for the analysis of aroma compounds [PL7]

Jean-Luc LE QUERE

Direct-injection mass spectrometry (DIMS) techniques have evolved into powerful methods to analyse volatile organic compounds (VOCs) without the need of chromatographic separation. In food-related applications, they have been used as on-line techniques for monitoring processes, quantification of VOCs, aroma release studies, profiling and fingerprinting foodstuffs. Combined to chemometrics, they have been used to solve sample categorization issues based on volatilome determination. They have also been used for real-time *in-vivo* aroma release experiments, in a method known as breath-by-breath analysis or the "nosespace" technique.

Starting with the notion of MS-based electronic nose, this presentation will be essentially devoted to on-line chemical ionization MS (CI-MS) methods: atmospheric pressure chemical ionization MS (APCI-MS), selected ion flow tube MS (SIFT-MS), and proton transfer reaction MS (PTR-MS).

For years, APCI-MS has been the reference technique for *in-vitro* and *in-vivo* monitoring of flavour compounds. Some examples of the early work and from our own developments will be presented. SIFT-MS has been used extensively to study the chemistry of ion-molecule reactions, and determining k-rate coefficients. SIFT-MS has also found practical analytical use in breath analyses and determination of biomarkers. Recent improvement of the sensitivity in reduced size instruments has allowed worth mentioning studies relating aroma analyses.

A special attention will be paid to PTR-MS, and particularly to the powerful PTR-time of flight-MS (PTR-ToF-MS) instrument. Recent results obtained by our group will be presented for *in-vitro* (profiling dark chocolates with different organoleptic properties), *ex-vivo* (real-time monitoring of volatile metabolites resulting from nasal odorant metabolism), and *in-vivo* (nosespace analyses and real-time monitoring of nasal metabolism) applications.

Hair reveals what people conceal: biometric traits from the chemical analysis of human hair [PL8]

Glen JACKSON

This presentation reveals the profound ways in which isotope ratio mass spectrometry (IRMS) can provide information about the geographic origin and history of almost everything around us, including our fellow humans. The presentation explains the ways in which we are and are not exactly what we eat, and it explains how researchers use IRMS measurements of hair to determine the geographic origin and travel histories of animals and humans.

Building off these established capabilities, our research develops the ability to objectively classify the hair of different human donors into soft biometric grouping factors such as sex, age group and body mass index (BMI). Classification is accomplished either through the quantitation of amino acids in human hair matrix, which is comprised mainly of structural keratins, or through the precise measurement of naturally occurring stable isotopes of 13C/12C of each amino acid in the hair matrix. Our work also demonstrates that amino-acid-specific analysis of human hair can predict type II diabetes in the donors. in support of forensic entomology investigations, the same types of amino acid analyses can be used to link individual blowflies to their carrion (flesh) diets.

Authors

Glen P. Jackson [1, 2]

Ming Hsieh Distinguished Professor of Forensic & Investigative Science.

1. Department of Forensic and Investigative Science, West Virginia University, Morgantown, WV
2. C. Eugene Bennett Department of Chemistry, West Virginia University, Morgantown, WV

From analytical proteomics to biology or from biology to analytical proteomics ? [PL9]

Alain VAN DORSSELAER

The final goal of proteomics is obviously to improve knowledge in biology, but the analytical aspects of proteomics are from far not yet able to bring all data that biologists are dreaming of. A short history of the development of mass spectrometry and analytical proteomics will be presented and will illustrate the importance of a series of unexpected breakthrough on a period of more than 130 years. This will show that there is still a need, but also possibilities for other developments in analytical proteomics. Therefore, high standard analytical proteomics cannot be seen yet as a routine technic that biology laboratories could just “buy and use”. Efforts need to continue for future developments and innovative approaches in separation sciences, mass spectrometry and bioinformatics. Because of the huge scope of knowledge, knowhow, manpower and financial investments required, tight collaborations between biologists and analysts/chemists/bioinformatics remain the best approach for high standard proteomics. These collaborations must be encouraged and supported by our universities and research institutions.

Oral communications

Parallel Session 1 : Ion Mobility

Structure determination of large isomeric oligosaccharides of natural origin through multi-pass and multi-stage cyclic traveling wave ion mobility mass spectrometry [KL1]

Hélène ROGNIAUX

Introduction

Carbohydrate isomers with identical atomic composition cannot be distinguished by mass spectrometry (MS). By separating the ions according to their conformation in the gas phase, ion mobility (IM) coupled to MS is an attractive approach to overcome this issue and extend the limits of MS in structural glycosciences. Some instrumental developments have recently improved the separation capacity and multi-function capability of ion mobility platforms [1-3]. We will show the potential of one of these technical advances for resolving the structure of complex natural polysaccharides.

Methods

A cyclic traveling wave IM cell integrated in a quadrupole/time-of-flight mass spectrometer was used. This instrument allows multi-pass ion trajectories, with an improvement in the separation capacity increasing over passes. It also allows performing multi-stage experiments, with pre and post-IM fragmentation [3]. Experiments were conducted on a complex mixture of oligoporphyrans, derived from the enzymatic digestion of the cell wall of a red alga.

Results

Although no CCS reference value (collision cross section) is available for oligoporphyrans or their fragments, we have been able to solve their complete structure by combining the arrival time distribution (ATD) profiles and the m/z of some specific fragments. This was done for oligoporphyrans up to 1500 Da and included the positioning of the methyl ether and sulfate groups. The structures defined by IM-MS/MS are in good agreement with those found in the past, but which used more demanding analytical approaches [4-5]. This study also revealed new structures, present at low intensity. Moreover, by comparing the abundance of the different isomers released by the enzyme, we drew unpublished conclusions about the specificity of the enzyme, particularly about its accommodation tolerance for anhydro bridges. Finally, a refined separation of two isoforms with close velocity was obtained in 64 passes in the cyclic IM cell, with an estimated resolution in ion mobility of approximately 800, confirming the structures attributed to these two isoforms.

Conclusions

The ATDs of selective fragments under "routine" runs (4 passes) on the cyclic IM instrument led to resolve the complete structure of several oligoporphyran isomers within few second experiments. The capability to selectively isolate isoforms and refine their separation over longer paths permitted to strengthen some structural attributions. Therefore, this recently marketed cyclic IM instrument appears as an attractive platform in structural glycosciences.

Novel Aspect

The combination of ATDs and m/z of specific fragments, with high resolution in ion mobility, led to the complete resolution of the structure of complex carbohydrate isomers.

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Keywords

High-resolution ion mobility, carbohydrates, isomers

Authors

Ropartz David [1], Fanuel Mathieu [1], Ujma Jakub [2], Giles Kevin [2], Rogniaux Hélène [1]

1. UR1268 Biopolymères Interactions Assemblages, INRA, F-44300 Nantes (France)
2. Waters Corporation, Wilmslow (UK) (Royaume-Uni)

Temperature-controlled IMS-IMS measurements for conformational thermodynamics [OC1]

Aurélien LE FEVRE

Introduction

Natural or synthetic molecular systems, such as biomolecules or molecular machines, often possess the ability to adapt their tridimensional structures to respond to environmental perturbations. Better understanding these mechanisms requires the development of experimental tools adapted to the complexity of such systems while being sensitive to their conformational dynamics and thermodynamics. We present a new approach to characterize the dynamics and the thermodynamics of isomerization reactions based on mass spectrometry (MS) and ion-mobility spectrometry (IMS).

Methods

We investigated isomerization reactions through tandem-IMS measurements, using a homemade setup [1]. Namely, one isomeric form of a molecular ion is selected based on a first IMS separation. After excitation by collisions, a second IMS separation is used to monitor potential structural changes. Moreover, selected ions can be trapped for a controlled amount of time in the region between the two IMS cells, which allows measuring isomerization kinetics [2].

Results

We modified our setup to allow controlling the temperature of the ions either during their drift through the mobility cells, or while they are trapped between the two drift cells. Thermalization is achieved through low energy collisions with the drift gas, which is used as a heat bath. We show preliminary results on thermally induced isomerization on model systems. Namely, reference molecules were studied, which display well-characterized isomerization reactions such as bradykinin [3], or [Ru(bpy)2(dmso)2] [4].

Two directions were explored in this work to access the energetics of isomerization reactions. On the one hand, we subjected thermalized ions to collisional activation in order to monitor the temperature-dependance of the collision energy threshold for isomerization. On the other hand, we obtained preliminary results on the temperature-dependance of the kinetics of isomerization for IMS-selected ions.

Conclusions

We obtained preliminary results on a novel version of a tandem-IMS setup, which opens new ways to direct measurements of the energetics of isomerization reactions. As a direct perspective, more systematic temperature-dependant kinetics measurements will be used as an alternative and direct way to determine Arrhenius activation energies for isomerization.

Novel Aspect

We use an original tandem-IMS MS setup paired with precise temperature control and measurement, for a novel gas phase conformational thermodynamics study.

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Keywords

IMS, Conformational thermodynamics, conformational dynamics

Authors

Le Fèvre Aurélien [1], Comby-Zerbino Clothilde [2], Dugourd Philippe [2], Chirot Fabien [1]

1. Institut des Sciences Analytiques (France)
2. Univ Lyon, Université Claude Bernard Lyon 1, CNRS, Institut Lumière Matière, F-69622, LYON, France (France)

Circular dichroism mass spectrometry of nucleic acids ions [OC2]

Frédéric ROSU

Introduction

Structural studies of biomolecular ions using native mass spectrometry is an area of increasing interest. Ion spectroscopy represent the next area which can be exploited for mass-spectrometry-based biomolecular structure determination. IRMPD or Forster Resonance Energy Transfer (FRET) have been successfully demonstrated in the gas phase. Here, we present the first example of circular dichroism measurements on biomolecular ions within a mass spectrometer, using electron photodetachment as a reporter.

Methods

d and l forms of the nucleic acids that form a tetramolecular G-quadruplex [(TG4T)4.NH4] was used. Different charge states produced by ESI were mass selected and trapped in a bruker amazon coupled to a tunable UV laser. Single pulse of either left or right handed circularly polarized light (CPL) is used to irradiate the ions at different wavelengths, generating electron detachment. The difference in the yield of action produced by right or left CPL is determined.

Results

We record the pulse energies throughout acquisition of mass spectra to allows a proper normalisation of electron detachment yields.

Circular dichroism measurements were performed by recording the electron detachment yield for both right and left handed CPL as a function of the wavelength. Here, the polarization is switched every 90 seconds to minimize the influence of fluctuations in pulse energy and alignment. These measurements are performed for the enantiomers of the G-quadruplexes.

It was found that there was an inversion of the circular dichroism signal (defined as the difference in the yield of electron detachment with right and left CPL) for the enantiomers. TDDFT calculations have been performed to correlate the G-quadruplexes structures (different stacking between G-quartet) and the CD spectra.

This demonstrated, for the first time, that CD measurements of large biomolecular architectures are possible within a mass spectrometer. Furthermore, the shape of the circular dichroism curve obtained is very similar to that observed in solution.

Conclusions

We demonstrated that measurements of circular dichroism on nucleic acids using absorption in solution and electron detachment in the gas phase are reporting on the same phenomenon. Hence, it is possible to directly compare structural properties of biomolecules in solution and in the gas phase using a single phenomenon.

Novel Aspect

We present here the first measurement of circular dichroism of large biomolecular polyanions using electron detachment as a reporter.

Keywords

Ion spectroscopy, Native mass spectrometry, Nucleic acids

Authors

Rosu Frederic [1], Daly Steven, Gabelica Valérie [2]

1. CNRS, Univ. Bordeaux (France)
2. Acides Nucléiques: Régulations Naturelles et Artificielles (ARNA) (France)

Intact- and middle-level Collision Induced Unfolding experiments to decipher gas-phase unfolding mechanism of hybrid and canonical mAbs [OC3]

Oscar HERNANDEZ-ALBA

Introduction

Most currently approved mAbs for clinical treatment of several diseases are selected from three human IgG isotypes (1, 2, and 4) [1]. Since different isotypes also differ in their ability to support secondary immune functions, there is a real interest in developing new strategies for a better understanding of structure-function relationship. Here we aim at developing new CIU-based strategies at intact and middle level to tackle structural differences/similarities on biotherapeutic mAbs not only from different isotypes, but also for hybrid IgG formats.

Methods

All the therapeutic mAb-based formats were deglycosylated and buffer desalted against 100 mM ammonium acetate at pH 7.0. Native CIU experiments were acquired on a hybrid SynaptTM G2 HDMS coupled to an automated chip-based nanoelectrospray device. Ions were progressively accelerated by increasing the acceleration voltage in the trap prior to IM separation by 5 V steps from 0 to 200 V. CIUSuite 2 [2] software was used to generate and compare all the CIU fingerprints.

Results

CIU experiments were used to differentiate and characterize the gas-phase stability of several iso-cross-section mAbs isotypes. At the intact level, several differences are distinguished between the three mAb isotypes in terms of unfolding energies and number of unfolding transitions. However, no clear-cut evidences could be obtained to assign the CIU fingerprints to specific mAb isotypes. Subsequently, middle-CIU experiments were performed on the F(ab')2 and Fc subunits, giving rise to a higher number of unfolding transitions, allowing to assign specific unfolding signatures to each individual isotype class. In this case, middle-CIU experiments provided more diagnostic fingerprints to efficiently characterize/categorize mAb isotypes. CIU experiments were also conducted to evaluate the stabilization of the mAb structure upon payload conjugation [3] or to study the differences of the unfolding mechanism of different IgG4 formats after Ser to Pro mutation in the hinge region [4].

Conclusions

Altogether, our study highlights the suitability of CIU experiments, in particular at the middle-level, for the characterization of the gas-phase unfolding dynamics of different therapeutic mAbs formats. We envision that specific signatures characteristic of each individual mAb isotype from different IgG-subunits can be used to characterize/categorize new IgG-based biotherapeutics.

Novel Aspect

Development of middle-CIU experiments to provide signature unfolding transitions in the gas-phase to characterize and categorize the isotype of different therapeutic mAbs.

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Keywords

Middle-CIU, Ion mobility, mAb isotypes

Authors

Hernandez Alba Oscar [1], Botzanowski Thomas [1], Wagner-Rousset Elsa [2], Colas Olivier [2], Beck Alain [2], Cianférani Sarah [1]

1. Laboratoire de Spectrométrie de Masse BioOrganique (France)
2. Centre d'Immunologie Pierre-Fabre (France)

Parallel Session 2 : Clinical Mass Spectrometry

Towards in-vivo molecular diagnostics of esogastric cancer with SpiderMass real-time, mini invasive analysis [KL2]

Nina OGRINC

Introduction

Esogastric cancer is the 4th most diagnosed cancer. Its extent can't be deduced from the CT-scan or endoscopy so the conventional practise is an intraoperative pathological exam which can be long and challenging. Therefore, we need technology allowing real-time diagnostics using molecular signatures. The water-assisted laser desorption/ionization mass spectrometry has demonstrated the capability to analyse biopsies ex vivo and correctly classify tumour types and grade. Here, we present ex vivo SpiderMass analysis combined with MSI to asses and classify esogastric cancer.

Methods

Fresh-frozen biopsies were supplied by the FREGAT database and sectioned for H&E (5µm), SpiderMass (20µm) and MALDI-MSI (12µm). The SpiderMass analyses were performed using a mini invasive IR-laser microprobe connected to the Q-TOF instrument in both polarities. The data was processed via supervised Abstract Model Builder and a CNN algorithm. The 12µm sections were coated with Norharmane (7mg/mL in CHCl3:MeOH, 2:1) and analyzed with RapifleX at 50 µm.

Results

The samples were H&E stained and annotated by a pathologist. Based on the annotations we selected ROI subtypes and analyze them with SpiderMass. The raw data files were directly imported into the AMX software. All of the imported spectra were used to build a PCA and LDA-based classification models. The PCA analysis decreases the dimensionality of the datasets and generates a list of features showing the largest variance within the dataset. Further on, the features were subjected to supervised LDA through user assigned classes: poorly cohesive carcinoma (SRC and NOS type cells), adenocarcinoma, healthy tissue and mucosal tissue. The LDA is used for a second space transformation, in which it minimizes the intra- (within) class variance and maximizes the inter- (between) class variance. The cross-validation was performed by removing a part of the cohort and re-building the model. A comparison of the classification models was made using deep learning CNN. Additional cross-validation was performed by dual polarity mass spectrometry imaging.

Conclusions

The new developed pipeline allows for rapid biomarker detection, guided surgery and real-time analysis of esogastric cancer.

Novel Aspect

Our aim is to improve intraoperative diagnostisis of esogastric cancer.

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Keywords

Water-Assisted Laser Desorption Ionization Mass Spectrometry, Cancer diagnostics, Esogastric cancer

Authors

Ogrinc Nina [1], Caux Pierre-Damien [1], Lintis Alexandru [2, 3], Renaud Florence [4, 3], Seddiki Khawla [5], Wisztroski Maxence [1], Droit Arnaud [5], Piessen Guillaume [2, 3], Salzet Michel [1], Fournier Isabelle [1]

1. Protéomique, Réponse Inflammatoire, Spectrométrie de Masse (PRISM) - U1192 (France)
2. Department of Digestive and Oncological Surgery, University Lille, Claude Huriez University Hospital, Lille Cedex, France (France)
3. Jean-Pierre Aubert Research Center, Neurosciences and Cancer, University Lille, UMR-S 1172-JPArc, Lille, France (France)
4. Department of Pathology, Biology Pathology Center, University Lille, University Hospital, Lille, France (France)
5. Department of molecular medicine, Faculty of medicine, Laval University, Quebec, Canada (Canada)

Multiplexed detection and quantification of the protein effectors of antibiotic resistance in clinical strains of Pseudomonas aeruginosa and Enterobacteriaceae by Scout-MRM – Application to positive blood cultures [OC4]

Romain CARRIERE

Today, approximately 6 million people die each year from bacterial infections. Among them, hospital-acquired infections caused mainly by pathogens of the ESKAPE family are of great concern. Thus, death incidences result both from a considerable downshift in the discovery of new antibiotics and by the appearance of strains expressing multiple resistance mechanisms caused by the selection pressure during antibiotherapy and by horizontal gene transfer between species.

The current diagnosis methods are time-consuming and thereby threatening since the patient's morbidity rate increases by 7% per hour in the absence of appropriate treatment.

In this context, we developed a unique 45-minute analytical method allowing the quantification a large panel of proteins (27) involved in antibiotic resistance mechanisms in Pseudomonas aeruginosa and Enterobacter spp. This methodology consists of a bacterial lysis, then a 10-minute enzymatic digestion under ultrasounds, followed by a targeted mass spectrometry method performed on a triple quadrupole (6500, Sciex) using Scout-MRM targeted analysis mode.

This analytical method allowed the relative quantification of a multitude of proteins in 137 bacterial strains. Bona fide proteotypic peptides (i.e. peptides unique to the protein of interest) were

experimentally selected and transition areas were normalized using ribosomal peptides common to all bacterial strains. This approach makes it possible to estimate the relative quantity of bacteria per strain which eventually allowed the quantification of overexpressed proteins within the latter.

That being said, our Scout-MRM method has enabled the quantification of different efflux systems (MexA/B-OprM for example) or β-lactamases of plasmid or chromosomal types such as: TEM, SHV, GES, OXA, CTX-M, VIM and AmpC, to mention a few.

In some cases, it has even been possible to identify whether these proteins had a simple activity or an extended-spectrum activity (ESBL). In addition, the protein encoded by the MCR-1 gene, responsible for colistin resistance (discovered in 2015) and outer-membrane protein TolC have also been quantified.

To summarize, this analytical method made it possible to estimate the bacterial numbers in 137 different strains using normalization by bacterial quantotypic peptides. This strategy eases the use of mass spectrometry for answering biological questions at no additional cost and without pipetting errors because of calibrant addition. Hence, analytical specificity and sensitivity were reached in this study, making our method an attractive alternative as a time-effective multiplexed protein assay.

Subsequently, this method has been applied to positive blood cultures and implemented in hospital-based health facilities for a routine clinical practice.

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Keywords

Antibiotic resistance, ESKAPE, Scout-MRM

Authors

Carrière Romain [1], Mouton Nicolas [1], Macavei Iulia [1], Dauwalder Olivier [2], Vandenesch Francois [2], Bonnet Richard [3, 4], Robin Frédéric [3, 4], Plésiat Patrick [5], Lemoine Jérôme [1]

1. Institut des Sciences Analytiques (France)
2. Centre International de Recherche en Infectiologie (France)
3. CNR "Résistance aux antibiotiques" (France)
4. Laboratory of Bacteriology, CHU Clermont-Ferrand, 63000, France (France)
5. Laboratoire Chrono-environnement - UFC (UMR 6249) (France)

Quantitative determination of IGF-I in blood using DBS and VAMS after solid phase extraction microelution and analysis by LC-HRMS for doping control purposes [OC5]

Cynthia MONGONGU

Insulin-like growth factor (IGF-I) is banned by the World Anti-Doping Agency (WADA) for its promoting properties on tissues. IGF-I serum concentration is also a biomarker of the Growth Hormone misuse by athletes trying to improve their physical performance. WADA, which monitors anti-doping activities in the world, currently authorizes two methods for the quantification of IGF-I in serum : an immunoassay on a specific bioanalyzer and a mass spectrometric analysis based on a bottom-up approach using two IGF-I specific peptides after proteins precipitation, reduction and alkylation of analytes followed by tryptic digestion.

Today, all these tests require invasive venous blood samples for athletes. In addition, testing authorities have to manage a fast and controlled temperature shipment at a high cost. Recent works have shown the interest of using dried blood on finger-prick blood collection supports such as Dried Blood Spot (DBS) and Volumetric Absorptive Microsampling (VAMS™) for quantification of various molecules including IGF-I. Micro volumes can be used, much smaller than a classical venous blood collection. Another important advantage is the very good stability of the analytes collected on these supports, which facilitates storage and allows transport at ambient temperature and therefore at a lower cost.

In this work we propose a fast and accurate protocol for IGF-I quantification based on a top-down analysis of the intact protein in dried blood sample collected on DBS and VAMS™ after a solid phase extraction. The use of 15N-labelled IGF-I standard is necessary in the analytical process for a reliable quantification. The sample preparation consists of desorption of the analytes of interest followed by a solid phase extraction on a 96-well microelution plate using a mixed-mode polymeric sorbent with anion-exchange groups. The eluate is diluted after extraction and directly analyzed by HPLC/HRMS. This approach avoids the trypsic digestion commonly used in protein analysis that is time-consuming. High resolution analysis of the intact IGF-I molecule is performed with good sensitivity and selectivity. Using 20 µL of dried blood, the developed method allows the quantification of IGF-1 within the physiological concentration range (50 to 500 ng/mL). A comparison between DBS and VAMS is ongoing to evaluate the support offering the best analytical performance. After complete validation, a comparison between dried blood and serum collected at the same time will be performed. The developed approach might be an alternative to the current quantitative analysis for anti-doping purposes.

Keywords

IGF-I, dried blood, HRMS

Authors

Mongongu Cynthia [1], Marchand Alexandre [1], Coudoré François [2], Audran Michel [1], Buisson Corinne [1]

1. AFLD, Département des analyses, 92290 Châtenay-Malabry (France)

2. Laboratoire de Neuropharmacologie, INSERM UMRS 1178, 92290 Châtenay-Malabry (France)

De novo sequencing of patient-derived monoclonal antibody light chains by top-down and bottom-up proteomics [OC6]

Mathieu DUPRE

Introduction

In multiple myeloma diseases, monoclonal immunoglobulin light chains (LC) are abundantly produced, with the consequence in some cases to form deposits affecting various organs, such as kidney [1], while in other cases the light chains remain soluble up to concentrations of several g/L in plasma. The sequence determinants of the solubility of light chains are not understood. To that end, more data, linking the biophysical and biochemical properties of patient-derived light chains to their sequences are needed. Here we establish a de novo sequencing workflow for patient-derived LCs.

Methods

LCs were isolated from urine samples of several patients with multiple myeloma. For bottom-up experiments, peptides were generated using several enzymes (trypsin, pepsin, Nepenthes fluid extract [2]) and analyzed in LC-MS/MS on an Orbitrap QExactive plus. PEAKS and ALPS [3] tools were used to process the data. For top-down proteomics, intact LCs were analyzed on an Orbitrap Fusion Lumos with different activation methods including UVPD.

Results

Monoisotopic masses of all LCs were first measured in LC-MS on the Orbitrap Fusion Lumos. Top-down MS/MS targeted experiments were then conducted with HCD, CID, EThCD and UVPD fragmentation modes and data were searched with both Uniprot and ALC (Amyloid Light Chain) databases. None of the experimental data correctly matched database entries. We therefore digested all samples with specific and unspecific enzymes and all peptide digests were analyzed by LC-MS/MS. Data were searched with PEAKS to generate de novo tags that were further processed with ALPS to generate overlapping sequences. Manual alignment was thus performed to concatenate the produced partial sequences to obtain complete light chain sequences in agreement with the intact mass measurements. These putative sequences were then matched against the top-down data to select the best candidate. Finally, high sequence coverage could be achieved and confirmed all sequences.

Conclusions

We performed complete de novo sequencing of 25 kDa LCs without database search combining proteomics approaches with appropriate data analysis. We highlighted that protein digestion with both trypsin and Nepenthes fluid extract could be enough to produce overlapping peptides able to generate the best LCs candidates. We also showed that combining activation methods for the fragmentation of intact LCs is a key-feature to fully characterize their sequences.

Novel Aspect

Complete de novo sequencing of light chains extracted from urine of patients with multiple myeloma without database search.

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Keywords

Immunoglobulin light chains, de novo sequencing, proteomics

Authors

Dupré Mathieu [1], Duchateau Magalie [1], Sternke-Hoffmann Rebecca [2], Boquoi Amelie [3], Fenk Roland [3], Rey Martial [4], Haas Rainer [3], K. Buell Alexander [5], Chamot-Rooke Julia [4]

1. Unité Spectrométrie de Masse pour la Biologie, Institut Pasteur, CNRS USR2000, Paris, France (France)
2. Institute of Physical Biology, Heinrich-Heine-University of Duesseldorf, Germany (Allemagne)
3. Department of Hematology, Oncology and Clinical Oncology, Heinrich-Heine-University Düsseldorf, Germany (Allemagne)
4. Unité Spectrométrie de Masse pour la Biologie, Institut Pasteur, CNRS USR2000, Paris, France (France)
5. Department of Biotechnology and Biomedicine, Technical University of Denmark, Denmark (Danemark)

Parallel Session 3 : Bio-Chemo-Informatics and Statistics

Striking the current metaproteomics dogma for deeper characterization of microbiota [KL3]

Jean ARMENGAUD

Introduction

Metaproteomics is the analysis of complex samples to describe how they function. The data may give novel taxonomical information based on peptide information. The current dogma in metaproteomics is i) to rely only on taxon-specific peptides for extracting the taxonomical information, and ii) to interpret MS/MS data with metagenomics data acquired on the same sample. We propose a new metaproteomics pipeline that allows a quick identification of any microorganism present in the sample based on the whole dataset, without need of additional costly metagenomics information.

Methods

We developed in python an in-house pipeline to assign taxonomical information to each detected peptide against a generalist database and for the deconvolution of this complex signal. In parallel, we developed a procedure to regularly update and curate the database to avoid taxonomic misassignment. We also optimized the sample preparation for extracting proteins of any organisms and the tandem mass spectrometry acquisition to maximize the results.

Results

We discovered the principle of a mathematical signature describing the number of peptide sequences shared with all other organisms calculated by modeling and phylogenetic relationships. This principle allows deciphering the precise content of a sample by the linear combination of such signatures applied on any experimental metaproteomic dataset. A sample can thus be described by its peptide-specified taxa and their respective relative ratios defined from the global peptide information. Its efficiency is exemplified with artificial mixtures. We developed the informatic pipeline to interpret quickly MS/MS datasets in terms of taxonomy, obtain response linearity regarding the label-free quantitation of biomass contributions, and establish the most appropriate protein sequence database for each sample. Several examples will be commented such as a deep characterization of human feces and a comparative analysis of sentinel animal intestines.

Conclusions

This methodology paves the way to accurate label-free quantitative metaproteomics without the need of metagenomic information. It has been proved robust with artificial mixtures of microorganisms and has been applied to a large panel of samples of interest in terms of clinics, biotechnology, and environment.

Novel Aspect

A new procedure for interpreting MS/MS metaproteomic dataset allows characterizing any sample in terms of taxonomy and improve the functional characterization.

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Keywords

Metaproteomics, bioinformatics, Microbiota

Authors

Pible Olivier, Culotta Karen, Gouveia Duarte, Hayoun Karim, Jouffret Virginie, Miotello Guylaine, Steinmetz Gérard, Alpha-Bazin Béatrice, Grenga Lucia, Armengaud Jean [1]

1. Laboratoire Innovations Technologiques pour la détection et le diagnostic (Li2D) (France)

Proteomics and metabolomics data integration for deep phenotyping [OC7]

Alyssa IMBERT

Introduction

Proteomics and metabolomics provide comprehensive information about the molecular phenotype. Since the interaction of proteins and metabolites is critical for biological regulation and signaling, the integration of both types of data is of high interest. However, the few combined studies described so far, however, are restricted to targeted analysis.

The ProMetIS project, driven by the five national infrastructures for mouse phenotyping (PHENOMIN), genomics (France Génomique), proteomics (ProFI), metabolomics (MetaboHUB) and bioinformatics (IFB), aims at developing innovative methods and pipelines for combined phenotyping by proteomics and metabolomics.

Methods

As a first case study, the liver and plasma of knock-out mice for the "Linker for Activation of T Cells" gene (LAT) were analyzed by multiple proteomics and metabolomics LC-MS platforms, resulting in a total of ten omics datasets, in addition to standard clinical and behavioral metadata.

Results

A common (consensus) statistical pipeline for univariate and multivariate (intra-omics) analysis was defined and significant features were identified in most of the omics datasets. Interestingly, such discrimination was not observed with the clinical metadata only. Pathway enrichment and mapping of both enzymes and metabolites are currently being performed to provide new insights about the biological interpretation. In parallel, multi-blocks analysis, with the regularized generalized canonical correlation analysis method (rGCCA [1]), enables to select combined signatures which best discriminate the two phenotypes.

Conclusions

Altogether, these results show the added value of global molecular approaches for the comprehensive characterization of the phenotype, and provide a methodology for the combined and standardized analysis of proteomics and metabolomics data.

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Keywords

Combined and standardized analysis, data integration, high-throughput phenotyping

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1. CEA, LIST, Laboratory for Data Sciences and Decision, MetaboHUB (France)
2. Institut Français de Bioinformatique - UMS CNRS 3601 (France)
3. LSMBO-DSA-IPHC (France)
4. Exploring the Dynamics of Proteomes (EDyP), BGE/U1038, INSERM/CEA/Université Grenoble Alpes (France)
5. Institut de Pharmacologie et Biologie Structurale (IPBS), UMR5089 CNRS - Université de Toulouse (France)
6. Institut Clinique de la Souris (France)
7. INRA, UMR 1019 Unité de Nutrition Humaine, CRNH Auvergne (France)
8. Service de Pharmacologie et d'Immunoanalyse, Laboratoire d'Etude du Métabolisme des Médicaments, CEA, INRA, Université Paris Saclay, MetaboHUB, F-91191 Gif-sur-Yvette (France)
9. Institut Pluridisciplinaire Hubert Curien (France)
10. Génomique Métabolique, Genoscope, Institut François Jacob, CEA, CNRS, Univ Evry, Université Paris-Saclay, 91057 Evry, (France)
11. CEA, LIST, Laboratory for Data Sciences and Decision, MetaboHUB (France)
12. Laboratoire des Signaux et Systèmes (France)
13. Plateforme de Bioinformatique/Biostatistiques (France)
14. Institut Clinique de la Souris (France)
15. Université Clermont Auvergne, INRA, UNH, Plateforme d'Exploration du Métabolisme, MetaboHUB Clermont (France)
16. CEA Grenoble (France)
17. Laboratoire des interactions plantes micro-organismes (France)
18. Exploring the Dynamics of Proteomes (EDyP), BGE/U1038, INSERM/CEA/Université Grenoble Alpes (France)
19. Exploring the Dynamics of Proteoms Laboratoire de Biologie à Grande Echelle (BGE) UMR_S 1038 Inserm/CEA/UGA (France)
20. Exploring the Dynamics of Proteomes (EDyP), BGE/U1038, INSERM/CEA/Université Grenoble Alpes (France)
21. Université Clermont Auvergne, INRA, UNH, Plateforme d'Exploration du Métabolisme, MetaboHUB Clermont (France)
22. UMR1331 Toxalim (France)
23. Institut de pharmacologie et de biologie structurale (France)
24. Génomique Métabolique, Genoscope, Institut François Jacob, CEA, CNRS, Univ Evry, Université Paris-Saclay (France)
25. Institut Français de Bioinformatique - UMS CNRS 3601 (France)
26. Laboratoire de Biologie à Grande Échelle (France)

27. Institut Clinique de la Souris (France)
28. Plateforme Métabolome Bordeaux - MetaboHUB (France)
29. Laboratoire d'Etude du Métabolisme des Médicaments, MetaboHUB-Paris (France)
30. Exploring the Dynamics of Proteomes - Large Scale Biology Laboratory BGE UMR_S 1038 (France)
31. Institut Clinique de la Souris (France)
32. Université Clermont Auvergne, INRA, UNH, Mapping (France)
33. Institut de pharmacologie et de biologie structurale (France)
34. DRF/Institut Joliot/SPI/LEMM, Université Paris-Saclay, CEA-Saclay (France)
35. Laboratoire de Spectrométrie de Masse BioOrganique, Département des Sciences Analytiques, Institut Pluridisciplinaire Hubert Curien, Strasbourg, France (France)
36. Laboratoire d'Analyses Bioinformatiques pour la Génomique et le Métabolisme (France)
37. CEA, LIST, Laboratoire Analyse de Données et Intelligence des Systèmes, MetaboHUB (France)

MetGem software for the generation of molecular networks based on the t-SNE algorithm [OC8]

Nicolas ELIE

Introduction

Metabolomic studies based on MS produce large amount of data requiring dedicated chem/bio-informatics tools to explore them. Whereas many algorithms are available for the data treatment at MS1 level, methodologies for MS2 data are scarce. One of the most popular is the generation of molecular networks (MNs) on a collection of MS2 spectra available on the online GNPS platform. We introduce here dedicated software, called MetGem (metgem.github.io), allowing the generation of GNPS-like MNs together with a t-distributed stochastic neighbor embedding (t-SNE) based visualization.

Methods

Analyzes were performed with a Dionex Ultimate 3000 RSLC system equipped with an Accucore C18 column (2.1 × 100 mm; 2.6 µm) coupled to a Q-TOF Agilent 6540 mass spectrometer. Data dependent acquisition mode was optimized to generate MS2 spectra. Datasets were processed using MZmine 2.

MetGem is written using Python programming language. Time critical parts of the software were accelerated using Cython. t-SNE implementation used is from scikit-learn package.

Results

Starting from the .mgf file, all spectra detected are compared to each other using the GNPS-based cosine score calculation system. These results are stored in a square matrix gathering together high-dimensional objects, i.e. MS2 spectra, whose dimensions contain the similarities (cosine score values), taken pairwise, between all the spectra of the dataset. As it is difficult to apprehend these high-dimensional objects and visualize them in a meaningful manner, several manifold learning algorithms have been developed for dimensionality reduction purposes and pattern recognition. The idea developed herein was to feed the t-SNE algorithm with a pairwise similarity matrix. Considering that only small distances are reliable in high-dimensional spaces, t-SNE aggregates local data points closer in the lower-dimensional space. It then tends to extract better-clustered local groups of point. Thus, it allows distinguishing easily patterns lying in different manifolds by simple visual analysis and in an unsupervised way.

Conclusions

The performances and advantages of the t-SNE output have been evaluated on two datasets, i.e. LC-MS2 analyses of fractions from a supercritical CO2 extraction of S. lineata leaves and a bark extract of C. peltatum. In both cases, t-SNE allows us to annotate more nodes compared to the GNPS-based cosine score calculation system and thus a deeper exploration of the experimental datasets was demonstrated.

Novel Aspect

With the development of MetGem software, we fulfilled the need of a dedicated, user-friendly, local software for MS2 networks generation.

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Keywords

Molecular networks, cheminformatics, software

Authors

Elie Nicolas [1], Olivon Florent [1], Grelier Gwendal [1], Roussi Fanny [1], Litaudon Marc [1], Touboul David [1]

1. Institut de Chimie des Substances Naturelles (France)

Replacing target-decoy competition to stabilize the FDR control of peptide identification in MS-based discovery proteomics [OC9]

Thomas BURGER

Introduction

In bottom-up discovery proteomics, database search engines (Mascot, Andromeda, X! Tandem...) can provide random false identifications. To avoid corrupting peptide identification lists, one relies on a False Discovery Rate (FDR), i.e. a statistical quantity that slightly overestimate the real proportion of false identifications in the dataset, so as to keep their number under control. Practically, FDR computation is achieved thanks to the Target Decoy Competition (TDC) approach [1].

Methods

We have evaluated the stability of the TDC-induced FDR by varying different search parameters on a dataset made of 10 replicates of a bacterial lysate analyzed on a high resolution instrument. Unexpectedly, and in contradiction with the asymptotical properties provided by the statistical theory [2], we observed an instability of important magnitude.

Results

We have observed that the stability of the FDR estimate decreases when the mass tolerances were narrowed (as a consequence, the more resolute the instruments, the less reliable the FDRs). Even if TDC remains valid from a theoretical viewpoint, its practical implementation and use in a context where it delivers stable and reliable results, especially when using high resolution instruments, requires many manual cares that have never been expected so far. These manual cares are difficult to reconcile with proteomics platform constraints, and are bound to increase with the improvement of instrument resolutions. Fortunately, we demonstrate it is possible to rely on an alternative procedure, which is simpler to implement, faster to compute, while providing more accurate FDRs, so that it constitutes a nice alternative to TDC.

Conclusions

For sake of more confident peptide identification lists (and subsequent biological conclusions), we end up with the conclusion that TDC should simply be abandoned and replaced by another FDR estimation procedure. Moreover, this provides a posteriori explanations on several controversies concerning low validated quality spectra [3], [4].

Novel Aspect

This work put into question decade-old statistical guidelines and proposes simple and accurate alternatives.

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Keywords

False discovery rate; target-decoy; peptide ID validation.

Authors

Couté Yohann, Bruley Christophe [1], Burger Thomas [2]

1. CEA, BIG, Biologie à Grande Echelle, F-38054 Grenoble, France (France)
2. CEA Grenoble (France)

Parallel Session 4 : Instrumentation

Andromede: MeV-Nanoparticle-SIMS for surface analysis [KL4]

Thanh-Loan LAI

Andromede (ANR-10-EQPX-23) is a new IN2P3/CNRS multidisciplinary facility in operation since November 2017. Its main asset is its wide range of available beams from protons to gold nanoparticles. The multi-charged atomic and molecular ion beams are produced with an electron cyclotron resonance source (ECR) MicroGAN provided by Pantechnik. The metallic cluster and nano-particle ion beams are delivered by the LMIS column named NAPIS (Nano Particle Ion Source) provided by OrsayPhysics. These heavy ion beams are accelerated to high energy by a NEC Pelletron® 4 MeV electrostatic accelerator then analyzed and deflected at 1,29° by magnetic dipole. This beam line is dedicated to cluster, molecule and nanoparticle beams for surface analysis, secondary ion mass spectrometry, material modifications, molecular physics, astrochemistry.

The main goal of the Andromede project is to create a new imaging mass spectrometer using MeV heavy clusters. Indeed, in SIMS, the use of high energy cluster ions in the MeV range increases secondary molecular ion yields for all samples by 100 times compared to keV cluster ions. The use of massive clusters permits another gain which allows the detection of a few tens molecular ions per projectile impact. The design of this new instrument will include a high resolution mass spectrometer ($M/\Delta M \sim 10000$) and an Electron and Proton Emission Microscope, allowing the localization of the NP impacts with sub-micrometre accuracy.

After a quick overview of the obtained results concerning the huge yields of secondary ion emission per impact, sputtering process and crater formation in different materials of various thicknesses, specific advantages of nanoparticle impacts for surface analysis in a large variety of scientific application will be presented:

1. In collaboration with the accelerator division of LAL we started the analysis and the characterization of copper, aluminum and stainless steel surfaces from various preparations (cleaning, electron bombardment…) to apprehend secondary emissions processes that disrupt the accelerators fields.
2. In collaboration with the IAS "Astrochemistry and Origins" group, analyses of mineral analogues of carbonaceous chondrite were conducted to detect the presence of organic molecules of molecular weight of about 1000 u, added by infiltration. This approach simulates the organic / mineral mixture of extraterrestrial carbonaceous meteorites and will lead to future meteorite analysis.
3. In collaboration with I2BC "Endotoxins, Structures and Host responses" team, we started the analysis and characterization of lipopolysaccharides (LPS) constituting the endotoxins bacteria responsible for infection in the host. Protocols for detecting LPS via lipid A (MW ~ 1300-1800 Daltons) have been validated. Ionic yields are 100 times higher than those obtained with commercial probes using 25 keV bismuth clusters.
4. The research group of Prof. E.A. Schweikert from Texas A&M University uses halogenated tags for the detection and co-localization of conjugated protein antibodies. In the framework of the long-standing collaboration with this group we extended this study using 12 MeV Au4004+ projectiles delivered by the Andromede facility. Three molecules with different halides have been tested, individually or mixed, as markers. Using the Andromede facility, we demonstrated the capability to measure several proteins co-localized on a surface in a single projectile impact, corresponding to an area below 1000 nm2. Interestingly in a mixture of all three tags, each tag was detected with 100 percent probability, thus it is possible to detect and count tagged protein antibodies.

Keywords

MeV-Nanoparticle, SIMS, surface analysis.

Authors

Lai Thanh-Loan [1], Della Negra Serge [1], Jacquet Dominique [1], Lesrel Jean [1], Ribaud Isabelle [1], Bilgen Suheyla [2], Mercier Bruno [2], Sattonnay Gael [2], Baklouti Donia [3], Arribard Yann [3], Augusto Luis [4], Chaby Richard [4], Tissieres Pierre [4], Eller Michael John [5], Verkhoturov Stanislav V. [5], Schweikert Emile A. [5]

1. Institut de Physique Nucléaire d'Orsay (France)
2. Laboratoire de l'Accélérateur Linéaire (France)
3. Institut d'astrophysique spatiale (France)
4. Institut de Biologie Intégrative de la Cellule (France)
5. Department of Chemistry, Texas A&M University College Station, TX 77843-3255 (États-Unis)

Benefits of Py-GCxGC/MS for the analysis of complex samples from cultural heritage [OC10]

Michel SABLIER

Introduction

Analytical pyrolysis has been explored as early as the 50s for polymer analysis [1] but is known to generate many thermal degradation products rending difficult the complete characterization of the resulting chromatograms. Using a technique providing enhanced resolution and separation power may offer the possibility to resolve such complex systems. However, Py-GCxGC/MS applications are scarce [2].

We propose to illustrate here how Py-GCxGC enlarges the potentialities for the exhaustive description of complex sample fingerprints typical to Py-GC in Cultural Heritage studies.

Methods

Pyrolysis was performed using FrontierLab furnace pyrolyzers. Analyses were conducted with a GC/MS Shimadzu QP2010-Plus, and a GCxGC/MS Shimadzu QP2010-Ultra mass spectrometer equipped with a two-stage thermal modulator Zoex II. Quantities of samples used were of few tens of µg. The chromatographic separation was conducted on nonpolar columns (30 m 5%Phe-95%Me) completed by a moderately polar (2,8 m 50%Phe-50%Me) capillary column for GCxGC/MS.

Results

We are illustrating the benefits provided by the use of Py-GCxGC/MS compared to Py-GC/MS through the analysis of reference and archaeological samples of cultural heritage materials. Two examples will be presented: (i) East Asian papers samples – for the identification of their markers of origin, (ii) lacquer sap samples – for the differentiation of Asian lacquers.

Py-GCxGC/MS showed how the gain in sensitivity and resolution inherent to the technique allowed a chemotaxonomic approach for the differentiation of very similar East Asian papers using multivariate data analysis [3]. It will be shown how the use of Py-GCxGC/MS profiles can help in the optimization of treatment for data issued from Py-GC/MS combined with the automatic deconvolution software AMDIS [4].

For Asian lacquers, the separation of each main group of the lacquer saps components through a bidimensional separation made the recognition of each peak group in all species lacquer samples easy, especially with an extracted ion chromatogram procedure without the use of derivation [5].

Conclusions

The chosen examples clearly demonstrated the great potential of Py-GCxGC/MS in refining the knowledge of samples through (i) an increased sensitivity and separation power, (ii) an

exhaustive fingerprint allowing the differentiation of samples of similar composition, and (iii) applications for data treatment (chemometrics and automatic deconvolution software) [4,5]. Potentially, this approach can renew today the analysis of well-known polymers of yesterday.

Novel Aspect

First applications of Py-GCxGC/MS in cultural heritage studies

Demonstration of the benefits of Py-GCxGC to differentiates complex samples combining statistics and MS.

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Keywords

Pyrolysis, GCxGC/MS, Cultural Heritage

Authors

Sablier Michel [1], Han Bin [2]

1. Centre de recherche sur la Conservation USR 3224 du CNRS, Muséum National d'Histoire Naturelle (France)
2. Department of Archaeology and Anthropology, University of Chinese Academy of Sciences (Chine)

BLI-MS: coupling BioLayer Interferometry with Mass Spectrometry [OC11]

Vincent JUNG

Introduction

Biolayer Interferometry is a label free technology which allows to determine the association constant between two interacting macro-molecules and to visualize their kinetic of interaction in real time. In order to do so, a bait (ligand) has to be immobilized on a biosensor while the prey (analyte) is in solution.

We aim to combine BLI interaction measurement with mass spectrometry in order to identify the proteins interacting with the bait in a complex mixture.

In this work we provide a proof of concept of the feasibility of BLIMS.

Methods

For BLI analysis we used an Octet RED96 (Fortebio). The bait was a biotinylated GFP antibody (Ab) to bind GFP tagged protein in a total cell lysate and CD9/81/63 antibodies to bind exosomes in urines. The analyte was accumulated with 50 consecutives cycles of association/elution (association 10 min, washes 10 sec twice and elution in 10mM glycine pH=2). The proteins were digested by FASP or S-Trap and analyzed with a nanoRSLC-Orbitrap Qex Plus (Thermo Scientific).

Results

In order to understand the sensitivity of the BLI-MS approach, we loaded GFP antibodies and performed a BLIMS experiment using concentrations of pure GFP ranging from 0.3 nM to 20 nM. The BLI association signal is proportional to the amount of GFP put into the well (LOD = 0.3 nM), as well as the number of GFP peptides identified by MS analysis of the GFP bound to the biosensor (1 peptides at 0.3 nM, 2 peptides at 0.6 and 1.25 nM, 6 peptides at 2.5 nM and 12 peptides at 5 and 10 nM).

To check the specificity of the method we performed BLIMS on complex mixture. We tested 100 µg and 10 µg of fibroblast lysate containing a GFP-tagged protein. We detected the GFP with 13 peptides in 100 µg and 4 peptides in 10 µg.

Finally, we challenged the BLI-MS workflow with the detection of urinary exosomes in 200 µl of urine using CD9/81/63 antibodies specifically recognizing these vesicles. The BLI signal showed an association, suggesting we captured some exosomes. Moreover, after MS/MS analysis, we were able to detect peptides of CD9.

Conclusions

This work provides the proof of concept on the feasibility of BLI-MS for protein-protein interaction studies. We show that BLI-MS allows for the enrichment of a protein of interest

and its identification in a complex mixture, even at very low concentration (up to 0.3 nM) allowing, potentially, the detection of protein-protein association and identification of the unknown targets in complex matrixes. Furthermore, the method can be of interest in the study of vesicles as we are able to capture some exosomes in an un-diluted biological fluid.

Novel Aspect

We combine BLI technology with MS analysis in order to follow protein-protein interaction and identify the protein partner.

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Keywords

Biolayer Interferometry, BLI-MS

Authors

Jung Vincent [1], Lipecka Joanna [2], Chhuon Cerina [3], Chiara Guerrera [4]

1. Proteomics platform 3P5-Necker, Université Paris Descartes - Structure Fédérative de Recherche Necker (France)
2. Plateforme Protéomique 3p5-Necker, Université Paris Descartes-Structure Fédérative de Recherche Necker, Inserm US24/CNRS UMS3633 (France),
3. INSERM (France)
4. Proteomics platform 3P5-Necker, Université Paris Descartes - Structure Fédérative de Recherche Necker (France)

Implementation and evaluation of a homemadenanoflow interface for capillary electrophoresis-mass spectrometry coupling [OC12]

Anthony LECHNER

Introduction

Since the first successful online coupling of capillary electrophoresis-mass spectrometry (CE-ESI-MS) using sheath flow interface [1], several application, from small molecules to large biomolecules, were reported [2]. However, the constant search for performance in terms of sensitivity and resolution involves the development of new CE-ESI-MS interfaces. Based on the work of the Dovichi's group [3], we implemented a homemade nanospray sheath flow interface at the laboratory and evaluated it.

Methods

Samples were analyzed using a PA800+ CE system (Sciex) coupled to a MicroQTOF (Bruker). CE-ESI-MS interface consisted to insert the separation capillary (70cm x 50 µm i.d.) in a borosilicate glass emitter filled with a sheath liquid. UV detection (214 nm) was implemented to control CE separation. ESI voltage was set at 1.8 kV and separation voltage at 30 kV. Gas supplies were deactivated. BSA and trastuzumab tryptic digest (2 µmol) were analyzed for interface evaluation.

Results

The first step of this study consisted to install CE-ESI-MS interface in the lab. Several parameters, as the diameter of emitter tip, reduction of the outer diameter of the capillary tip and sheath liquid nature were investigated. To assess performances of the interface, UV detection was deported prior to MS. No peak broadening was observed. Although 50 µm emitter tip diameter provided stable spray, only < 10 µm diameter enabled to conserve the top resolution and sensitivity. Concerning the capillary tip, reduction of the outer diameter of 135 µm by an acidic etching, allowed to drastically minimized the dead volume of the emitter tip. Increase of separation resolution using this capillary demonstrated the decrease of diffusion phenomena and then the increase of separation performances. Finally, different sheath liquids were tested based on the literature [4]. MeOH:H2O (50:50 v/v) + 0.5% FA was found to be the best in terms of sensitivity and efficiency. Evaluation of the interface was performed by analyzing 20 fmol of BSA and trastuzumab tryptic digest.

Conclusions

Homemade nanospray sheath flow interface, based on Dovichi's work, was implemented in the laboratory. Several parameters as diameter of the emitter tip and the capillary tip, and sheath liquid composition, were optimized. Analytical performances were evaluated in terms of sensitivity and resolution using a bottom up

approach. Within 20 fmol of protein digest, more than 80% sequence coverage was obtained demonstrating efficiency and robustness of the interface

Novel Aspect

Homemade fabrication of nanoflow interface for CE-MS coupling was demonstrated. Moreover, an additional UV detection was implemented for interface evaluation.

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Keywords

Capillary electrophoresis, CE-ESI-MS coupling, Instrumental development.

Authors

Lechner Antony [1], Sakka Eminé, François Yannis-Nicolas, Leize-Wagner Emmanuelle

1. Laboratoire de Spectrométrie de Masse des Interactions et des Systèmes (France)

Parallel Session 5 : Proteomic Sciences 1

Proteomics for hunting molecular targets to combat biofilms and proteomics again for deciphering associated molecular mechanisms [KL5]

Pascal COSETTE

Pseudomonas aeruginosa (PA) is a Gram-negative bacterium, opportunistic pathogen, involved in a large number of nosocomial infections. This microorganism is also the main infectious agent involved in bronchopulmonary infections in cystic fibrosis patients. This pre-eminence is partly due to the ability of PA to form biofilms, which confers an increased resistance to antibiotics to the bacterial cells.

A first differential proteomic analysis allowed to demonstrate the existence of a specific proteome when the bacterium grows in the biofilm mode, with about 40 proteins, specifically accumulated when bacteria adhere to a surface. Among these proteins, the hypothetical protein PA3731 was particularly investigated. In phenotypic assays, this protein was shown to be involved in biofilm formation, rhamnolipids production, resistance to tobramycin and swarming mobility.

Bioinformatic research showed that the pA3731 gene belongs to a cluster of 4 genes ranging from pA3729 to pA3732 (BAC system), which may act concertedly. This hypothesis was the starting point of a new piece of work. First, by mutating the 4 above mentioned genes, the involvement of the whole BAC system in biofilm formation, antibiotic resistance and rhamnolipid production was established. In order to highlight common traits of these mutations, endo- and exoproteome studies highlighted regulation of proteins associated to antibiotic resistance, to quorum sensing and to type-VI secretion system.

Authors

Saffiedine Brahim, Ben Mlouka Mohamed Amine, Hardouin Julie, Dé Emmanuelle [1], Jouenne Thierry [1], Cosette Pascal [1]

1. CNRS UMR 6270 (France)

Critical role of a phosphorylation in sheath assembly and function of an atypical type VI secretion system [OC13]

Chiara GUERRERA

Introduction

The bacterial pathogen Francisella tularensis possesses a non-canonical type VI secretion system (T6SS) that is required for phagosomal escape in infected macrophages, but the molecular mechanisms triggering T6SS assembly and contraction remain largely unknown. KCl stimulation has been previously used to trigger assembly and secretion of the T6SS in culture. In order to understand the mechanisms underlying this KCl-induced T6SS production, we performed a global proteomic and phosphoproteomic analysis on KCl-induced and non-induced bacteria.

Methods

Proteomics analysis was performed on bacteria stimulated with 5% KCl vs non stimulated. Phosphopepetides were enrichment on titanium dioxide tips (TiO2). All samples were analysed on nanoRSLC-Q Exactive PLUS (RSLC Ultimate 3000, Thermo Scientific, MA, USA). Data were analysed using MaxQuant. Assembled T6SS complex was enriched by optiprep gradient. In vivo and ex vivo validation experiments were performed on mutants ΔiglB, Y139F, Y139A mutants.

Results

By differential proteomics, we found here that the amounts of the T6SS proteins remained unchanged upon KCl stimulation, suggesting involvement of post-translational modifications in T6SS assembly. We hypothesized that protein phosphorylation might occur in Francisella and contribute to the dynamics of T6SS assembly-disassembly. By phosphoproteomic analyses, we identified more than one hundred peptides bearing serine/threonine/or tyrosine phosphorylated residues in the proteome of F. novicida. Of note, we identified a unique phosphorylation Y139 of IglB, a key component of the T6SS sheath. Substitutions of Y139 with alanine or phosphomimetics prevented T6SS formation and abolished phagosomal escape whereas substitution with phenylalanine delayed but did not abolish phagosomal escape in J774-1 macrophages. Altogether our data demonstrated that the Y139 site of IglB plays a critical role in T6SS biogenesis, suggesting that sheath phosphorylation could participate to T6SS dynamics.

Conclusions

In conclusion, our results revealed a critical role of IglB phosphorylation in bacterial survival. Phosphorylation of IglB, a key component of T6SS, plays a critical role in functional T6SS assembly and is necessary for bacterial phagosomal excape.

Novel Aspect

Phosphorylation in bacteria can play a critical role in bacteria phagosome escape.

Keywords

Bacteria, phosphorylation , T6SS assembly

Authors

Chhuon Cerina [1], Jason Ziveri [2], Henri Thomas, Alain Charbit [2], Guerrera Ida Chiara [1]

1. INSERM (France)
2. INSERM U1151 (France)

Functional proteomics of glioblastoma: investigation of tumor initiating cell secretome [OC14]

Serge URBACH

Introduction

In spite of recent advances, glioblastoma remains the most common primary brain tumor in adults with poor prognosis. One major reason for the failure of current therapies is the presence of a small proportion of self-renewing glioma stem-like cells (GSCs), which differentiate into glioma cells (DGCs) constituting the whole tumor mass. In light of previous findings suggesting a role of GSC-secreted factors in tumor formation, the present study was undertaken to characterize the GSC secretome (in comparison with the DGC secretome) to identify new targets for glioblastoma therapy.

Methods

Secretomes from three GSC lines and their corresponding differentiated cells were collected, concentrated by ultrafiltration followed by trichloroacetic acid precipitation and resolved into five fractions by SDS-PAGE. Proteins from each fraction were digested in-gel with trypsin and the resulting peptide samples were analyzed using nano-flow liquid chromatography coupled to a Q-Exactive Plus mass spectrometer.

Results

A total of 3,428 proteins were identified using the Andromeda search engine with a FDR <1%. Label-free quantification using Maxquant (v.1.5.5.1; Cox & Mann, 2008) followed by statistical analyses of data using Perseus (v1.6.1.1; Tyanova et al., 2016) showed a good correlation between protein abundances among the different samples, with a majority of proteins exhibiting similar levels in the supernatant of GSCs and DGCs. Nevertheless, 177 proteins showed significant differences in abundance in GSC versus DGC secretome (GSC/DGC ratios <0.5 or >2 and p values < 0.01), 36 proteins more abundant in GSC secretome and 141 more abundant in DGC secretome. Among those proteins, we initially considered Fibromodulin (FMOD, Mondal et al, 2017), a protein exhibiting higher abundance in DGC secretome and known to promote glioma cell migration. Functional analyses revealed that FMOD is essential for glioblastoma tumor growth and promotes angiogenesis but is not required for sphere formation and differentiation.

Conclusions

We propose a model whereby GSCs differentiate into DGCs that can secrete large amounts of FMOD, which promotes tumor angiogenesis, thus favoring glioblastoma growth.

Novel Aspect

- Deciphering the GSC specific secretome signature.
- Validation and functional characterization of selected molecules from GSC specific secretome signature.

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Keywords

Glioblastoma stem cell, Secretome, Label free

Authors

Urbach Serge [1], Sengupta Shreoshi [2], Decourcelle Mathilde [3], El Koulali Khadija [3], Vigy Oana [1], Séveno Martial [3], Marin Philippe [1], Somasundaram Kumar [2]

1. Institut de Génomique Fonctionnelle (France)
2. Department of Microbiology and Cell Biology, Indian Institute of Science (Inde)
3. BioCampus Montpellier (France)

Dynamic analysis of the VAV1 interactome in primary T cells reveals a crosstalk between the TCR and CD226 [OC15]

Anne GONZALEZ DE PEREDO

Introduction

The T-cell receptor (TCR) pathway is essential for the development and function of T cells, and involves a complex network of signaling cascades, tightly regulated to ensure immune tolerance. Although many proteins involved in TCR signaling have been identified, the general organization of the TCR signalosome and its temporal dynamics remain incompletely understood. Using original affinity purification (AP) methods [1,2], we performed the proteomic characterization of protein complexes dynamically associating around VAV1, a key TCR signaling molecule.

Methods

We specifiaclly used here genetically modified mice that express the VAV1 bait protein bearing a C-terminal One-Strep tag (OST) to purify the protein complexes that assemble around the bait at different time points following activation of primary CD4+ T cells. Quantitative analysis of samples was performed by LC-MS/MS on a LTQ-Orbitrap.

Results

Label-free quantitative comparison of complexes purified from OST mice with appropriate controls prepared from WT mice allowed the characterization of specific binding partners, and the monitoring of their binding kinetics over 600 seconds of TCR activation. Major kinetic clusters contained interactors that bound to VAV1 between 30 s and 2 min, including kinases and adaptor proteins involved in signal initiation and propagation, while late clusters were mainly composed of phosphatases likely involved in the termination of TCR responses. We also detected a very early, transient, and previously unreported interaction with CD226, a costimulatory molecule of immune cells. Further studies indicated that engagement of CD226 induces the tyrosine phosphorylation of VAV1 and synergizes with TCR signals to specifically enhance IL-17 production by human primary CD4 T cells [3].

Conclusions

The combination of mouse genetics with AP-MS provided a detailed picture of the assembly and the internal organization of key molecular complexes formed around VAV1 upon TCR engagement in primary T cells. This study revealed that a VAV1-based, synergistic cross-talk exists between the TCR and CD226 during both physiological and pathological T cell responses.

Novel Aspect

Characterization of endogenous signaling complexes, directly in primary T cells, in a time-resolved manner, provided new insights into T cell signalling.

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Keywords

AP-MS, label-free quantification, TCR signalling

Authors

Gaud Guillaume , Roncagalli Romain , Chaoui Karima , Colacios Céline , Kassem Sahar , Monsarrat Bernard , Burlet-Schiltz Odile , Gonzalez De Peredo Anne [1], Malissen Bernard , Saoudi Abdelhadi

1. Institut de pharmacologie et de biologie structurale (France)

Parallel Session 6 : Structural Characterization

From molecular recognition to cellular function: Pex5p receptor phosphorylation controls peroxisomal matrix protein import [KL6]

Friedel DREPPER

Introduction

Peroxisomes are multi-purpose organelles dynamically adapting their functions to environmental changes and metabolic needs. They rely on the posttranslational import of nuclear-encoded peroxisomal proteins. The cytosolic receptor protein Pex5p recruits matrix proteins carrying a peroxisomal targeting signal (PTS) 1 to the peroxisomal membrane, where it forms a transient import pore together with Pex14p, followed by cargo import and release into the matrix. So far, however, little is known about the regulation of this highly dynamic process.

Methods

Using high-resolution MS, we mapped 22 in vivo phosphorylation sites of Pex5p. Phosphomimicking mutations resulted in decreased import efficiency for the artificial cargo protein GFP-PTS1. Native MS and gas-phase collisional activation experiments were performed to study in detail formation and stability of a receptor-cargo complex in vitro.

Results

As a result of our native MS studies we determined distinct differences in binding affinities between cargo and phosphosite mutant compared to wild-type Pex5p. These results were compared to the dissociation constant of the complex measured by isothermal titration calorimetry and, furthermore, to high-content fluorescence microscopy data to screen for effects on native peroxisomal proteins in cells.

Conclusions

Altogether, our data show that site-specific phosphorylation of Pex5p in its tetratricopeptide repeat domains involved in cargo recognition modulates the binding of individual PTS1 cargo proteins and thereby their import efficiency into peroxisomes to different degree.

Novel Aspect

By combining phosphoproteomics, native MS and biophysical methods we show that phosphorylation of Pex5p precisely modulates peroxisomal matrix protein import.

Keywords

Native MS, collisional activation, protein import

Authors

Fischer Sven [1], Maskit Shiran [2], Buergi Jerome [3], Mastalski Thomas [4], Maier Renate [1], Platta Harald W. [4], Wilmanns Matthias [3], Schuldiner Maya [2], Zalckvar Einat [2], Oeljeklaus Silke [1], Drepper Friedel [5, 1], Warscheid Bettina [5, 1]

1. Institute of Biology, University of Freiburg (Allemagne)
2. Department of Molecular Genetics, Weizmann Institute of Science, Rehovot (Israël)
3. EMBL Hamburg (Allemagne)
4. Institute of Biochemistry and Pathobiochemistry, Biochemistry of Intracellular Transport Mechanism, Ruhr-University Bochum (Allemagne)
5. Signalling Research Centres BIOSS and CIBSS, Freiburg (Allemagne)

Negatively charged liganded gold clusters: where is the charge? Insight from IMS-MS [OC16]

Clothilde COMBY-ZEBRINO

Introduction

Liganded noble metal nanoclusters display interesting physico-chemical properties [1], which make them good candidates for numerous applications, including the design of new imaging probes or therapeutic vectors. However, those properties are highly size-dependent, and a precise characterization of the size and structure of the clusters is mandatory to guide synthesis strategies.

Methods

We use tandem-ion-mobility-spectrometry (IMS) [2] and mass spectrometry (MS) to monitor the diversity of the clusters in terms of composition and structure, as well as their stability. In this work, we used IMS-resolved high-resolution MS measurements to determine how the charge on the cluster is shared by the metal core and the ligands. We studied Au25 clusters stabilized by 18 mercaptobenzoic acid (MBA) ligands in their ortho, meta and para forms [3].

Results

MS measurements in the negative mode show that, while the synthesis yields very pure samples in terms of composition, the detected cluster ions display a mixture of different charging patterns. Namely, for a given charge state, the observed isotope pattern clearly reveals different de-protonation patterns of the ligands co-exist (e.g. [Au25(MBA)18-qH+±m]n-, with -1<=m<=1, and m+q=n). This suggest that the share of the charge supported by the gold core is accordingly different, and then that different oxidation states of the core are observed. Interestingly the relative contribution of these different charging schemes is strongly affected by the substitution of the ligands.

We were then able to partially separate the different species present by high-resolution IMS measurements. Using tandem-IMS we could isolate cluster ions with a pure charge repartition pattern. Our results provide clear evidence that spontaneous electron-detachment may occur in the gas-phase, depending on the charge state and on the nature of the ligand.

Conclusions

Our results show that high-resolution IMS-MS measurements allow detailed characterization of the charge repartition on liganded clusters, as well as separation and isolation of the different species in mixture. Such level of characterization is mandatory, since the properties of the core are highly affected by its charge. In this context, the observation of spontaneous electron detachment species is of central importance. Indeed, the existence of such a phenomenon may dramatically affect the interpretation of MS data in terms of the oxidation state of the clusters in solution.

Novel Aspect

High resolution IMS-MS is an emerging characterization tool in the field of nanoclusters [3, 4], and it is the first time that electron-detachment has been observed by tandem-IMS. Moreover, such separation of species with different charging patterns in a mixture has never been reported to our knowledge.

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Keywords

IMS, gold clusters, electron detachment

Authors

Comby-Zerbino Clothilde [1], Bertorelle Franck [1], Dugourd Philippe [1], Chirot Fabien [2], Antoine Rodolphe [1]

1. Univ Lyon, Université Claude Bernard Lyon 1, CNRS, Institut Lumière Matière, F-69622, LYON, France (France)
2. Univ Lyon, Université Claude Bernard Lyon 1, CNRS, Ens de Lyon, Institut des Sciences Analytiques, F-69100, VILLEURBANNE, France (France)

Distribution study of paracetamol and its metabolites in rat whole body after on-tissue chemical derivatization by MALDI Imaging Mass Spectrometry [OC17]

Mira MERDAS

Introduction

MALDI Imaging Mass Spectrometry (IMS) is increasingly used to study the tissue distribution of small exogenous molecules. However, the lack of sensitivity often encountered remains a strong limitation of the technology and novel procedures must be developed to enhance that sensitivity. Thus on-tissue chemical derivatization (OTCD) [1] is a promising technique to improve the ionization of targeted chemical functions. Here, we developed an OTCD of phenol function present on paracetamol and some of its metabolites.

Methods

We used 2-fluoro-1-methyl pyridinium p-toluene sulfonate (FMPTS) [2] as a derivatization reagent on whole body tissue sections of rats treated with 300 mg/kg of paracetamol and scarified one hour after intravenous administration. Signal intensity of derivatized and underivatized compounds was measured in positive and negative ionization modes. Deposition of concentration ranges of standards allowed the quantification of analytes in various organs.

Results

The first tests performed on standards showed that derivatization by FMPTS strongly improves the detection of paracetamol and both its cysteine and mercapturate metabolites in positive ionization mode. Interestingly, derivatization is not required for the detection of the sulfate and glucuronide conjugates of paracetamol that are well detected in negative ionization mode. These conditions were then applied on whole body tissue sections prepared from treated rats. Derivatized paracetamol was detected in all organs. However derivatized cysteine and mercapturate metabolites were essentially detected in kidney and guts, whereas sulfate and glucuronide conjugates presented a different distribution. The analytes were further quantified in various organs by MALDI imaging using a method that considers the variation of signal extinction between organs. Inter- and intra-reproducibilities were evaluated by applying the same methodology on three biological replicates.

Conclusions

OTCD appeared to be indispensable to the detection of paracetamol and its cysteine and mercapturate metabolites in whole-body rat sections by MALDI imaging. We were thus able to study the distribution of the paracetamol and its metabolites and quantify them by MALDI IMS.

Novel Aspect

Development of new method to enhance the detection sensitivity of various molecules by MALDI IMS provides complementary information that is relevant in drug discovery.

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Keywords

On-tissue chemical derivatization, MALDI, imaging mass spectrometry

Authors

Merdas Mira [1, 2], Lhumeau Antoine [1], Vanbellingen Quentin , Lagarrigue Mélanie , Umbdenstock Thierry , Da Violante Georges , Pineau Charles

1. Technologie Servier (France)
2. Protim (France)

Glycoproteomic study of Saccharomyces cerevisiae yeast cell wall mannoproteins [OC18]

Marie YAMMINE

Introduction

Yeast cell wall (YCW) is composed of an inner polysaccharide layer to which are bound mannoproteins that form the outer layer and are the second most abundant component of YCW (40%, w/w). These mannoproteins show particular functional properties related to their molecular structure [1], but they have been little investigated. This work aims to study the structure of YCW mannoproteins.

Methods

SDS-extracted YCW obtained by mechanical disruption of yeast samples [2] was O- and N-deglycosylated using NH4OH and PNGase F activity respectively, adapted to eFASP method [3]. Proteins were identified by nanoESI-LC-MS/MS using proteome discoverer 2.2 to query against SGD (Saccharomyces cerevisiae S288C strain). In addition, Uniprot was used to determine proteins subcellular localization. O-glycans were detected by LC-MS and FT-ICR MS after chemical derivatization with 4-aminobenzoic acid butyl ester (ABBE).

Results

The proteomic study performed after deglycosylation showed an increase in the number of identified mannoproteins compared to that performed without deglycosylation (46 vs 27 covalently bound YCW mannoproteins). This work allowed us to identify 46 YCW covalently-bound mannoproteins among 1635 identified proteins that are majorly annotated as membrane localized proteins, thus proving the reliability of our method of extraction. The isolated O-glycans were labeled by aminative reduction reaction with ABBE were detected by mass spectrometry, whether by nanoESI-LC-MS/MS coupled to a nanoflow HPLC U3000 RSLC instrument or by FT-ICR MS in direct infusion.

Conclusions

This work has allowed us to prove the reliability of the YCW extraction method. In addition, the deglycosylation protocol adapted to the eFASP method has shown its advantage concerning increasing the number of identified mannoproteins, while isolating the O- and the N-glycans simultaneously. We were able to detect isolated O-glycans after labeling with ABBE by mass spectrometry proving the efficiency of the deglycosylation protocol.

Novel Aspect

This work is considered to be the first describing a glycoproteomic methodology using deglycosylation protocol adapted to an eFASP method applied on extracted YCW.

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Keywords

Saccharomyces cerevisiae Cell Wall, mannoproteins, mass spectrometry

Authors

Yammine Marie [1], Bray Fabrice [1], Socolsky Cecilia [2], Mouly Isabelle [2], Rolando Christian [1]

1. Université de Lille, USR 3290 – MSAP – Miniaturisation pour la Synthèse, l'Analyse et la Protéomique, 59000 Lille, France. (France)
2. Lesaffre International, 137 rue Gabriel Péri, 59700 Marcq-en-Baroeul, France (France)

Parallel Session 7 : Structural Biology

Study of the largest and most heterogeneous macromolecular complex by HDX-MS, bringing new important mechanistic insights in proteasome regulation [KL7]

Julien MARCOUX

Introduction

The 20S proteasome is a multi-catalytic protease composed of 4 heptameric rings. It degrades proteins in a controlled fashion, thereby directly regulating intracellular concentration of cytokines and hub proteins. Alteration of its activity can lead to pathologies including cancers, heart and auto-inflammatory diseases. Its activity can be regulated by replacing its constitutive catalytic subunits and/or by interacting with different activators. However, whether its catalytic subunit composition favors the interaction with a particular regulator is still unclear.

Methods

Here, we utilized Hydrogen-Deuterium eXchange coupled to Mass Spectrometry (HDX-MS) to investigate the impact of the catalytic subunit composition of the 20S proteasome on its structure and association to specific activators. Human standard (std20S) and immuno (i20S) proteasomes were deuterated alone of after incubation with the PA28αβ or PA28γ activators. Samples were analyzed on a commercial Waters setup (Twin HTS PAL coupled to SynaptG2Si).

Results

We successfully optimized the classical HDX-MS workflow in terms of sample preparation, chromatography and MS acquisition to work on both poorly concentrated and very heterogeneous protein complexes. We developed a web application called HDX-Viewer [1] to instantly visualize the raw data of these large complexes, directly from DynamX outputs. Deuteration rates of the three PA28 monomers (forming the PA28αβ or PA28γ heptamers) indicated both common features suggesting a similar mode of activation but also local discrepancies.The std20S and i20S clearly showed a faster deuteration on the solvent-exposed surface of the α-ring compared any other ring-interface. Furthermore, we identified flexible regions that are available for interaction with the ~200 Proteasome-Interacting-Proteins described so far. Comparison of the std20S Vs. i20S deuteration highlight subtle but meaningful discrepancies. Similarly, binding of the PA28 regulators influences the deuteration of the 20S proteasomes.

Conclusions

Deuteration of the std20S Vs. i20S suggests a first "inner to outer ring allosteric change". Changes in the inner β-rings upon regulator binding were interpreted as an "outer to inner ring allosteric change". Altogether, the α-ring region that was more dynamic in the std20S Vs. i20S was also the most protected by activators. Our results thus highlight how the

incorporation of different catalytic subunits can alter the proteasome affinity to different regulators.

Novel Aspect

Study of the largest macromolecular complex by HDX-MS, representing a methodological breakthrough and bringing invaluable insights in the 20S proteasome regulation.

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Keywords

Hydrogen-Deuterium eXchange MS, Structural MS, Proteasome

Authors

Lesne Jean [1, 2], Parra Julien [1], Zivkovic Dusan [1], Menneteau Thomas [1, 3], Chavent Matthieu [1], Locard-Paulet Marie [1, 4], Bousquet Marie-Pierre [1], Schiltz Odile [1], Marcoux Julien [1]

1. Institut de pharmacologie et de biologie structurale (France)
2. Centre de Biochimie Structurale [Montpellier] (France)
3. University College of London [London] (Royaume-Uni)
4. Center for Protein Research, Copenhagen (Danemark)

Structure of Aβ42 pore-forming oligomers [OC19]

Eduard PUIG

Introduction

Alzheimer's disease (AD) is the most common form of dementia. It was first described in 1906 by Alois Alzheimer. Later on, in 1984 George Glenner and Colin Masters isolated the amyloid-beta (Aβ) peptide from a human brain and associated it to the disease. Since then the amyloid hypothesis has been a rather controversial matter due to the variety of aggregation forms that Aβ can adopt, thus turning it into a highly complex target. Therefore, understanding the links between protein aggregation and neurotoxicity, and specially obtaining the 3D structures of the aggregates responsible for neurotoxicity is key to design effective diagnostic and therapeutic strategies. Unfortunately, this remains one of the most important unresolved issues in the field.

The group of Dr. Carulla has been working on the hypothesis that Aβ interacts with the cell membrane leading to ionic dyshomeostasis. In order to study this scenario, the group has changed the paradigm and treated Aβ as a membrane protein and applied well known methodologies used to characterize this family of proteins to study Aβ. By doing so, the group has been able to prove that Aβ is able to form a type of oligomer in the presence of detergent micelles which adopts a very specific and defined structure with characteristics of a β-barrel assembly and functions as a pore. They refer to these type of oligomer as Pore-Forming β-Barrel Oligomer (PFβO). This work was recently published in PNAS [1] and the group is currently working towards its 3D structure.

Methods

As a starting point, we have used detergents to study the oligomerization process in a membrane mimetic environment. Micelles compared to other more native-like biomimetics environments based on lipids, will enable the application of novel mass spectrometry (MS) strategies [2] and well-established solution NMR techniques [3] thus providing high-resolution structural information. The group of Dr. Carulla identified the micelle to Aβ ratio ([Aβ]:[M]) to be an important factor in the oligomerization process of Aβ. Throughout the work done we have optimized not only the ratio but also other conditions such as the buffer and the pH to modulate the preparation of samples enriched in defined oligomer populations. To go further into structural detail, we have been setting up an ion mobility mass spectrometry (IM-MS) protocol in order to establish the stoichiometry of the oligomers. Unfortunately, only non-ionic detergents can be used in MS analysis this is why we performed and extensive screening to transfer the oligomers from DPC to MS friendly detergents. More precisely, we have used size-exclusion chromatography (SEC) coupled to IM-MS. This strategy has allowed us to establish the stoichiometry of distinct oligomer species present in the sample as a function of their elution time through a SEC column. We have also used cross-linking MS strategies to confirm the intact mass of these oligomers and to obtain structural restraints. Altogether these strategies have contributed to the 3D structural characterization of βPFO-type oligomers.

Results

Before starting our collaboration with Dr. Sarah Cianferani's group, we had analysed the samples by previously buffer exchanging them to MS-compatible buffer solution before the MS analysis. Thanks to this collaboration we implemented a new approach based on coupling size exclusion chromatography (SEC) directly to a Synapt G2 [4]. This approach allowed us to establish that higher molecular weight oligomers are better preserved and therefore better detected as we increase the signal to noise ratio. This gave us the opportunity to study different points of the SEC chromatogram and therefore understand better the composition of our samples and our system. For the standard βPFO samples, we have been able to obtain specific charge states for the octamer and tetramer species.

In parallel to complement the native-MS results, we have also worked with Dr. Stéphane Chaignepain from CBMN in Bordeaux to develop a method to analyse chemically cross-linked βPFOs by MALDI-TOF. After a process of trials and optimizations we established a zero-length cross-linking conditions which allowed us to cross-link the βPFOs and detect again tetramer and octamer such as in the native-MS approach. As it will be shown in the presentation, this approach has been extended to another βPFO preparation.

Proteomic analysis of cross-linked peptides enabled us to establish that βPFO cross-links through the N-ter flexible ends. Moreover we were able to build potential octamer models to be further validated with the IMS data. For this we calculated the theoretical collision cross-section (CCS) from the models and compared it to the experimental CCS [5]. This strategy led us to confirm that the tetramer's flexible parts collapsed into the β-sheet in the gas phase. In the case of the octamer we observed that two of these sheets collapsed into a β-sandwich.

Conclusions

- We found that increasing the [Aβ42]:[DPCm] ratio led to the formation of larger βPFO oligomers, thus referring to the two preparations as βPFOsmall and βPFOlarge.
- Solution NMR experiments indicated that βPFOsmall and βPFOlarge share similar structural features due to their transient same fingerprint and the two defined environments for Met-35 side-chain.
- Interconversion between βPFOsmall and βPFOlarge suggests a plausible mechanism of evolution in size that is correlated by an increase in the Aβ concentration.
- Electrical recordings in planar lipid bilayers showed that βPFOlarge formed well-defined type of pore with less current fluctuations than the ones formed by βPFOsmall.
- The selected MS-compatible detergents preserved the oligomerization state of βPFOsmall and allowed the optimal transmission of oligomer ions in the gas phase.
- Stoichiometry determination showed that βPFOsmall is mainly enriched in tetramers and βPFOlarge is mainly enriched in octamers. Moreover, this result points at tetramers as the building block for βPFO formation.
- Tetramers and octamers showed high kinetic stability to gas phase activation. More concretely, results indicated that the tetramer broke through a process of asymmetric dissociation into trimers and highly-charged monomers.
- Zero-length cross-linking with subsequent analysis by either SDS-PAGE or high-mass MALDI-MS indicated as native MS, that βPFOsmall is mainly enriched in tetramers and βPFOlarge is mainly enriched in octamers.
- Proteomic analysis of the zero-length cross-linked peptides indicated that the stabilization is achieved through the flexible ends of βPFO.

- Modelling of the Aβ42 tetramer structure guided by collision cross-sections derived from IMS indicated the collapse of the flexible ends into the hydrophobic core of the β-sheet. As for the octamer, results suggested the collapse of two tetramers with the flexible ends collapsed into the β-sheets.

Novel Aspect

We report the firs 3D structure obtained by NMR of a membrane-associated amyloid beta oligomer. Moreover, we support the structure with IM-MS data defining the stoichiometry of the oligomers formed by this peptide. We implement a new methodology to in-line detergent-exchange samples for native-MS studies and report a significant increase in the signal-to-noise ratio. Finally we also show that these type of oligomers incorporate into lipid-bilayers forming pores, thus we establish a potential link of these species with the amyloid pore hypothesis. This hypothesis suggests the membrane disruption as a potential mechanism of Alzheimer's disease.

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Keywords

Structure, Amyloid-beta, native-MS

Authors

Puig Eduard [1], Botzanowski Thomas [2], Ciudad Sonia , Chaignepain Stéphane [3], Cianferani Sarah [2], Carulla Natàlia [3]

1. Institute for Research in Biomedicine, IRB Barcelona, Spain (Espagne)
2. Institut Pluridisciplinaire Hubert Curien (France)
3. Chimie et Biologie des Membranes et des Nanoobjets (France)

Benzophenone photoreactivity in a lipid bilayer to probe cell-penetrating peptides/lipid membrane interactions by affinity photocrosslinking coupled to mass spectrometry [OC20]

Leïla BECHTELLA

Introduction

Cell-penetrating peptides (CPP) can cross cell membranes and deliver biologically active molecules into cells. Understanding their internalization mechanisms is of first interest for an appropriate use in the medical field or for biotechnological applications. Model membranes are useful systems to study interactions between membrane-active peptides and lipids. Affinity photocrosslinking coupled to mass spectrometry, using benzophenone (Bzp)-functionalized peptides, was used to study the non-covalent interactions of CPPs and lipid membranes at a molecular level [1].

Methods

Upon irradiation, a peptide functionalized with a Bzp photoprobe can be covalently bound to lipid fatty acid chains in its immediate environment [2]. The method was optimized using various phospholipids, saturated or unsaturated: DMPG (14:0), DPPG (16:0), DOPG (18:1 cis $\Delta 9$), 18:1 (trans $\Delta 9$) PG and DLoPG (18:2 cis $\Delta 9$, 12). We significantly increase photocrosslinking yields, ions production and detection, resulting in high signal-to-noise (S/N) MS spectra.

Results

We observed all the classical and less common Bzp reactivities described in the literature by direct MS analysis: C=C double bond formation on saturated fatty acids, covalent adducts formation via classical C-C bond and Paternò-Büchi (P-B) oxetane formation followed or not by fragmentation (retro-P-B) and photosensitisation of unsaturated lipids leading to lipid dimers. We revealed highly informative secondary reactions occurring during UV irradiation, whose products were unequivocally identified by MALDI-TOF and ESI-Orbitrap MS and MALDI-TOF/TOF MS/MS. All these reactions can occur concomitantly in a single biological system: a membrane-active peptide inserted within a phospholipid bilayer. Careful analysis of the MS spectra allowed to characterise the position of insertion of the CPP on the fatty acyl chain without the need of MS/MS. These results confirm that these CPPs favour interaction with fluid disordered regions of the membrane and do not insert deeply [3].

Conclusions

This work shows how to exploit in an original way the different reactivities of Bzp in the context of a lipid membrane. We propose an analytical workflow for the interpretation of MS spectra, giving access to information on the CPP/lipid interaction at a molecular level such as depth of insertion or membrane fluidity in the CPP vicinity. An application of this workflow illustrates the role of cholesterol in the CPP/lipids interaction.

Novel Aspect

This work represents a noteworthy improvement for the characterisation of interacting partners such as CPPs and membrane lipids using Bzp photocrosslinking.

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Keywords

Benzophenone photoreactivity, Lipid membrane, Cell-penetrating peptides

Authors

Bechtella Leïla [1], Kirschbaum Carla [1], Cosset Marine [1], Clodic Gilles [2], Matheron Lucrèce [2], Bolbach Gérard [1], Sagan Sandrine [1], Walrant Astrid [1], Sachon Emmanuelle [1, 2]

1. Laboratoire des biomolécules (LBM) (France)
2. Plateforme de spectrométrie de masse et protéomique IBPS (France)

An innovative in vivo cross-linking mass spectrometry workflow for proteome-wide studies [OC21]

Martial REY

Introduction

Cross-linking (XL) coupled to mass spectrometry (MS) is being increasingly used for in vivo studies [1, 2]. However multiple challenges have to be faced, especially for cross-linked peptide enrichment and data processing. Here we present a new pipeline for in-vivo XL-MS based on a trifunctional cross-linker (NNP9) [3, 4] allowing a single step purification of cross-linked peptides via click-chemistry and data processing with a high-end algorithm (MassSpec Studio) [5]. Applied to Neisseria meningitidis, our workflow leads to thousands of cross-linked peptides in a single LC-MS/MS run.

Methods

We labeled 1010 bacteria with 600 nmoles of NNP9 for 3 hours. Proteins were extracted with 8 M urea and washed on a filter. After trypsin digestion, labeled peptides were covalently bound on agarose beads via click-chemistry. After extensive washing, labeled peptides were released upon UV exposure.

The enriched fraction was analyzed by nanoLC-MS/MS on Q-Exactive Orbitrap. Data were processed with MassSpec Studio using Neisseria meningitidis protein database.

Results

To set-up our in-vivo XL-MS workflow, we first developed an enrichment protocol for cross-linked peptides using click chemistry on photo-cleavable beads to ensure specificity and efficiency. After optimization, we achieved more than 95 % efficiency for the binding/release steps of modified peptides.

After optimization of the HCD parameters (intensity threshold, AGC and NCE), we could identify 6,200 labeled peptides, including 2,200 cross-linked ones in a single 4-hour LC-MS/MS run. Only 80 non-modified peptides (1.2 % of the total identification) were obtained showing the great efficiency of our single step purification workflow. We also checked the quality of the cross-linked peptides identified: 95% of the 250 ribosomal cross-links obtained were found to be shorter than 30 Å, which is in perfect agreement with the size of our cross-linking reagent.

Conclusions

We combined a powerful one-step purification based on click-chemistry with new search engine to create a simple in vivo XL-MS workflow for proteome-wide interactome studies. We could identify 2,200 cross-linked peptides in a single run. The distance restrains of known large protein complexes perfectly agree with the length of the cross-linker, validating the quality of the data.

Our new approach represents a huge step forward in the field of XL-MS and interactomics.

Novel Aspect

We present a new integrated in-vivo XL MS workflow based on single step purification and identification only based on MS² spectra by high-end dedicated search engine.

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Keywords

Proteome-wide interactomics, Cross-linking mass spectrometry, Click-chemistry

Authors

Rey Martial [1], Dhenin Jonathan [1], Kong Youxin [2], Dupré Mathieu [1], Duménil Guillaume [2], Chamot-Rooke Julia [1]

1. Unité Spectrométrie de Masse pour la Biologie, Institut Pasteur, CNRS USR2000, Paris, France (France)
2. Pathogenèse des infections vasculaires, Institut Pasteur, INSERM – ERL1225 , Paris, France (France)

Parallel Session 8 : Geochemistry / Environment / Polymers

Laser Desorption Ionisation coupled to FTICR for analysis of whole soils [KL8]

William KEW

Natural organic matter (NOM) is a diverse and highly complex mixture, playing an important role in numerous environmental processes including carbon cycling. The analysis of NOM by high resolution mass spectrometry is key to understanding the chemistry and functions of this mixture. Routinely, analysis is performed on sample extracts as the ionization source of choice is electrospray ionization (ESI). However, extractions are biased, and ESI mass spectra of extracts may not be fully representative of soils. Here, we present Laser Desorption Ionization for analysis of whole soils.

Data were acquired on a Bruker Solarix FTICR MS equipped with 15 Tesla magnet. For ESI and atmospheric pressure photoionization (APPI), soil samples were extracted with either water, methanol, or sodium hydroxide and cleaned up via solid phase extraction. For LDI, samples were affixed to a polished steel target with copper tape or spotted directly (for extracts). Ionization sources used were those commercially available from Bruker, with the laser operating at 355 nm.

LDI spectra of reference extract samples show the commonly observed normal distribution of masses with multiple peaks at each nominal mass, and thousands of peaks detected across the mass range m/z 200 to 1000. Analysis of whole soils with varying levels of total organic carbon (TOC) shows a diverse range of responses, with low carbon soils producing spectra with correspondingly low signal. Mineral or species, with mass defects from .7 to .95 were observed, especially in samples with lower carbon. In both positive and negative modes, ions were produced singly charged as a mixture of (de)protonated ions, radical ions, and adducts (sodium, potassium, or chloride). When compared to the ESI and APPI spectra of the soil extracts, LDI produced more complex spectra, with formula assignment made more complex. The types of compounds ionized by the three methods also was diverse, with LDI ionizing more condensed aromatic type structures than observed with ESI.

Analysis of complex natural mixtures by just one method only shows a narrow aspect of the chemistry involved. To more completely characterize these environmental samples, a range of sample preparation and analysis techniques are required. Here, LDI expands the available toolbox for NOM analysis by allowing high resolution mass spectrometry to be performed on whole soils, without pretreatment or extraction.

Comprehensive analysis of LDI FTICR MS for analysis of a range of whole soils and compared to solution state methods.

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Keywords

Complex mixtures, FTICR, environmental chemistry

Authors

Kew William [1], Chu Rosalie [1], Paša-Tolić Ljiljana [1]

1. Pacific Northwest National Laboratory (États-Unis)

Robust proteomics workflow for the high throughput identification and classification by MALDI FT-ICR MS of paleontological bones indiscernible by their anatomy [OC22]

Fabrice BRAY

Introduction

Proteomics has become attractive for fossil analysis and has become an alternative to DNA analysis which is limited by DNA amplification present in ancient samples and its contamination [1,2]. A limiting point for the analysis of fossils is the use a low quantity of material to avoid damaging the samples. The analysis of extinct species and non DNA sequencing leads to difficulties for the identification of species. The development of a proteomic workflow to answer the questions of the identification of taxa with extremely small quantities and the extinct species is a challenge [3].

Methods

Our corpus consists of bones from Scladina (150,000 BC, Ardenne, Belgium). One milligramme of bones was demineralized with TFA solution. Insoluble bone powder and acid fraction were digested by eFASP method. Then digested peptides were analyzed by MALDI FT-ICR on a Bruker SolariX XR 9.4 Tesla and nanoLC MS/MS Q-Exactive. Raw data from MALDI FT-ICR were analyzed with an in-house software which classified samples by origins by comparing them to samples of known species.

Results

The comparison of MALDI FT-ICR spectra of fossils and know samples allowed to classify samples by similarity. The classification by similarity of spectra allow to validate the paleoanthropological identification and facilitate the identification. This information facilitates the interrogation of LC-MSMS data against targeted databases. Proteomics approach show for all samples and fractions that the majority of detected peptides match on collagen I alpha 1 and collagen I alpha 2. The average coverage of collagen I alpha 1 and collagen I alpha 2 are 64%. The identified genus taxa correspond to the anatomical analysis realized by paleontologist. For for samples, Panthera tigris altaica are identified as specific species. The alignments of the collagen I alpha 1 and I alpha 2 sequences of the species Felis catus, Panthera pardus, Panthera tigris and Acinonyx jubatus (Feline species present in the NCBi database) show a high similarity. PEAKS analysis identify amino acids substitution on Panthera tigris altaica.

Conclusions

These results show that the amino acids substitution are different in function of geological position of the bones from the same species and a possible evolution of the collagen sequence. This new methodology allows an analysis of fossil bones by obtaining information on the collagen sequence, the modifications and the substitutions of the amino acids. The classification of the MALDI spectra makes it possible to group the samples by similarity and as well as targeting databases for proteomics analysis by LC-MSMS.

Novel Aspect

Proteomics, evolution, classification without database, amino acids substitution, high resolution MALDI FT-ICR MS

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Keywords

Paleontology, collagen, MALDI FT-ICR MS

Authors

Bray Fabrice [1, 2], Flament Stéphanie [1], Auguste Patrick [3], Rolando Christian [4, 1]

1. Miniaturisation pour la Synthèse, l'Analyse et la Protéomique - USR 3290 (France)
2. Miniaturisation pour la Synthèse et l'Analyse Protéomique (France)
3. UMR 8198 Evolution, Ecologie et Paléontologie CNRS - Université de Lille (France)
4. USR 3290 MSAP, Université Lille 1, Sciences et Technologies (France)

Non-targeted analysis applied to archaeological samples for a fast composition overview [OC23]

Jasmine HERTZOG

Composition characterization of archaeological sample is essential to increase our knowledge on ancient civilisations regarding their cultural habits, the contemporary trade routes allowing the importation of specific materials or the status of the buried person. One of the preferred method for the analysis of these samples is gas chromatography coupled to mass spectrometry (GC-MS). It ensures to evidence organic components such as pine tree resins, beeswax, or vegetal oils. In this study, a sample from a quite late 8th century BC, burial context from Cumae (Naples, Italy), was analysed by non-targeted electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry, in negative-ion mode (ESI FT-ICR MS). This approach enables to obtain the global sample composition description but also to made hypotheses based on the achieved raw formulae. Eventually, targeted GC-MS analysis was performed to identify compounds involved in the sample and therefore to confirm or invalidate previous hypotheses.

For FT-ICR MS analysis, the sample was crushed, dissolved and diluted in methanol. The achieved solution was directly infused in the mass spectrometer. For GC-MS analysis, a common sample preparation was performed, which consists in trimethysilylation derivatization followed by dissolution in dichloromethane, prior injection over the GC non-polar column [1].

The high-resolution of the FT-ICR mass spectrometer allows obtaining mass spectra of thousands features. In addition, the high mass accuracy provided by this instrument (mass error < 1 ppm) ensures to assign a unique CcHhNnOoS molecular formula to each m/z signal. To have a global view of the sample composition, the thousands assignments were plotted according to their hydrogen-to-carbon vs. oxygen-to-carbon ratios to generate a van Krevelen diagram [2]. Depending on the plot location, it is possible to distinguish fatty acids, terpenoids or sugars. Furthermore, CcHhNnOoSs assignments were compared to raw formulae of known archaeological biomarkers for possible matching [3,4]. Thus, several matches were obtained with archaeo-biomarkers of pine resin (abietic acid derivates), birchbark (betulin derivates), sandarac resin (pimaric acids), and fatty acids. As no structural information is provided by FT-ICR MS, only hypotheses can be made. Therefore, GC-MS was employed to verify these latter suggestions and it confirmed the presence of the previous components and beeswax and vegetal oil.

FT-ICR MS can be regarded as a powerful tool for the rapid and global description of archaeological samples. Moreover, both sample preparation and assignment process are not tedious. If FT-ICR MS analysis reveals interesting hypotheses for the scientific and archaeological communities, then GC-MS analysis can be carried out to confirm them.

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Keywords

Archaeometry, natural substances, Fourier transform ion cyclotron resonance mass spectrometry.

Authors

Hertzog Jasmine [1, 2], Fujii Hitomi [3], Babbi Andrea [4], Lattuati-Derieux Agnès [3], Schmitt-Kopplin Philippe [1, 2]

1. Analytical Food Chemistry, Technische Universität Muenchen, 85354 Freising, Germany (Allemagne)
2. Analytical BioGeoChemistry, Helmholtz Zentrum Muenchen, 85764 Neuherberg, Germany (Allemagne)
3. Centre de Recherche et de Restauration des Musées de France, Research Department, Palais du Louvre, 75001 Paris, France (France)
4. Graduiertenkolleg 1876, Johannes Gutenberg-Universität Mainz, 55122 Mainz, Germany (Allemagne)

Analysis of bitumen by high performance thin layer chromatography coupled to laser desorption ionization Fourier transform ion cyclotron resonance mass spectrometry [OC24]

Oscar LACROIX

Introduction

Bitumens are highly complex mixtures generally obtained as a residue of petroleum distillation [1]. The different compounds of bitumens can be separated by the saturates, aromatics, resins, and asphaltenes (SARA) fractionation, which is based on polarity and solubility of the compounds [2]. The aim of this study was to develop and optimize a direct coupling between HPTLC and laser desorption ionization (LDI) Fourier transform ion cyclotron resonance mass spectrometry (FTICR) for the characterization of bitumen.

Methods

Bitumen samples were solubilized in methylene chloride at a concentration of 10 mg.mL-1. 1µL was applied on HPTLC plate of cellulose using an automatic TLC sampler 4. A mixture of heptane and ethanol 80/20 (v/v) was used as elution solvent. Mass spectrometry experiments were performed on a FTICR Solarix 12T instrument equipped with a laser desorption ionization source. The data were internally calibrated and processed with DataAnalysis and OriginPro softwares.

Results

Initial comparison of silica and cellulose stationary phases have shown that a much higher signal was obtained with cellulose. Several solvents were tested on maltene and asphaltene fractions showing that elution of the maltenes was obtained with heptane/ethanol 80:20. Under this conditions from bitumen samples, two zones were separated on the HPTLC plate. The HPTLC plate was directly analyzed by LDI-FTICR. The separation of the two fractions was underlined by the formation of fullerene ions on the non-eluted zone (RF=0) due to the presence of asphaltenes [3]. Furthermore, compound classes N2, N2O1, N2S1, and N1S2 are predominant in this zone. Ionic interactions occurred between heteroatoms present in the most polar molecules and the hydroxyl groups of the cellulose. On the eluted zone (RF=1), compound classes CH, S1, S2, O1, O2, and O1S1 were mostly found. These compounds had poor interactions with cellulose and good affinity for the solvent mixture. DBE vs #C plots highlighted that the compounds with higher DBE values were on the non-eluted zone.

Conclusion

We demonstrated that silica stationary phase, which is widely used for heavy crude oil separation, is not adapted for online coupling with mass spectrometry. This mineral phase has too strong interaction with molecules containing polar substituents, preventing desorption by the LDI source. Here we presented a method with organic phase of cellulose which allowed to characterize two fractions of bitumen using online coupling with LDI-FTICR.

Novel aspect

HPTLC with a stationary phase of cellulose coupled with LDI-FTICR is a new and original approach to characterize fractions of bitumen.

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Keywords

HPTLC, FTMS, Bitumen

Authors

Lacroix-Andrivet Oscar [1], Hubert Roux Marie [1], Mendes Siqueira Anna Luiza [2], Bai Yang [2], Afonso Carlos [1]

1. Chimie Organique et Bioorganique : Reactivité et Analyse (France)
2. TOTAL, Centre de recherche de Solaize (France)

Parallel Session 9 : Proteomic Sciences 2

Quantitative determination of Staphylococcus aureus enterotoxins in complex food matrices by a multiplex immunocapture mass spectrometry [KL9]

Donatien LEFEBVRE

Introduction

Staphylococcal food poisoning outbreaks (SFPO) are caused by the ingestion of food contaminated with staphylococcal enterotoxins (SEs) produced by strains of Staphylococcus. To date, 27 SEs are described in the literature but only 5 of them, can be routinely detectable via commercially available immunoassays. Proteomic tools for quantification and confirmation of a wide range of toxins are crucial in a context of public health. In this project, we propose to develop an immunocapture mass spectrometry (MS) approach for the detection and quantification of SEs in food matrices.

Methods

Samples from SFPO were incubated with magnetic beads coated with toxin-specific antibodies. After toxin extraction, on-bead trypsin digestion was performed and recovered peptides were analyzed by LC-MS on a Q-Exactive mass spectrometer. Peptide separation was achieved by UHPLC in 20 minutes on a C18-300A column. Using the parallel reaction monitoring (PRM) mode, 35 peptides from 8 distinct SEs were monitored to ensure unambiguous detection and quantification.

Results

During the investigation of many SFPO outbreaks and despite clear symptoms of SE intoxication, it is quite usual not to find any of the five most common SEs. Furthermore, the presence of strains containing genes encoding other SEs has been demonstrated by various genomic tools in such kind of food products. We have therefore developed a sensitive and specific approach for the detection of 5 usual SEs and 3 additional SEs in milk samples thanks to multiplex immunocapture [1], this method is based on selective capture by antibodies and targeted high resolution MS/MS. The method is based on selective capture by antibodies. A total of 36 peptides were selected for the quantification, with 3-5 peptides/toxin. Isotopically labeled peptides were used as internal standards for improved detection reproducibility and accurate quantification. Preliminary results indicated a limit of detection at 0.1 ng / mL in milk, which is in perfect agreement with toxic levels (20-1000 ng / person).

Conclusions

Preliminary results demonstrated that the implemented multiplex immuno-MS method enables the robust, specific, and sensitive of 8 SEs in milk samples. The implemented approach is now being applied to other complex food matrices (cheese, meat...). In parallel, a complementary antibody-free assay is also evaluated as an alternative for the detection and quantification of SEs for which no specific antibodies are available.

Novel Aspect

We report the first MS-based approach enabling the simultaneous detection and quantification of 8 SEs in the low ng/mL range in complex food matrices.

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Keywords

Staphylococcal Enterotoxins, MS-Multiplex, Immunocapture

Authors

Lefebvre Donatien [1], Blanco Valle Kevin [2], Feraudet Tarisse Cécile [3], Merda Déborah [2], Vingadassalon Noémie [2], Simon Stéphanie [3], Fenaille François [1], Hennekinne Jacques-Antoine [2], Nia Yacine [2], Becher François [1]

1. CEA, DRF, Service de Pharmacologie et d'Immunoanalyse, Laboratoire d'Etude du Métabolisme des Médicaments, Gif-sur-Yvette, France. (France)
2. Laboratory for food safety, ANSES, Université Paris-Est, Maisons-Alfort F-94700, France (France)
3. CEA, DRF, Service de Pharmacologie et d'Immunoanalyse, Laboratoire d'Etude et de Recherche en Immunoanalyse, Gif-sur-Yvette, France (France)

Quantifying the cellular redoxome : effect of MnSOD mimic, a potential metallodrug against Inflammatory Bowel Diseases [OC25]

Martha ZOUMPOULAKI

Introduction

Superoxide Dismutases (SODs) are metalloenzymes involved in the cellular antioxidant protection pathway controlling reactive oxygen species (ROS) [1]. However, the SOD defenses are weakened in intestinal epithelial cells from patients suffering from inflammatory bowel diseases (IBDs), leading to an increase in ROS [2,3]. To complement for this SOD deficiency, we have investigated the effect of the manganese complex Mn1 (Figure B), a SOD mimic with intracellular anti-superoxide and anti-inflammatory activity [4,5].

Methods

The effect of Mn1 is studied on the proteins of intestinal LPS-stressed epithelial cells (HT29-MD2), as a model of oxidative stress and inflammation, using OcSILAC (a bottom-up redox proteomics strategy) which couples a differential labelling approach to SILAC quantification. The changes in protein expression, as well as the oxidation state of cysteines, main targets of protein oxidation, are quantified by nanoLC-MS/MS.

Results

A kinetic study (15 min to 6 h +/- LPS/Mn1) was realized leading to different up- and down-regulated proteins for different incubation times of LPS. The highly impacted quantified proteins were involved in the respiratory and electron transport chain, as well as the regulation of inflammation and oxidative stress (such as MnSOD). The co-incubation of LPS with Mn1 reduced the increased number of oxidized peptides and prevented the up-regulation of MnSOD (after 6 h), as expected for a SOD mimic (Figure C).

Conclusions

Our novel strategy allows to quantify the redoxome for challenging proteins of interest such as the mitochondrial MnSOD. Furthermore, the kinetic study allows to follow the different proteins implicated in the oxidation and inflammation pathways over time. The comparison between the data obtained with or without incubation with SOD mimic Mn1 suggests that Mn1 could be a potential antioxidant and anti-inflammatory metallodrug.

Novel Aspect

Application of OcSILAC strategy to quantify the effect of SOD mimics

Post-translational modifications of Cys in a model of oxidative stress mediated inflammation.

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Keywords

Redoxomics, MnSOD, Inflammatory Bowel Diseases

Authors

Zoumpoulaki Martha [1, 2], Chiappetta Giovanni [2], Quévrain Elodie [1], Diebolt Samuel [2], Shakir Shakir [2], Seksik Philippe [3], Delsuc Nicolas [1], Policar Clotilde [1], Vinh Joelle [2]

1. Laboratoire des Biomolécules (France)
2. Spectrométrie de Masse Biologique et Protéomique (SMBP) (France)
3. Centre de Recherche Saint-Antoine (CRSA) (France)

Development of quantitative targeted proteomics for the diagnosis of Lyme borreliosis [OC26]

Liz Paola Cantero Mendieta

Introduction

Lyme borreliosis (LB) is caused by the transmission of Borrelia burgdorferi sl. Bacteria are inoculated in the skin, multiply locally and can spread to different organs. In order to improve the diagnosis of LB in terms of rapidity and specificity, targeted SRM-MS was first developed in mouse skin. The proof of concept of early diagnosis was then established in human with the quantification of two Borrelia proteins1. Recently, we compared the sensitivities of SRM-MS and PRM-MS. In addition, we are now in the process of validating these approaches on a large human cohort.

Methods

The LLOQ in human skin were determined using 56 NAT and 56 SIS peptides, belonging to six Borrelia proteins. We also examined the impact on LLOQ of the sample preparation (prefractionation, in-gel or in-solution digestion). To validate targeted proteomics for the diagnosis of LB, we are currently analyzing 70 skin biopsies collected from patients with early LB (erythema migrans), and we are comparing with two other direct diagnosis methods, PCR and Borrelia culture.

Results

Among the 56 targeted peptides, belonging to six proteins (flagellin, OspC, DbpA, GAPDH, enolase and lipoprotein gi|365823350), 30 peptides were quantified by both methods. Nineteen peptides have better LLOQ using SRM-MS (as low as 1 fmol), while seven have better LLOQ using PRM-MS (as low as 0.06 fmol). Our results show the complementarity of SRM-MS and PRM-MS, which was further confirmed by the analysis of the human cohort. We observed that targeted proteomics enables the direct detection of up to four Borrelia proteins in patients, as well the typing of Borrelia thanks to species-specific peptides. As a whole, mass spectrometry-based detection of Borrelia is in good agreement with PCR analysis. Moreover, targeted proteomics appears to be more sensitive for 30% of biopsies analyzed up to now. Indeed, SRM-MS and PRM-MS detect more proteins in infected tissues than PCR which only targets flagellin gene.

Conclusion

SRM-MS and PRM-MS enable the specific detection and quantification of Borrelia proteins in human skin and both targeted approaches are complementary for the diagnosis of LB. In our study, the detection of bacterial proteins using targeted mass spectrometry has the advantage of a better sensitivity and a multiplexing capacity. These results are promising for the improvement of the direct diagnosis of disseminated LB2 and the extension to other tick-borne diseases.

Novel Aspect

This study constitutes an improvement of the direct detection of bacterial proteins in the skin of patients, and paves the way to novel diagnosis approaches.

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Keywords

Lyme borreliosis, SRM-MS, PRM-MS

Authors

Cantero Paola [1], Gulsari Vildan [1], Boulanger Nathalie [2, 3], Jaulhac Benoit [2, 3], Hansmann Yves [4, 3], Lenormand Cédric [5, 3], Zilliox Laurence [2], Kieffer Pierre [6], Ehret-Sabatier Laurence [1]

1. Laboratoire de Spectrométrie de Masse BioOrganique, Institut Pluridisciplinaire Hubert Curien (UMR 7178) (France)
2. Centre National de Référence Borrelia (France)
3. EA7290 : Virulence bactérienne précoce : groupe Borrelia (France)
4. Service d'infectiologie (France)
5. Clinique Dermatologique (France)
6. Centre Hospitalier de Mulhouse (France)

Tricks to overcome challenges in metaproteomics of the human intestinal microbiota [OC27]

Céline HENRY

Introduction

Owing to the growing recognition of the gut microbiota as a main partner of human health, we are expecting that metaproteomic of intestinal microbial communities will increase. This should help reveal the microbial functions that are disturbed in a number of diseases, discover predictive/prognostic markers and move towards modulations of the microbiota as curative or preventive measures. However, given the tremendous complexity of the intestinal ecosystem, each step of gut metaproteome analysis is challenging and only a few labs over the world are joining this research field.

Methods

We adopted a five-step approach : (i) extraction of microbiomes and cell lysis; (ii) separation of cytosolic and membrane proteomes then trypsin digestion; (iii) analysis on Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer in DDA mode; (iv) identification by iterative interrogation of the gut metagenomic database MetaHIT 9.9 [1] with X!Tandem; (v) grouping of proteins with X!TandemPipeline [2] and quantification with MassChroQ [3].

Results

Here are two examples of outcome results focused on technical considerations to characterize human gut metaproteomes: the ProteoCardis cohort [4] (188 individual cytosolic and envelope-enriched metaproteomes from controls and patients at different stages of cardiovascular disease), and the MICI-Pep cohort [5] (20 individual envelope-enriched metaproteomes from well-characterized intestinal bowel inflammation phenotypes). With our approach, we identified more than 280,000 and 332,000 unique peptides in the ProteoCardis cytosolic and envelope fractions, respectively, corresponding to nearly 55,000 and 62,000 non-redundant protein subgroups. In the highly dysbiotic MICI-Pep cohort, we identified more than 230,000 unique peptides corresponding to more than 43,500 non-redundant protein subgroups. Taxonomic and functional annotations led to identify and quantify of more than 4 000 COG and KEGG functional categories and more than 31,000 taxonomic groups. From these data, we extracted peptide and protein signatures of disease states.

Conclusions

In addition to bringing unprecedented insights into the functions truly expressed by the intestinal microbiome, such middle-high throughput approaches make it possible to discover variables or cluster of variables that efficiently classify patients into clinical groups. If validated for their predictive value in at risk patients through targeted proteomics, these peptide and protein candidates could be used as new biomarkers, targets or therapeutic molecules.

Novel Aspect

Preparative and analytical aspects of gut metaproteomes in large-middle size cohorts.

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Keywords

Gut metaproteome, Human, Disease, High-throughput

Authors

Henry Celine [1], Bassignani Ariane [2, 3, 4], Langella Olivier [2], Monnet Veronique [1], Juste Catherine [4]

1. Plate-forme PAPPSO, Micalis Institute, INRA, AgroParisTech, Université Paris-Saclay (France)
2. PAPPSO, GQE - Le Moulon, INRA, Univ. Paris-Sud, CNRS, AgroParisTech, Université Paris-Saclay (France)
3. US1367 MetaGenoPolis, INRA, Université Paris-Saclay, 78350, Jouy-en-Josas, France (France)
4. Micalis Institute, INRA, AgroParisTech, Université Paris-Saclay, 78350 Jouy en Josas (Franc

Parallel Session 10 : Lipidomics / Metabolomics

Molecular networking and ion mobility complementarity in metabolites identification of a Fagara heitzii extract [KL10]

Isabelle SCHMITZ-AFONSO

Introduction

Studying secondary metabolites of plants is of great interest for medicine and biotechnology. Based on LC-MS/MS analysis, metabolite identification is an important work due to the amount of data generated. Therefore, molecular networking has been developed to associate families of compounds based on their MS/MS fingerprint and to implement database searches [1,2]. In addition, coupling liquid chromatography-ion mobility-mass spectrometry (LC-IM-MS) increases the efficiency of separation and brings an additional parameter of characterization [3,4].

Methods

The study was carried on methanol extracts of Fagara heitzii, a tree from the Rutaceae family. This medicinal plant is widely used in central Africa for the treatment of many diseases but is not fully characterised [5]. Each extract was analyzed by ultra-high performance liquid chromatography coupled to tandem - mass spectrometry (MS-MS) or ion mobility - mass spectrometry (IM-MS), with a hybrid quadrupole-time of flight analyzer, equipped with an ion mobility cell.

Results

At first, MS-MS parameters were optimized in the objective to build molecular networks using the Global Natural Products Social Molecular Networking platform. Peak picking was realized with mzmine. Molecular networks allowed us to compare the fragmentation profiles of the different compounds and enhanced metabolite search. As example, a family of compounds belonging to tetrahydropalmatine has been identified. In parallel, LC-IM-MS data were acquired to determine the collision cross sections (CCS) of each compound detected in the plant extract. As CCS is an intrinsic property of a given compound, it could increase reliability of compound annotation in metabolomics by comparing experimental CCS with reference of calculated values. Therefore, experimental CCS were measured for reference compounds, and theoretical CCS values were calculated for some metabolites to confirm the hypothesis given by the molecular networks.

Conclusions

Molecular networks helped to determine different families within Fagara heitzii extracts. In addition, annotation was carried out with CCS determination of all compounds. Development of software (such as UNIFI®, Waters) to extract multidimensional data (retention time, drift time, m/z and intensity) will help to implement this approach. Thus, compounds could be annotated with different confidence levels, as described by the Metabolomics Standards Initiative.

Novel Aspect

Molecular networking and collision cross sections determination showed their complementarity to enhance plant metabolome discovery.

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Keywords

Molecular networking, ion mobility, plant metabolome

Authors

Schmitz-Afonso Isabelle [1], Prévost Candice [1], Elomri Abdelhakim [1], Afonso Carlos [1]

1. Chimie Organique et Bioorganique : Réactivité et Analyse (COBRA) (France)

Convenient production of deuterium-labelled internal standards for large scale quantitative metabolomics [OC28]

Kathleen ROUSSEAU

Introduction

Metabolomics consists in studying the small molecules with molecular weight below 1.5 kDa present in a biological sample. Metabolome analysis requires the use of advanced analytical techniques such as high-resolution mass spectrometry (HRMS).

In HRMS, the use of stable isotope labelled compounds remains the gold standard method for absolute quantification in matrix. To overcome current limitations regarding standard availability and cost, we implemented a fast and versatile strategy to generate deuterium-labelled metabolites by metal-catalysed H/D-exchange reaction.

Methods

The one-step labelling process consisted in reacting authentic standards with D2 / D2O in the presence of a metal serving as catalyst (Ru, Pd, Pt). H/D exchange reaction screening was performed using either a parallel synthesis system allowing up to twenty-four simultaneous labelling or a flow chemistry apparatus. For each reaction product, the isotope incorporation rate was assessed by LC-HRMS using an Orbitrap FusionTM or a Q-ToF mass spectrometer.

Results

The deuterium labelling methodology was evaluated on fifty-five authentic standards corresponding to biologically relevant endogenous metabolites and belonging to different chemical families. These metabolites were essentially functionalised molecules belonging to amino acid, organic acid or poly-hydroxylated metabolite classes. The use of metal catalysts was also investigated to efficiently and robustly incorporate deuterium atoms at stable position(s) through C-H activation. So far, thirty-six out of fifty-five metabolites were efficiently labelled with deuterium: no remaining unlabelled material was detected (%M0<1%) on the HRMS spectra of the deuterium-labelled product, one major isotopologue was obtained, the metabolite remained stable during the labelling reaction. No isotopic effects were observed neither on analyte chromatographic retention nor on ionization efficiency for any of the produced thirty-six deuterium-labelled compounds.

Conclusions

The one step metal-catalysed H/D exchange proved to be an efficient and easy way to produce labelled internal standards. Such labelled compounds will then be used for the large-scale quantification of endogenous metabolites in biological matrices such as plasma or urine. Labelled-metabolites can also be used to better identify and elucidate the structure of unknown metabolites.

Novel Aspect

This labelling technique allows rapid and convenient access to stable deuterium-labelled metabolites for further quantitative LC-HRMS-based metabolomic studies.

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Keywords

Isotopic labelling, High-Resolution Mass Spectrometry, Quantification

Authors

Rousseau Kathleen [1], Michieletto Jessica [1], Kiw Yu Min [1], Feuillastre Sophie [2], Pieters Grégory [2], Junot Christophe [1], Fenaille François [1], Damont Annelaure [1]

1. DRF/Institut Joliot/SPI/LEMM, Université Paris-Saclay, CEA-Saclay (France)
2. Laboratoire de Marquage par le Tritium (France)

Supercritical-Fluid Chromatography coupled to High-Resolution Mass Spectrometry (SFC-HRMS) for qualitative and quantitative analysis of N-acylhomoserine lactones implied in quorum-sensing [OC29]

Hoang Thi Phuong Thuy

Introduction

Supercritical-Fluid Chromatography (SFC) [1] using CO2 as solvent is considered as a green analytical technic because it employs a non-toxic recycling product of the petroleum industry [2, 3]. This technique offers lot of advantages such as good chromatographic resolution, short analysis temps and avoiding degradation of analysts [4]. It has been therefore more and more applied in research laboratory and in industry.

Methods

We propose here to investigate SFC coupled to High-Resolution Mass Spectrometry (SFC-HRMS) for qualitative and quantitative analysis of N-acylhomoserine lactones (HSLs), signal molecules responsible for the cell-to-cell communication in the kingdom of bacteria [5]. After a large column screening, chromatographic analysis condition together with MS parameters to reach the best sensitivity were studied and established.

Results

Column screening step figured out Hypercarb® (Thermo Scientific) column which showed the best separation of HSLs. Optimal analysis conditions by using ethanol containing 50 µM of ammonium acetate as co-solvent, column temperature at 60 °C, back-pressure regulator (BPR) at 140 bar, fragmentor at 120 V, gas temperature at 350 °C and Vcap value of 3500 V were established. The quantification method of 31 N-acylhomoserine lactones was then confirmed and used to study HSLs production of one gram-negative endophytic bacterium Paraburkholderia sp. BSNB-0670 isolated from an amazonian palm tree Astrocaryum sciophilum. Sixteen known HSLs together with three new were detected. Based on established MS/MS fragmentation pathways, structures of new HSLs were proposed. Quantification of some abundant HSLs was also carried out.

Conclusions

Development of a method for qualitative and quantitative analysis of N-acylhomoserine lactones using SFC-HRMS was established and validated. Our results demonstrate the performance of SFC-HRMS in analysis with a short analysis temps, high sensitivity and reproducibility.

Novel Aspect

In comparing to others methods using for detection and quantification of N-acylhomoserine lactones, SFC-MS is more environment-friendly and highly efficient.

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Keywords

Supercritical-Fluid Chromatography, N-acylhomoserine lactone, Paraburkholderia sp.

Authors

Hoang Thi Phuong Thuy [1], Barthélemy Morgane [1], Lami Raphaël [2], Eparvier Véronique [1], Touboul David [1]

1. CNRS, Institut de Chimie des Substances Naturelles, UPR2301 (France)
2. Laboratoire de Biodiversité et Biotechnologies Microbiennes (France)

Untargeted profiling of toxicologically relevant reactive metabolites using all ion MS/MS and specific data filtering [OC30]

Laurent DEBRAUWER

Introduction

Human exposure to toxic food contaminants is a major societal concern. Current approaches based on food consumption data crossed with targeted food contamination or biomonitoring data are restricted to exposure evaluation only on some families of compounds. Recent untargeted exposomics related approaches [1] allowing to identify unknown compounds are better suited for a wide exposure assessment to mixtures of compounds. Furthermore, among these numerous unknown metabolites, focusing on toxic compounds should provide more relevant data to link exposure and health.

Methods

We propose an untargeted UPLC-HRMS method to specifically profile toxic electrophilic metabolites, which are mainly detoxified by conjugation to glutathione and excreted as mercapturic acid conjugates (MAC) as final urinary metabolites.

Based on a characteristic neutral loss of MACs, all ion MS/MS (MSE) was used on a Waters Synapt G2-Si mass spectrometer to detect all species displaying this feature, and therefore to specifically highlight reactive metabolites.

Results

As a proof of concept, this approach has been applied to the issue of linking red meat consumption and colorectal cancer development. Heme iron contained in red meat is known to catalyse lipid peroxidation in the intestinal tract, leading to the formation of toxic alkenals possibly involved in colorectal cancer development [2,3], and leading to MACs upon metabolisation. Urines of rats fed various oils with heme iron or free iron supplementation were analysed by UPLC-MS. MACs profiling was performed on QC samples by monitoring their specific neutral loss using the HRMSE acquisition mode and the UNIFI software. Signals of detected conjugates were then measured in all urine samples using the HRMS mode and XCMS software.

Thus, dozens of MACs could be detected without a priori, including DHN-MA and other known and expected conjugated aldehydes. Based on complementary structural analyses (ion mobility, MSn experiments). Our approach also allowed the detection of other MACs derived from unexpected aldehydes, as well as of other chemical classes.

Conclusions

The proposed approach allows specific profiling of the toxicologically relevant MACs metabolites. Furthermore, multivariate statistical analyses carried out on the MACs extracted dataset enables a much better group segregation compared to a classic untargeted metabolomic approach. Therefore, our approach not only allows highlighting metabolites of

lipo-peroxidation, but also opens the way to the untargeted detection of toxicologically relevant compounds.

Novel Aspect

Untargeted profiling of mercapturic acid metabolites representative of toxic compounds, applied on the study of aldehydes generated from lipid peroxidation.

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Keywords

High resolution mass spectrometry, all ion MS/MS, reactive metabolites

Authors

Mervant Loic [1, 2], Costantino Robin [1, 2], Martin Jean-François [1, 2], Debrauwer Laurent [1, 2], Guéraud Françoise [2], Jamin Emilien [1, 2]

Axiom Platform, MetaToul-MetaboHUB, National Infrastructure of Metabolomics and Fluxomics, F-31027 Toulouse, France (France)

Toxalim, UMR 1331, Université de Toulouse, INRA, ENVT, INP-Purpan, Université de Toulouse 3 Paul Sabatier, F-31027 Toulouse, France (France)

Posters

Instrumentation et Développement de Méthode

Direct insertion probe and atmospheric pressure ionization coupled to high-resolution mass spectrometry for the description of lignocellulosic biomass [P1]

Introduction

Lignocellulosic biomass is a complex mixture whose chemical analysis is a challenge because lignins and cellulose are highly reticulated polymers that are poorly soluble and usually require chemical, enzymatic or thermal degradation for their analysis. We chose to emulate evolved gas analysis (EGA) by using the thermal degradation of lignocellulosic biomass occurring in a direct insertion probe (DIP). We used and compared DIP with atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) coupled to high resolution mass spectrometry.

Methods

Ground pellets of beech were introduced in the DIP capillary without sample preparation, and the capillary was roughly sealed with a piece of quartz fibers filter (QFF). APCI and APPI source parameters such as temperature, drying gas and nebulizer gas flow, were optimized so as to allow the analysis of the ground pellet sample within 5 minutes. Analyses were performed in the positive mode. A 12T FT ICR (SolariX XR, Bruker Daltonics) was used as mass analyzer.

Results

Source and transmission parameters were first optimized to avoid space charge in the analyzer. A resolving power of roughly 200 000 was achieved at *m/z* 400 with a transient length of 0.699 s (1.43 Hz acquisition rate). For the two sources, two main ion distributions were observed, that most likely corresponded to lignin monomeric and dimeric degradation products. A time-resolved analysis revealed first the formation of low mass volatile compounds then molecules of higher mass.

Due to the change of ion intensities with time, small shifts of the *m/z* values were observed for most peaks from scan to scan. This phenomenon required re-calibration of each scan to ensure a reliable molecular attribution. For this purpose, the mass spectra, pre-calibrated using Bruker Data Analysis, were exported and re_calibrated using MATLAB scripts. These scripts apply lock-mass corrections to each scan, using a set of well identified ions in the samples. Molecular attributions with minimal errors could then be obtained for the summed mass spectra.

Conclusions

Comparison of replicates of APPI and APCI showed 70% of attributions were common to at least three out of the five replicates and molecular attributions were dominated by oxygenated series from CxHyO2 to CxHyO7, in both sources. Classical marker species, such as paracoumaryl, coniferyl, and sinapyl alcohol were found among these oxygenated molecules.

Van Krevelen plots revealed aliphatic compounds to be more abundant for APCI whereas APPI revealed predominantly unsaturated/aromatics compounds.

Novel Aspect

DIP-APCI/APPI FTICR-MS methodology for the quick molecular -level analysis of biomass samples.

Keywords

Biomass, direct introduction, FTICR

Authors

Castilla Clément [1], Lavanant Hélène [1], Rüger Christopher [1], Afonso Carlos [1]

1. Normandie Univ, UNIROUEN, INSA Rouen, CNRS, COBRA (France)

Characteristics of MALDI-imaging on a new dual ion source QTOF with TIMS separation [P2]

Introduction

MALDI Imaging has a broad range of applications in Omics research. However, a gap exists between desired mass resolution capabilities and the acquisition speed of current instrumentation. We present initial results from the timsTOF flex system; a timsTOF Pro QTOF mounted with a high-throughput, high spatial resolution MALDI source and stage.

Methods

A timsTOF Pro was mounted with a MALDI source and 10 kHz smartBeam 3D laser featuring electronically controlled spot positioning and beam profile for imaging. Performance in ESI mode was evaluated by analyzing a commercially available HeLa digest (Pierce) using DDA PASEF approach. Tissue samples for MALDI-MSI were mounted on conductive glass slides, and coated with matrix using standard protocols on a TM Sprayer (HTX Technologies, Chapel Hill, NC, USA). MALDI ion mobility imaging experiments were acquired on the system at a mobility resolution of 150 1/K0.

Results

High spectral quality MALDI Imaging data could be acquired at a rate of up to 20 pixels/second in both positive and negative mode. A sagittal rat brain section consisting of approximately 370,000 pixels took ~5 hours to measure. Spatial resolution of 20 µm was confirmed by matching ion signals to specific cells and structures in rat brain. In experiments designed to stress the system, 20 hours of image acquisition or ~1.5 million pixels showed no decline in imaging dataset quality and a mass deviation of RMS 2.06 without lock mass. Trapped ion mobility imaging measurements removed isobaric interferences in lipid imaging.

Proteomics analysis s was used to assess if the dual source design and MALDI Imaging experiments affected LC-MS/MS performance. Injections of 200ng HeLa revealed over 5000 protein groups identified; this figure is maintained over the course of measuring 20 million MALDI pixels.

Conclusions

The timsTOF fleX allows for fast, high-spatial resolution MALDI acquisition, and robust ESI performance.

Authors

Schmit Pierre-Olivier [1], Goedecke Nils [2], Kaspar-Schoenenfeld Stephanie [2], Cornett Shannon [3], Ly Alice [2], Woods Lucy [2], Raether Oliver [2]

1. Bruker Daltonique (France)
2. Bruker Daltonik GmbH, Bremen (Allemagne)

3. Bruker Daltonics (États-Unis)

A Cyclic Ion Mobility-Mass Spectrometry System for Structural Elucidation of Isomers [P3]

Introduction

Improvements in the performance and availability of commercial instrumentation have made ion mobility – mass spectrometry (IM-MS) an increasingly popular approach for the structural analysis of ionic species as well as for separation of complex mixtures. Here, a new research instrument is presented which enables complex experiments, extending the current scope of IM technology.

Methods

The instrument is based on a Waters SYNAPT G2-Si IM-MS platform, with the IM separation region modified to accept a cyclic ion mobility (cIM) device. In addition to single and multi-pass separations around the cIM, providing selectable mobility resolution, the instrument design and control software enable a range of 'multi-function' experiments such as: mobility selection, activation, storage, IMSn and importantly, custom combinations of these functions.

Results

In this presentation, instrument performance, functionality and flexibility will be showcased in a study of isomeric pentasaccharides. Using high cIM resolution we demonstrate the separation of three isomeric pentasaccharides and, moreover, that three components are present for each compound. Using IMSn we show that structural differences between product ions reflect the precursor differences in some cases but not others. These findings are corroborated by a heavy oxygen labelling approach. Using this methodology, the identity of fragment ions may be assigned. This enables us to postulate that the two main components observed for each pentasaccharide are anomeric forms. The remaining low abundance component is assigned as an open-chain form.

Conclusions

Demonstration of the unique capabilities of cyclic Ion Mobility High Resolution Mass Spectrometry for the analysis of a range of isobaric species and the structural elucidation of isomers.

Novel Aspect

The instrument is a cyclic ion mobility-enabled quadrupole time-of-flight (Q-cIM-oaToF) mass spectrometer. The instrument design and control software enable a range of 'multi-function' capabilities.

Keywords

High Resolution in Ion Mobility, IMSn, Mobility Selection, Cyclic IMS

Authors

Ujma Jakub [1], Ropartz David [2], Giles Kevin [1]

1. Waters Corporation (Royaume-Uni)
2. UR1268 Biopolymères Interactions Assemblages, INRA, F-44300 Nantes (France)

Toward automation of Collision Induced Unfolding experiments through online Size Exclusion Chromatography coupled to native Mass Spectrometry [P4]

Introduction

Native ion mobility (IM) and collision induced unfolding experiments (CIU) play a key role in the characterization of biotherapeutics [1]. CIU detects subtle structural differences of monoclonal antibodies (mAbs) based on their gas-phase unfolding patterns and stabilities [2], by sequential increase of collision voltages (CV). However, CIU lacks in automation and remains tedious and time-consuming, hampering its routine use. We thus present here an automated CIU data acquisition workflow using size-exclusion chromatography (SEC) coupled to native IM-MS for mAb analysis.

Methods

For the online SEC-native IM-MS instrumentation, an Acquity UPLC H-class system (Waters), with an Acquity BEH SEC column, was hyphenated to a Synapt G2 HDMS (Waters) mass spectrometer. For each chromatrographic peak, trap collision voltages were ramped from 0 to 200 V using MassLynx 4.1. CIU data were analyzed with CIUSuite 2, by automatically extracting arrival time distribution of specific ions at each CV.

Results

Automated CIU data acquisition using SEC-native IM-MS was evaluated for several intact mAbs of different isotypes. CIU fingerprints obtained with our automated workflow are compared to manually performed experiments.

Online automated SEC-CIU experiments present several benefits over manual CIU, among which i) improved and fast desalting efficiency compared to manual buffer exchanges used for classical CIU experiments [3]; ii) drastic reduction of the overall data acquisition time process from 2 hours to 30 minutes along with iii) maintaining CIU key features (number of transitions and CIU50 values) to reach similar CIU fingerprints.

Conclusions

Our results demonstrate the possibility of automating CIU experiments using an online SEC-CIU coupling. Our strategy provides an automated workflow, from sample preparation to CIU data interpretation. This fast isotype screening method proved its efficiency for mAb analysis and could be adapted to the analysis of other systems.

Novel Aspect

Automation of CIU experiments through the online hyphenation of size exclusion chromatography (SEC) to native IM-MS.

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Keywords

Collision Induced Unfolding, Native Ion Mobility Mass Spectrometry, Biotherapeutics

Authors

Deslignière Evolène [1], Ehkirch Anthony [1], Botzanowski Thomas [1], Hernandez Alba Oscar [1], Beck Alain [2], Cianférani Sarah [1]

1. Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO) (France)
2. IRPF - Centre d'Immunologie Pierre-Fabre (CIPF) (France)

Off-line coupling of CE-UV with MALDI: an efficient instrumentation set-up for bioanalytical compounds [5]

Introduction

Capillary electrophoresis is increasingly used for the separation and analysis of biological compounds. CE instruments are usually equipped with UV detection. However, hyphenation with mass spectrometry (MS) was recently developed. Most of the time, coupling CE with electrospray ionisation (ESI) MS is not straightforward due to the adverse CE conditions such as low flow rate, salt or surfactants and constant capillary voltage required for CE separation. MALDI (matrix assisted laser desorption ionisation) MS can allow to encompass most of these issues notably thanks to its great tolerance to salt.

Methods

In the present study, we modified an existing instrument, a P/ACE System MDQ from Beckman Coulter, to be able to spot directly the compounds coming from the CE separation on a MALDI plate and analyze them by MALDI MS. In a first step, the CE instrument was adapted in order to accommodate the MALDI plate. Then, the spotting process was investigated and optimized with salting molecules and aromatic compounds.

Results

Pressures, volumes and times were modified to determine: (i) the lower spotted amount which will be detectable, (ii) the best conditions for separation and deposition, (iii) the minimum amount of molecule quantity required for MALDI detection. Repeatability and robustness were also considered.

Conclusions

This newly optimized CE-MALDI off-line method was developed on commercial instrument with very few internal modifications. This technic giving two simultaneous identifications, UV and MS could be thus applied on various types of molecules such as active pharmaceutical ingredients, lipids or sugars.

Novel Aspect

MALDI-MS in off-line coupling with CE on existing commercial instrument for biological applications.

Keywords

capillary electrophoresis, mass spectrometry, method development

Authors

Muracciole Bich Claudia [1], Costales Lucia Teresa, Montels Jérome, Ladner Yoann, Enjalbal Christine [2], Perrin Catherine

1. Institut des Biomolécules Max Mousseron [Pôle Chimie Balard] (France)
2. Université de Montpellier, PT-LMP, IBMM UMR 5247 CNRS (France)

Evaluation of a tims-Q-TOF instrument for targeted proteomics [P6]

Introduction

Targeted proteomics approaches are now commonly used, either to perform targeted biomarker candidate discovery or to validate candidate biomarkers. In this study, we are performing an evaluation of the potential of the nano-ESI tims Q-TOF architecture for targeted proteomics approaches. More specifically, we are willing to check if the time and space concentration of ions that results of the Trapped Ion Mobility Spectrometry (tims) separation process can translate into increased sensitivity and specificity performances for targeted proteomics approaches.

Methods

An equimolar mixture of 259 quantified synthetic peptides labelled with stable isotopes (AQUA) was diluted in a 100ng/µl human cell line digest. The dilution series covered 6 concentration levels ranging from 31.25 amol/µl to 25 fmol/µl. All samples were separated by nano-HPLC with a 60 min gradient and analysed on a high-resolution timsTOF Pro instrument (Bruker Daltonics) operated in data dependent PASEF mode or an exploratory targeted TIMS-PRM acquisition modes. The global sensitivity, selectivity and detectability of the different acquisition modes was evaluated with the latest version of the Skyline ™ software.

Results

The PASEF acquisition of cell lysate digest spiked with 25fmol, 6,25fmol, 1562.5amol, 500amol, 125amol and 31.25 amol of the AQUA mixture allowed to identify 253/235/205/104/48 and 4 of the original 259 AQUA peptides, respectively. Using an exploratory tims-PRM approach with a 100 ms tims trapping time, and prior to any collision energy optimization, 110/168/205/213 and all of the AQUA peptides could be quantified at a the 31/125/500/1562/6250 amol level, respectively. Increasing the tims trapping time allowed to increase the detected S/N ratio. The latest results of the optimization process will be presented.

Conclusions

We have demonstrated a real (yet) unexploited potential of the tims-Q-TOF architecture for targeted proteomics approaches.

Keywords

PRM, Targeted Proteomics, timsTOF

Authors

Lesur Antoine [1], Longworth Joseph [1], Schmit Pierre-Olivier [2], Bernardin Francois [1], Dittmar Gunnar [1]

1. Luxembourg Institute of Health (Luxembourg)

2. Bruker Daltonique (France)

GPU-based signal processing optimization for 1&2D FT-ICR MS data [P7]

Introduction

Most of the progress in fields such as proteomics, metabolomics, pharmacology or forensic sciences depend on the use of mass spectrometry. Fourier-transform ion-cyclotron-resonance-spectrometer (FT-ICR) is the most accurate method to distinguish between two close but distinct masses thanks to a high resolution. Until now, 2D FT-ICR developments was blocked for several reasons, chief among them are limitations in memory size and processing power of the computers (in regard to the quantity of data generated). Indeed, the size of a 2D FT-ICR spectrum may reach more than one Terabyte. This work aims at taking advantage of the computer sciences progress, in particular GPU parallel programming, to speed up the signal processing regardless of the signal size.

Methods

Our implementation was timed on two Intel Xeon CPU (12 cores each) endowed with a single NVIDIA GeForce RTX 2080 Ti GPU. We translated the existing algorithms in C/CUDA with CUDA Toolkit 10.0. We worked with single precision (32-bit floating point) values rather than double precision (64-bit floating point) and consequently had a better speedup. The program was tested and validated with different GPU/CPU hardwares, with only about 1% precision variation in comparison with an equivalent pure CPU program. First we implemented a GPU-optimized 2D Fast Fourier Transform (FFT) algorithm with optionnal padding and with apodization, for any signal size. Then we adapted an iterative denoising algorithm for GPU to reconstitute the skipped points in Non-Uniform Sampling (NUS) signal acquisition.

Results

We adapted existing FT-ICR signal processing algorithms in order to process the signal on a massively parallel environment, with a generic implementation in regard to the size of the signal, CPU RAM, type and number of CUDA-capable GPUs. Challenges come from memory management and execution time. Indeed, it took previously more than three weeks to compute the spectrum of a 64 kpoints per 512 kpoints signal (128 GB) on 4 Intel Xeon CPU at 2,6 Ghz (12 cores each) when we had to reconstitute the missing points with urQRd. Moreover, it was impossible to process big signals (e.g. 1 TB) if there wasn't enough CPU RAM, because our previous implementation didn't process the signal chunk by chunk, in an optimized way. Our 2D FFT implementation based on cuFFT library is a multi-threaded implementation that uses an execution pipeline per GPU during the calculus. It computes the results chunk by chunk in a methodic way so that to be generic. Indeed, 2D FFTs may be computed on a 2D signal applying two successive steps, that are 1D FFT's on each line, and then 1D FFT's on each column.

Conclusions

When the signal is inferior to the CPU RAM we transpose the intermediate results on GPU in order to benefit from row-major-order and coalescent memory accesses in the rest of the program. But when the signal is too big to stay in CPU RAM, intermediate results are stored

on the hard-drive and read back again, chunk by chunk, in a transposed way. We reached an acceleration speedup of 45 for the processing without denoising and we accelerated denoising process by a factor of 100. Furthermore, we are now able to process every spectrum size as long as there is enough memory to store the required data on the hard-drives

Novel Aspect

GPU programming, data processing optimization, 1&2D FT-ICR, denoising algorithm, acceleration

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Keywords

GPU (Graphics Processing Unit), Non Uniform Sampling, Real time processing

Authors

Haegelin Marc [1], Bray Fabrice [1], Jeannin-Girardon Anne [2], Collet Pierre [2], Rolando Christian [1]

1. Miniaturisation pour la Synthèse, l'Analyse et la Protéomique - USR 3290 (France)
2. Laboratoire des sciences de l'ingénieur, de l'informatique et de límagerie (France)

Performance evaluation of a modified Quadropole Orbitrap mass spectrometer [P8]

Introduction

The increasing importance of understanding how, when and where proteins are expressed, together with interaction with other proteins and what functions they perform, is pushing advancements in mass spectrometric instrumentation. Here we evaluated a new Orbitrap Exploris 480 mass spectrometer for proteomics applications, with focus on data dependent acquisition (DDA) and data independent acquisition (DIA). Additionally, we assess the use of the FSDM processing algorithm on TMT11plex labeled samples.

Methods

The label free experiments (LFQ) were done with different amounts of yeast digest spiked in a constant background of HeLa digest at ratios of 2, 5 and 10. The TMT experiments were done using HeLa digest labeled with TMT11plex mixed in a ratio of 1:1, with yeast digest were spiked in different concentration. The peptides were analyzed on Orbitrap Exploris 480 mass spectrometer. Data files were processed using Thermo Scientific™ Proteome Discoverer™ 2.3 software.

Results and Conclusions

The initial results from the comparison of DDA methods between the Q Exactive HF-X and the Orbitrap Exploris gives a yield of approximately 10% more peptide and protein identifications when using the same sample and experimental conditions. For the DIA experiments, we were able to reduce the sample amount by a factor of 2 and still achieve the same results as on the Q Exactive HF-X. Furthermore, to demonstrate the qualitative cabilities of the Orbitrap Exploris, a two proteome mixture was analyzed. We determined the precision and accuracy for LFQ using yeast spiked into a constant HeLa background on the Orbitrap Exploris MS and found the highest deviation in accuracy to be only around 10% with high precision. A dedicated algorithm, phase-constrained spectrum deconvolution method (FSDM), has been implemented to reach higher resolution in shorter times. This is especially beneficial when using reporter ion quantitation such as TMT11plex, as it enables the use of shorter transients to achieve the same mass resolution relative to conventional FT based approaches without sacrificing data quality. With the FSDM algorithm activated we were able to boost the total number of quantified proteins in a HeLa digest labeled with TMT 11plex, by 25 %, compared analysis with the FSDM deactivated.

Novel Aspect

In-depth evaluation of a modified quadrupole Orbitrap mass spectrometer for bottom-up proteomics.

Keywords

Orbitrap Exploris, DDA, DIA, TMT

Authors

Dauly Claire [1]

1. Thermofisher Scientific (France)

Host Cell Protein Analysis by LC-MSMS, challenge to identify low-abundance HCP peptides versus highly abundance peptides from drug product. [P9]

Introduction

Biopharmaceuticals are generated from biological sources, so, some of the low-level host cell proteins (HCPs) could remain in the final products. Due to their potential to affect product safety and efficacy, HCP level must be monitored. ELISA is the standard method of choice for quantifying HCPs; but with limitation. Therefore, LC/MS technologies have become a choice for HCP analysis. The main challenge during LC/MS analysis of HCPs exists in the coelution of low-abundance HCP peptides with the highly abundant peptides from the drug product.

Methods

The digested sample was fractionated into six fractions on an Agilent Reversed Phase (RP-S) cartridge using the AssayMAP Bravo. Digested sample was loaded onto each cartridge and eluted into six fractions using at pH 10 with a stepwise increase of CAN. LC separation was performed on an Agilent AdvanceBio Peptide Plus column. Each sample was analyzed on the 6545XT AdvanceBio LC/Q-TOF system using conventional Auto MS/MS method or an Iterative MS/MS method.

We demonstrated improvements on identification sensitivity by HPRP fractionation using RP-S cartridges. All the spiked-in proteins above 2 ppm were identified with high confidence, demonstrating an improvement in the identification sensitivity using HPRP fractionation The unfractionated sample was analyzed by three Iterative MS/MS runs, and the HPRP fractionated samples were analyzed by two Iterative MS/MS runs per fraction. The results show a more than three-fold increase using HPRP fractionation coupled with Iterative MS/MS (138 versus 38).

Results

We first compared Iterative MS/MS to Auto MS/MS using the LC/Q-TOF system without any offline fractionation. UPS2 is a complex protein mixture containing 48 human proteins at six concentrations ranging from 500 amoles to 50 pmoles. This spiking sample mimics the wide dynamic range of the HCPs present in the therapeutic proteins. It also allows us to evaluate the sensitivity and dynamic range of different methods being investigated in this study. Overall, Iterative MS/MS identified more unique peptide sequences per protein across a wide dynamic range. All the spiked-in proteins above 8 ppm level were identified with high confidence.

Conclusions

The AssayMAP Bravo using cartridges has brought unprecedented reproducibility, scalability and flexibility.

Iterative MS/MS improves protein identification coverage. Using LC-MS/MS coupled with Iterative MS/MS acquisition, all the spiked-in standard proteins above 8 ppm were identified with high confidence.

By adding HPRP fractionation using AssayMAP Bravo, all the spiked-in proteins above 2 ppm were identified with high confidence.

Keywords

HCP – Host Cell Protein, HPRP - High pH Fractionation, Iterative MS

Authors

Desmoulins Serge [1]

1. Agilent Technologies (France)

Hyphenation of Cation Exchange Chromatography to native Mass Spectrometry for therapeutic protein charge variants characterization (CEX-native MS). [P10]

Introduction

The analytical characterization of monoclonal antibodies (mAbs) requires the investigation of multiple critical quality attributes, among which the determination of charge variants (CV, acidic and basic) [1].

The main technique for CV assessment is cation exchange chromatography (CEX) for detection and quantitation of acidic and basic variants, but its main limitation is the lack of concommitant mass-based identification of the different species. Here, we present a direct hyphenation of CEX to native MS on an Orbitrap instrument for online mAb charge variant characterization.

Methods

The LC system consists of a UHPLC Acquity H-Class (Waters) system hyphenated to an Exactive Plus EMR Orbitrap mass spectrometer (Thermo Fisher Scientific). The CEX column used was a BioPro IEX SF strong cation exchange column (4.6 x 100 mm, 5 µm) from YMC. The mobile phase A consists in 20 mM ammonium acetate pH 5.6 and mobile B 140 mM ammonium acetate pH 7.4. The gradient was adapted to each mAb by increasing the composition of mobile phase B for 10 min at a flow rate of 0.25 mL/min.

Results

CEX-UV is considered today as the reference method for CV analysis, separating acidic and basic variants, as a result of post-translational modifications. This chromatographic method is not directly amenable to native MS due to the presence of high salt concentration in the mobile phase (MES buffer + NaCl). However, recent publications report the direct hyphenation of CEX to native MS using volatile mobile phase for a limited number of mAbs [2-4]. We report here the hyphenation of CEX to native MS using volatile mobile phases to keep best CEX performance (similar to those obtained with classical salt gradient conditions) for a large panel of mAbs. The use of high resolution Orbitrap instrument for CEX-native MS coupling was shown to be necessary to provide high mass accuracy for unambiguous charge variants identification. Reslizumab was analyzed through CEX-native MS methodology, highlighting basic variants such as lysine truncation (- 128 Da) and acidic variants with sialic acid (+ 309 Da) or pembrolizumab basic variant (oxidation +16 Da).

Conclusions

Altogether, our results demonstrate the ability of the CEX-native MS setup to provide a comprehensive characterization of mAb charge variants within a single run. Our CEX-native MS method affords simultaneous charge variants profiling and relative quantitation of acidic and basic variants (CEX) along with the unambiguous identification of each separated species through accurate intact mass measurement (native MS).

Novel Aspect

Online hyphenation of non-denaturing CEX chromatography to native MS for charge variant characterization of a wide range of mAbs using a high resolution Orbitrap instrument.

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Keywords

Cation Exchange Chromatography, Native Mass Spectrometry, Biotherapeutics

Authors

Ehkirch Anthony [1], Murisier Amarande [2], Beck Alain [3], D'atri Valentina [2], Fekete Szabolcs [2], Guillarme Davy [2], Cianferani Sarah [1]

1. Laboratoire de Spectrométrie de Masse BioOrganique (France)
2. School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, CMU, Rue Michel-Servet, 1, 1211 Geneva 4, Switzerland (Suisse)
3. IRPF (France)

In-depth Characterization of a Site-Specific Antibody-Drug Conjugate Generated Through Enzymatic Remodeling and Click Chemistry [P11]

Introduction

Antibody-drug conjugates (ADCs) are a promising class of biotherapeutics [1]. First-generation ADCs (lysine or cysteine conjugates) are heterogeneous compounds with a drug load distribution ranging from 0 to 8 drug/antibody and an average drug-to-antibody ratio (DAR) ~4. To control the position, distribution and average number of drugs, various site-specific strategies were developed [1].

We monitor the GlyCLICK conjugation process, a site-specific approach based on enzymatic remodeling of Fc-glycans and click chemistry, by combining native mass spectrometry (MS) methods.

Methods

Size Exclusion Chromatography (SEC)-native MS experiments were performed with a H-Class chromatographic system (Waters) hyphenated to a Synapt G2 HDMS mass spectrometer (Waters). The column used was an Acquity BEH SEC 4.6 x 150 mm, 200 Å, 1.7 µm (Waters).

Ion mobility-MS (IM-MS) and collision induced unfolding (CIU) data were recorded on the Synapt G2 HDMS (Waters). CIU fingerprints were generated using CIUSuite 2 software.

Results

We report on the characterization of a site-specific ADC by state-of-the-art MS methods, including native MS and its hyphenation to SEC, IM-MS and CIU. SEC-native MS analyses highlighted, within a single run, in a rapid and automated manner, highly homogeneous compounds with an average DAR of 2.0. While IM-MS failed in distinguishing very closely related conformations upon the conjugation process, CIU experiments at both intact and middle levels were performed to assess the gas-phase conformational stability of the bioconjugation process steps. Similar unfolding mechanisms between the different intermediates were observed, which demonstrated a preserved stability throughout the click reaction. Finally, CIU50 values revealed a higher stability of the intact click-ADC compared to first generation ADCs, brentuximab vedotin (BV) and trastuzumab emtansine (T-DM1). CIU results confirmed a global gas phase stabilization of the antibody upon drug conjugation.

Conclusions

Our study underlines the benefits of SEC- native MS and CIU experiments for the analytical characterization of site-specific ADCs. The efficiency of a site-specific click-chemistry based ADC bioconjugation process to produce a highly stable and homogeneous DAR 2.0 ADC compared to first generation ADCs was demonstrated.

Novel Aspect

Characterization of a site-specific ADC generated through enzymatic remodeling and click-chemistry with an average DAR 2.0 using SEC-native MS, IM-MS and CIU.

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Keywords

ADC, Native Ion Mobility Mass Spectrometry, SEC- native MS

Authors

Deslignière Evolène [1], Hernandez Alba Oscar [1], Ehkirch Anthony [1], Beck Alain [2], Toftevall Hanna [3], Nordgren Maria [3], Sjögren Jonathan [3], Cianférani Sarah [1]

1. Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO) (France)
2. IRPF - Centre d'Immunologie Pierre-Fabre (CIPF) (France)
3. Genovis AB, SE-220 07 Lund, Sweden (Suède)

A case study to identify the drug conjugation site of a site-specific antibody-drug-conjugate using middle-down mass spectrometry [P12]

Introduction

Middle-down mass spectrometry (MD MS) has emerged as a promising alternative to the classical bottom-up approach for protein characterization [1]. Classically, peptide mapping is performed for primary sequence and post-translational modification (PTM) assessments. MD MS can also be performed to provide in-depth sequence information, with the advantage of requiring less time and sample handling without affecting the quality of the final product [2-3]. We report here the use of middle-down analysis of a site-specific ADC for the identification of the conjugation site and PTMs.

Methods

A site specific antibody-drug conjugate containing four drug loads (D4 ADC) was digested with IdeS followed by denaturation and reduction for middle-down experiment. Data were acquired using a Thermo Scientific™ Fusion™ Lumos™ Tribrid™ mass spectrometer. Higher-energy Collision Dissociation (HCD), Electron Transfer Dissociation (ETD) and Ultra-Violet Photo-Dissociation (UVPD) were combined to identify the conjugation and glycosylation sites of the ADC.

Results

DARav and DLD of the ADC were assessed through RPLC-MS after ides digestion, pinpointing a highly homogeneous ADC. Middle-down MS afforded ADC primary sequence assessment with additional drug conjugation and glycosylation sites identification within the same analysis. ADC subunits (LC, Fd and Fc/2) were fragmented using three activation techniques: HCD, ETD, and 213 nm UVPD. The ion activation techniques were evaluated either as standalone fragmentation techniques or in combination to provide optimal sequence coverages, ADC drug conjugation and PTM site identifications. Primary sequence assessment (62%, 44%, and 50% for LC, Fc/2, and Fd subunits, respectively) along with conjugation site identification were obtained with unrivaled performances within a unique 10 min UVPD LC-MS/MS run, highlighting the promise of UVPD for MD MS. Finally, complementarity of UVPD, ETD and HCD techniques is clearly shown with 80-90% sequence coverage of all subunits.

Conclusions

Altogether, these results highlight the complementary of the MS/MS activation techniques, specially the sinergy between UVPD and ETD fragmentations, as well as the suitability of this approach for MD characterization of mAbs-related products. With the ongoing progress in mass spectrometry instrumentation and tailored software for automated scoring, MD MS will soon be mature for more routine use in biopharma companies for mAb-based formats characterization.

Novel Aspect

MD MS in combination with HCD, ETD, and UVPD fragmentation techniques to decipher the conjugation, and glycosylation sites of a third generation ADC.

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Keywords

Middle-down mass spectrometry (MD MS), Ultraviolet photo-dissociation (UVPD), Site-specific ADC

Authors

Hernandez Alba Oscar [1], Houel Stéphane [2], Hessmann Steve [1], Erb Stephane [1], Rabuka David [3], Huguet Romain [2], Josephs Jonathan [2], Beck Alain [4], Drake Penelope [3], Cianférani Sarah [1]

1. Laboratoire de Spectrométrie de Masse BioOrganique (France)|Thermo Fisher Scientific (États-Unis)
2. Catalent Biologics West, Emeryville, CA (États-Unis)
3. Centre d'Immunologie Pierre-Fabre (France)

Proteomic characterization of secretomes from selected fungal strains with high biotechnological potential [P13]

Introduction

Filamentous fungi are among the most potent degraders of lignocellulosic biomass due to

their ability to thrive in lignocellulose-rich environments; they produce a high number and a broad variety of enzymes that have different and complementary catalytic activities. A large screening of the fungal CIRM-CF collection (http://cirm.esil.univ-mrs.fr/) using robotic methods specifically developed in-house led to the identification of filamentous fungi that deconstruct efficiently polysaccharides. These investigations enabled the unbiased identification of fungal strains issued from biodiversity with high biotechnological potential. To understand their mechanism of action, some of these fungal strains were further investigated

using state-of-the-art genomic, transcriptomic and proteomic techniques.

Methods

In this context, collaboration between BBF and PAPPSO aims at characterizing the fungal extracellular enzyme production. Fungal strains are cultured on different inducers to favour the secretion of enzymes active on ligncellulosic biomass and the corresponding secretomes (the proteins present in the culture supernatant) are further analyzed using a bottom-up proteomics approach coupling nano liquid chromatography to high resolution mass spectrometry (nano LC-HRMS). X!Tandem Pipeline, a software developed by PAPPSO was used to perform proteins identification from peptide MS/MS spectra.

Results

Over the last ten years, hundreds of fungal secretomes have been analyzed. Each secretome contains an average number of 100 specific enzymes for a given growth condition, which means that several thousands of lignocellulose-acting enzymes have been identified. The analysis of *Fusarium verticillioides* secretomes is given as an example. Secreted proteins of *F. verticillioides* from induced or noninduced culture condition at 3 different kinetic times were analyzed in one injection on a Q-Exactive+ mass spectrometer (Thermo Scientific) summing up to 735 proteins identified in all samples. The comparative approach revealed the abundant secretion of copper-containing enzymes acting on polysaccharides in the culture condition containing metals.

Conclusions

The comparative analysis of fungal secretomes using advanced proteomics (PAPPSO) highlighted the cooperation between fungal enzymes for enhanced degradation of complex lignocellulosic substrates and some discrepancies in carbohydrate-active enzymes (CAZymes) sets dedicated to different types of biomass.

Novel Aspect

A robust proteomic approach was established and validated for large-scale proteomic analysis of fungal secretomes from biodiversity to understand the enzymatic mechanisms of biomass degradation in perspective of biotechnological applications.

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Keywords

Filamentous fungi, proteomic analysis, enzymes

Authors

Oliveira Correia Lydie [1], Ribeaucourt David [2], Saker Safwan [2], Lafond Mickaël [3], Navarro David [2], Berrin Jean-Guy [2]

1. PAPPSO, MICALIS Institute (France)
2. Biodiversité et Biotechnologie Fongiques (France)
3. BiosCiences (France)

Automated phosphopeptide enrichment: a sensitive and robust approach for clinically relevant samples [P14]

Introduction

Deregulation of protein phosphorylation is associated with most tumoral processes. Due to low abundance and stoichiometry, comprehensive phosphoproteome studies entail phosphopetide enrichments from large amount of starting material prior to LC-MS/MS analysis [1,2]. Automated workflows are being developed to increase efficiency and reduce variability. Here we compared manual metal oxide affinity chromatography (MOAC) with titanium (TiO2)-based phosphopeptide enrichment protocol with automated immobilized metal affinity chromatography (IMAC) with Fe(III) workflow.

Methods

Phosphopeptides from a human B-cell lymphoma cell line (BL2) were enriched using 2 different workflows: a manual TiO2-based strategy adapted from the EasyPhos protocol [3] and an IMAC-based strategy with Fe(III)-NTA cartridges coupled to the AssayMAP Bravo platform (Agilent technologies) [4]. Phosphopeptides were then run with a 90 min LC gradient on a timsTOF Pro (Bruker). Label-free quantification and phosphosite localization were performed with MaxQuant.

Results

BL2 protein extract was digested using Lys-C/trypsin and 150 µg of resulting peptides were subjected in triplicate to manual TiO2 or automated Fe(III)-IMAC based phosphopeptide enrichment. On average, the automated Fe(III)-IMAC workflow resulted in more quantified phosphosites than the manual TiO2 workflow (4595 vs. 2298 unique phosphosites, $p < 0.001$). The proportions of class I phosphosites were greater than 90% for both workflows. The percentages of S, T and Y enrichment were very similar. Since in biological experiments the amount of protein can be considerably smaller, we assessed workflow performances with 50 µg of the same starting peptide mixture. Automated Fe(III)-IMAC significantly outperformed manual TiO2 workflow with an average of 3912 vs. 1138 ($p < 0.001$) unique phosphosite quantified, respectively. Interestingly, the quantification was more reproducible with Fe(III)-IMAC from 50 µg than 150 µg of peptide mixture, suggesting the cartridge being saturated with 150 µg of peptides.

Conclusions

Increased attention is drawn toward the development/optimization of automated and miniaturized workflows to investigate the phosphoproteome of samples with limited amount of starting material. In this study we highlighted the potential of combining Fe(III)-NTA cartridge with the assayMAP Bravo platform. This automated phosphopeptide enrichment approach proved to be particularly efficient, reproducible and sensitive with limited amount of material.

Novel Aspect

Automated and miniaturized workflow will be particularly valuable to investigate phosphorylation deregulation in clinically relevant samples such as primary tumoral cells

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Keywords

Phosphoproteome, automation, miniaturization

Authors

Komeili Marziyeh [1], Schneider Justine [1], Moritz Chloé [1], Vallat Laurent [2] [3], Cianférani Sarah [1], Rolland Delphine [2] [3] [1], Schaeffer-Reiss Christine [1]

1. Laboratoire de Spectrométrie de Masse Bio-Organique (LSMBO) (France)
2. Laboratoire d'hématologie (France)
3. Interface de Recherche Fondamentale et Appliquée en Cancérologie (IRFAC) (France)

Nanobody-based APMS analysis of endogenous metabotropic glutamate receptor 2 interactome from mice brain [P15]

Introduction

Nanobodies [1] are Lama single chain antibodies whose small size is well-suited for accessing small and hydrophobic pockets in proteins. This technology is growing fast in the field of seven-transmembrane domain receptors (GPCRs), the largest family of proteins expressed at the cell surface targeted by nearly 40% of prescribed drugs. Metabotropic glutamate receptor 2 (mGlu2) is a GPCR targeted by a new generation of antipsychotics under clinical trial. If activators of mGlu2 receptors have shown promising results2, signaling of mGlu2 receptor and pathological perturbations still remain poorly characterized.

Methods

A subnanomolar affinity nanobody was obtained by engineering nanobodies from a bank directed against mGlu2 receptor. This nanobody was then used to immunopurify endogenous mGlu2 receptor and interacting proteins from two mice brain regions known to be implicated in the physiopathology of schizophrenia.: prefrontal cortex and hippocampus.

Results

LC-MS/MS identified 1856 proteins, including 142 which were significantly enriched by the mGlu2 nanobody compared with a non-relevant nanobody ($p<0.05$, $n=3$). Orthogonal validations and functional studies are ongoing.

Conclusions

The ability of nanobodies to recognize small (and hydropobic) epitopes invisible to classical antibodies is an important advantage to target GPCRs. They can used in affinity purification of endogenous GPCRs at a scale compatible with mass spectrometry analysis of in vivo interactome.

Novel Aspect

Nanobody technology represents an interesting alternative to classical antibodies. Once characterized, they can be produced in large amount for a relatively low cost.

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Keywords

Nanobody; in vivo interactome; GPCR, APMS

Authors

Philibert Clémentine [1], Oosterlaken Mathieu [1], Rondard Philippe [1], Pin Jean-Philippe [1], Marin Philippe [1], Vandermoere Franck [1]

1. Institut de Génomique Fonctionnelle (France)

Reproducible and Hands-off Sample Preparation Workflow for Mass Spectrometry with Focused, Acoustics-Based Ultrasonication [P16]

Introduction

Proteomics analyses typically begin with sample lysis and protein extraction, which step is responsible for most of the variability in proteomics data. The Adaptive Focused Acoustics (AFA) technology allowed us to develop a fast, hands-off, standardized and reproducible sample preparation method. This is of importance for MS-based studies which require optimal and reproducible workflows that allow a satisfactory coverage of the proteome and its modifications.

Objectives

We present here a protocol developed with ProtiFi LLc where the solubilization power of 5% SDS with the extreme sheer forces afforded by AFA is enough to reproducibly and fully extract proteins from all sample types from cell cultures to hard tissues, including FFPE.

Materials & methods

Different tissues were processed like pancreas, brain, bone, kidney harvested from mice, and human kidney FFPE blocks. Proteins were extracted in different buffer conditions using AFA and processed for MS including a clean-up step in ProtiFi S-Traps columns. Peptides were analyzed on a Fusion (Thermo) and data searched with Mascot to a 1% FDR.

Results

The combination of high SDS and AFA treatment before MS analysis showed 1) higher yields (up to 4X more) 2) improved ID rates (up to 100% more) and 3) lower CVs (5% +/-3%) when compared to other buffer/extraction technique combinations.

Conclusion

Several attempts have been made in the past for less cumbersome and more reproducible protocols, but no standardization has been reached. The combination of 5% SDS, AFA and S-Traps is a universal protein extraction solution. It reproducibly samples the entire proteome and consistently identifies the highest number of proteins (quantity and diversity). This combined system is also fully suited to automation and high-throughput with 96-well plates.

Authors

Autret Nicolas [1]

1. Covaris (Royaume-Uni)

A comparison of "universal sample preparations" for proteomics analysis: FASP, SP3 and S-TRAP [P17]

Introduction

The sample preparation is a critical step for an MS/MS analysis. Two challenges to overcome are the need to get rid of detergents necessary to solubilize proteins and that may impact the chromatographic separation as well as the reproducibility of the digestion. In this work we compare FASP, S-Trap and SP3, three sample preparations methods allowing an efficient removal of detergent.

Methods

FASP, S-TRAP and SP3 sample preparation protocols were tested on 1µg, 10µg and 30µg of total protein HELA lysates in SDS 5%. Protein concentration was measured using DC protein assay. Peptides were analysed on NanoLC chromatography coupled to Q Exactive Plus Orbitrap (estimated 500 ng) using 40min gradient, TOP 10 HCD, MS resolution at 70 000 FWHM and MS/MS resolution at 17 500 FWHM. Data were analyzed by Proteome Discoverer with Mascot search engine.

Results

On average, the number of identified protein for FASP is about 1400 +/- 34 proteins with 10µg and 30µg lysates (n=3), but it can falls to 1100 +/- 87 proteins with 1µg. Furthermore, there is 20% to 30% of peptides which have one missed cleavage for 1µg and 10µg but only 15% for 30µg of lysate.

For S-Trap, the number of proteins identified is ranged from 1300 to 1600 proteins, depending of the amount of proteins digested. The number of missed cleavages is around 20% independently of the quantity of proteins processed.

Finally, SP3 shows less reproducibility, the number of proteins varying from 1300 proteins to 1600 proteins but with little correlation with the amount digested. The number of missed cleavages peptides is roughly equivalent to S-Trap.

Conclusions

FASP is a robust method preparation but is not always as efficient for low amount of material (especially in large volumes). In the other hand, S-Trap is robust and reproducible and works well even at 1ug of protein digested. Furthermore, it has a greater peptide recovery capability in comparison to FASP, leading to higher number of proteins identified. SP3 is a method comparable to S-Trap in terms of number of proteins identified, but with inconsistent efficiency in detergent removal and protein digestion.

In conclusion, this study shows that, in own hands S-Trap outperforms FASP and SP3.

Novel Aspect

We compared three digestion methods in terms of number of proteins identified, sensitivity, digestion completeness, detergent removal efficiency and reproducibility.

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Keywords

FASP, S-Trap, SP3

Authors

Jung Vincent [1], Lipecka Joanna [2], Chiara Guerrera [3], Chhuon Cerina [4]

1. Proteomics platform 3P5-Necker, Université Paris Descartes - Structure Fédérative de Recherche Necker (France)
2. Plateforme Protéomique 3p5-Necker, Université Paris Descartes-Structure Fédérative de Recherche Necker, Inserm US24/CNRS UMS3633 (France)
3. Proteomics platform 3P5-Necker, Université Paris Descartes - Structure Fédérative de Recherche Necker (France)
4. INSERM (France)

A novel robust LCMS approach using micro pillar array columns (µPAC™) [P18]

Introduction

Bottom-up proteomics using 50 to 100 µm C18 packed capillaries coupled to high resolution mass spectrometers is used to analyze protein samples from tissues, body fluids or cell lysates. Typically, micrograms of samples are separated in 30 to 240 min nano LC gradients. However, ease-of-use and reproducibility of nanoflow LCMS using packed capillaries does not yet allow novice and routine use.

PharmaFluidics' µPAC™ technology (micro Pillar Array Column) is a unique and novel approach to a chromatographic support structure and builds upon micromachining chromatographic separation beds into silicon, with exceptional properties that result in excellent chromatographic performance with high resolution and high sensitivity.

Methods

The performance of PharmaFluidics' µPAC™ columns for sensitive analysis of limited copy number samples has been demonstrated in nanoflow mode, coupling a 200 cm µPAC™ column via a nanoflex source with a 10 µm silica emitter to a Thermo EasynLC 1200 pump and a Thermo Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer. However, due to the high permeability of the µPAC™, high throughput operation at capillary flow is also possible. Here we demonstrate this by coupling the µPAC™ via a Thermo EasySpray source to a Thermo Ultimate RSLC nano 3000 and a Thermo Q-Exactive HF-X MS under capillary flow conditions with a flow rate of 1 µl/min in short 30 to 90 minute gradient runs.

Results

Capillary flow conditions in proteomics are used for throughput and robustness reasons in quantitative analyses. A HeLa cell digest dilution series was used to investigate the effect of injected sample amount on the output in terms of protein identifications. For the highest concentration of 1000 ng/µl, over 4800 proteins could be identified in a single 90 minute gradient separation (120 minute run time). For the lowest concentration of 2 ng/µl, approximately 600 proteins could be identified in a single 30 minute gradient separation, once more highlighting the potential of µPAC™ Orbitrap LCMS workflows for limited sample proteomics experiments.

Conclusions

This set of experiments proves that a single µPAC™ column can be used over a wide range of flow rates, both nano and capillary flow can easily be applied on the same column. Consequently, it is a viable alternative to packed fused silica columns in proteomics experiments for both qualitative and quantitative analysis.

Novel Aspect

micro-Pillar Array Column technology

Keywords

micro-Pillar Array Column, Nano- and Capillary flow LC-MS, Robustness

Authors

Van Ling Robert [1]

1. PharmaFluidics (Belgique)

Maximize the output of routine proteome analyses by using a 50 cm long microfabricated nano LC column [P19]

Introduction

As an alternative to the conventional packed bed nano LC columns that are frequently used in bottom-up proteomics research, PharmaFluidics offers micromachined nano LC chip columns known as micro pillar array columns (µPAC™). The inherent high permeability and low 'on-column' dispersion obtained by the perfect order of the separation bed makes µPAC™ based chromatography unique in its kind. The peak dispersion originating from heterogeneous flow paths in the separation bed is eliminated (no A-term contributions) and therefore components remain much more concentrated during separation resulting in unprecedented separation performance [1]. The freestanding nature of the pillars also leads to much lower backpressure allowing a high operational flow rate flexibility with exceptional peak capacities [2].

Methods

Complementary to its landmark 200 cm long column which is ideally suited to perform comprehensive proteome research, a 50 cm long µPAC™ column is now available which can be used in a more routine research setting. With an internal volume of 3 µL, this column is perfectly suited to perform high throughput analyses with shorter gradient solvent times (30, 60 and 90 minute gradients) and it can be used over a wide range of flow rates, between 100 and 2000 nL/min.

Results

Recently performed experiments with 500 ng of HeLa cell digest indicate that an increase in protein identifications up to 50% and a gain of 70% in peptide identifications can be achieved when comparing the 50 cm µPAC™ column to the current state-of-the-art in packed bed columns.

Conclusions

The conventional packed bed columns (2 different vendors) used for this benchmarking experiment were 15 cm in length and were packed with sub 2 µm porous silica particles. LC pump pressures needed to operate these classical columns at a flow rate of 300 nL/min range between 200 and 300 bar, whereas only 40 bar was need to operate the 50 cm µPAC™ column at the same conditions

Novel Aspect

micro-Pillar Array Column technology

References

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Keywords

micro-Pillar Array Column, Nano- and Capillary flow LC-MS, Robustness

Authors

Van Ling Robert [1]

1. PharmaFluidics (Belgique)

A simple high pH fractionation on SPE cartridge increases peptide and protein identification by Mass Spectrometry [P20]

Introduction

Improving protein identification is a critical stake for proteomics. This can be achieved by longer LC-MS gradient or multidimensional chromatography, often using SCX chromatography for the first dimension. Here we compared a reference method for liquid digestion [1] with (i) a SCX LC-MS method, (ii) a basic C18-SPE LC-MS method and (iii) a basic C18-HPLC LC-MS method. The advantages and limitations of the different methods are discussed.

Methods

Tryptic digest of HeLa cell was used as model. After the liquid digestion, the C18-SPE cartridge usually used for the sample desalting was also used here as a prefractionation system. We compared classical SPE (0.1%TFA, 1 fraction) with either a low pH SPE (0.1% TFA, 3 fractions) or a high pH SPE (20 mM NH_4HCO_3, 3 fractions) or high pH C18 fractions (20 mM NH_4HCO_3, 4 fractions) or a SCX chromatography (0.1% TFA, 4 or 8 salts elution steps).

Results

The reference method identified in 3 repeated LC-MS of 2h nearly 23000 peptides (3300 proteins). With the same analysis time but no repetition, the SPE fractionation significantly increased the number of identified peptides, especially in basic conditions. This orthogonal fractionation at high pH allowed to identify 14000 new peptides (1600 new proteins). Lastly the SCX with 4 or 8 fractions presented the best results but dramatically increase the complexity of the experiment (*i.e.* additional desalting steps and long analysis time).

Conclusions

A simple one step high pH fractionation provides excellent orthogonality prior the nano LC-MS analysis. Indeed, this method increases peptide identification by 60% and provide a better protein coverage without extending sample preparation time or MS analysis time. This result could be relevant to whom want an easy and fast method for reaching a deeper proteome and a better protein coverage.

Novel Aspect

The poster proposes a comprehensive protocol for basic C18 spin column prior LC-MS/MS analysis; an easy and efficient method for the improvement of peptide identification.

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Keywords

High pH SPE, Peptide identification

Authors

Barroso Kim, Morlet Bastien, Ruffenach Frank, Raffelsberger Wolfgang, Negroni Luc [1]

1. Institut de Génétique et de Biologie Moléculaire et Cellulaire (France)

Onchip digestion coupled to nanoLCMS/MS [P21]

Introduction

In order to scale down the quantity of sample and the time needed for a protein analysis protocol by mass spectrometry, several microfluidic devices have been developed. A microfluidic reactor that enables rapid digestion of proteins before analysis by electrospray ionization mass spectrometry ESI-MS is generally based on the fixation of proteins or enzymes on solid phase. The solid phase loading in the microfluidic device and immobilized enzymes can be very tedious. Here, we present an alternative microfluidic tool named Chipfilter (CPF) for a full automation of sample preparation and protein digestion without any immobilization of substrate or enzyme.

Methods

The microfluidic system incorporates a 10 kDa cut-off molecular filter to retain proteins and wash small molecules like reactants, salts and detergents and relies on the FASP strategy [1]. The membrane is inserted between 2 reaction chambers and sealed so that pressure driven fluids will have to pass through the membrane to go from the first to the second chamber. Different kind of reactions can be done while proteins are retained on the membrane (alkylation, reduction, labeling) a rinsing step is done after each reaction. The microfluidic chip can be directly coupled to a LC-MS system in order to separate and analyze peptides released from the proteins directly by electrospray ionization mass spectrometry.

Results

This microfluidic device strongly decreases sample handling and is very versatile. Sample loss during preparation steps is lowered. The great advantage of this microfluidic device is the reduction of the gap between the sensitivity of LC-MS system and the starting material quantity threshold. As a proof of concept, the device was applied to the classical tryptic digestion procedure before shot-gun analysis. Because of miniaturization kinetics are improved with a reduced time for sample preparation and digestion steps. Whole yeast protein extract have been analyzed with the microfluidic device. A number of 1350 proteins have been identified from 500 ng starting material of protein extract from cell lysate within one-hour digestion time and 2-hours elution gradient using a full on-line device coupled to nanoLC MS/MS with a standard Q Exactive HF.

Conclusions

The microfluidic device allows from the same sample using the same reagents lower sample processing duration, higher sample recovery, higher proteome coverage, higher protein sequence coverage than FASP methods1.

Novel Aspect

Miniaturization of the whole sample preparation for bottom-up proteomics

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Keywords

Microfluidic, Proteomics, Miniaturization,

Authors

Ndiaye Massamba Mbacké [1], Vinh Joelle [2], Chiappetta Giovanni [3]

1. Laboratory of Biological Mass Spectrometry and Proteomics, CNRS USR3149, ESPCI Paris, PSL Research University (France)
2. Laboratory of Biological Mass Spectrometry and Proteomics, CNRS USR3149, ESPCI Paris, PSL Research University (France)
3. Spectrométrie de Masse Biologique et Protéomique (France)

Proteomic analysis of Plasmodium sporozoïtes limited samples [P22]

Introduction

With material-limited samples, it can be challenging to obtain sufficient amounts of proteins to generate high-quality mass spectrometric data. Sporozoïtes, the invasive stage of *Plasmodium (parasite responsible of Malaria)*, can only be recovered from hand-dissected mosquito salivary glands homogenized by grinding. Moreover, a purification step is then needed to obtain sporozoïtes purified from mosquito debris. For proteomic analysis, sporozoïtes represent limited samples.

Methods

We performed proteomic microscale sample preparation on limited sporozoïtes samples. Peptides were analyzed by the new and sensitive timsTOF Pro mass spectrometer (Bruker) coupled to the nanoElute HPLC.

Results

We identified a maximum of 1015 proteins with less material than observed in literature [1,2], and with no prior fractionation of the samples. Several sporozoïtes purification steps were tested and optimized to reduce mosquitoes proteins contamination from 60 to 30 %.

Conclusions

In conclusion, we identified proteins from sporozoïtes limited samples, with less effort on sample preparation and with less contamination.

Novel Aspect

The timsTOF Pro mass spectrometer enables the proteomic analysis of limited samples.

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Keywords

Plasmodium, sporozoïte, proteome

Authors

Pionneau Cédric [1], Hamada Soumia [1], Silvie Olivier [2], Marinach Carine [2], Chardonnet Solenne [1]

1. Sorbonne Université, INSERM, UMS 37 PASS, Plateforme Post-génomique de la Pitié-Salpêtrière (P3S), F-75013 Paris, France (France)
2. Sorbonne Université, Inserm U1135, CNRS ERL 8255, Centre d'Immunologie et des Maladies Infectieuses (Cimi-Paris) F-75013 Paris, France (France)

Comparison between SP3, STrap, Tube-Gel and in-solution methods for preparation of plant samples [P23]

Introduction

Sample preparation, from protein extraction to the obtention of a clean peptide mixture ready to be analyzed by mass spectrometry, is a key step of proteomic analysis. A good compromise has to be found between the quality of the final product, the cost and the preparation time. Because of the presence of different unwanted compounds (cell wall, chlorophyll, phenolic compounds, etc.), plant samples need specific methods. In addition, adaptations are needed for large scale experiments, that are increasingly required in system biology studies.

Methods

Two methods recently described in the literature, SP3 (Hughes *et al*, 2014) and STrap (Zougman *et al*, 2014), were compared to two methods used in our laboratory for large-scale experiments, the in-solution method and the miniaturized Tube-Gel method (Balliau *et al*, 2018). The compatibility of SP3 and Strap methods with three different solubilisation buffers : The Laemmli buffer, a SDS-Urea buffer and a buffer containing an acid labile surfactant (RapiGest from Waters), urea and thiourea. All preparations were performed from the same TCA-acetone dried extract of Arabidopsis leaves.

Results

Protein abundance variations were mostly due to the difference between the methods rather than between protein solubilisation buffers. Regarding the number of identified proteins, the Strap and SP3 methods were less efficient than the in solution-method. They also showed lower quantitative reproducibility than the in- solution and Tube-Gel methods. The STrap method proved to be particularly sensitive to buffer changes. It also showed the highest number of missed cleavages. The SP3 method was apparently not compatible with the buffer containing the acid labile surfactant, but it was only slightly affected by the switch between the SDS-Urea buffer and the Laemmli buffer. The number of missed cleavages was higher with the SP3 method than with the Tube-Gel and in-solution methods but lower than with the Strap method.

Conclusions

Although the results obtained with the in-solution method were globally better than with the others, the SP3 method performed well, it was is easy to use and it is inexpensive. It seems also easy to improve and it could then become a method of choice for the preparation of samples in large-scale experiments. We are continuing in this direction.

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Keywords

Proteomics, sample preparation, protein digestion

Authors

Balliau Thierry [1], Bruce Johanna, Blein-Nicolas Mélisande [1], Zivy Michel [1]

1. UMR Génétique Quantitative et Evolution - Le Moulon (France)

Microfluidic device for the automated preparation of a blood sample in proteomic analysis [P24]

MALDI-ToF mass spectrometry revolutionized microbiological diagnosis in hospitals. However, LC-MS methods for the determination of blood protein biomarkers are struggling to establish in medical biology. This difficulty is closely linked to the preparation of the sample that is time consuming and complex, especially with the target analysis of biomarker proteins of low abundance. This preparation also remains subject to the risk of the protocols performed manually and the technical impossibility for the care teams to guarantee a constant delay between the sampling blood and its analysis by laboratory.

Through the **PEP'S project**, these issues were addressed with the development of a **compact instrument and a microfluidic device (cartridge)** to integrate all the steps of sample preparation: from blood collection to storage of digested peptides. Five steps of preparation were integrated and performed in two hours: separation and calibration of the plasma, incorporation of standards of quantification, albumin depletion, enzymatic digestion and storage of the peptides.

The performance of the microfluidic system was evaluated by comparison with conventional manual preparations by quantifying 5 clinical biomarkers by targeted proteomic analysis (LC-MRM).

The system has been evaluated from healthy samples or blood samples mimicking pathological concentrations of protein biomarkers associated with different pathologies. All biomarkers were detected at pathological concentrations using the microfluidic device.

Portable, compact and easy to handle, PEP'S is designed to allow automated and rapid preparation of blood samples taken from the patient's bed, for mass spectrometry multiplex proteomic analyzes.

Authors

Gilquin Benoit [1], Cubizolles Myriam [2], Den Dulk Remco [2], Revol-Cavalier Frédéric [2], Alessio Manuel [2], Goujon Charles-Elie [3], Echampard Camille [2], Arrizabalaga Gorka [3], Louwagie Mathilde [4], Adrait Annie [4], Couté Yohann [4], Laurent Patricia [2], Navarro Fabrice [2], Cosnier Marie-Line [2], Brun Virginie [4]

1. Univ. Grenoble Alpes, CEA, LETI, Clinatec, F-38000 Grenoble (France)
2. Univ. Grenoble Alpes, CEA, LETI, Technologies for Healthcare and biology division, Microfluidic Systems and Bioengineering Lab, F-38000 Grenoble, FRANCE (France)
3. Univ. Grenoble Alpes, CEA, CEA tech, DIP, F-38000 Grenoble, FRANCE (France)
4. Univ. Grenoble Alpes, CEA, Inserm U1038, Exploring the Dynamics of Proteomes team, F-38000 Grenoble, FRANCE (France)

Protein crosslinking using bottom up and top down mass spectrometry: molecular evidence of restoration treatments applied to historic manuscripts [P25]

Introduction

Proteins from Cultural Heritage artworks are subjected to chemical modifications due to ageing, environmental factors and/or restoration procedures. Among these modifications, protein crosslinking may be induced by chemical agents. The present work shows a new investigation method based on the study of protein crosslinkings using bottom up and top down approaches, revealing the use of particular chemicals for restoration. The techniques were successfully applied to study the Coptic manuscripts, revealing a secret recipe of treatment underwent at the Vatican-Library.

Methods

The analytical methodology was developed on mock up made with model proteins and glues: lysozyme, parchment, bones and skin glues. Each sample was treated with formaldehyde and analyzed with nanoLC- Orbitrap Fusion Lumos (C18 for bottom up, monolithic for top down). Crosslinked peptides and proteins were identified. The historic samples were taken from the Coptic manuscripts (822-914 A.D.) provided by the Morgan Library & Museum and the Metropolitan Museum of Art.

Results

The study is based on structural elucidation of both crosslinked patterns and protein breakdown patterns. Focusing on bottom up experiments, various formaldehyde-induced crosslinkings have been observed in collagen proteins such as alpha1(III), alpha1(II), and alpha2(I). For example, in model samples, a lysine-glutamine crosslinked peptide of collagen alpha1(II) was identified between (135)GDRGDKGEKGAPGPR(139) and (120)GPPGPQGPAGEQGPR(134), using MS precursor ion ([M+4H]4+ 728.112) and MS/MS fragment ions (e.g. [b10b11 β]2+). Among information provided by intact protein analysis and top down experiments are the heterogeneous patterns of protein dimers and the breakdown patterns informing on chemical reactions that occurred. Applied to few micrograms of historic samples, several evidences of formaldehyde induced crosslinks were achieved between K-H, R-N, R-Q and K-R. An example is the lysine-histine crosslinking between (497)GPSGDPGKAGEK(508) and (509)GHAGLAGAR(517) peptides of collagen alpha2(I) ([M+3H]3+ 640.661).

Conclusions

This study shows how bottom up and top down approaches were used to characterize protein dimers and identify crosslinked amino acids resulting from a chemical treatment by the formaldehyde. This study represents the first chemical molecular evidence of the use of formaldehyde as restoration treatment in historic material; here, the Coptic manuscripts. These results revealed the recipe that was used by the Vatican Library.

Novel Aspect

Protein crosslinking and top down proteomics provide new information on chemical treatments underwent on Cultural Heritage samples

Keywords

Formaldehyde crosslinking, Bottom up and top down proteomics, Ancient proteins

Authors

Galluzzi Francesca [1], Rawlins Catherine [1], Arslanoglu Julie [2], Claverol Stéphane [1], Pozzi Federica [2], Fredericks Maria [3], Trujillo Frank [3], Tokarski Caroline [1]

1. Institut de Chimie & Biologie des Membranes & des Nano-objets (CBMN), Bordeaux (France)
2. The Metropolitan Museum of Art (États-Unis)
3. Thaw Conservation Center, The Morgan Library & Museum (États-Unis)

Contribution of mass spectrometry to the study of hair from South American mummies [P26]

Introduction

In recent years, the study of hair has become a major way to enrich the history of mummies. Current characterization of ancient capillary fibers is mainly based on elemental, structural and biological analyses. Thanks to mass spectrometry and to specific analytical developments, it is now possible to provide complementary information on mummy hair including radiocarbon dating, study of coatings and molecular preservation state (bottom-up proteomic approach). Studies of hair from South American mummies (Musée des Confluences, Lyon [1]) are reported here.

Methods

Radiocarbon dating was realized according to a specific protocol using accelerator mass spectrometry AMS [2]. Hair samples were cleaned up using different solvent baths that will be analyzed by GC-MS to determine a possible coating. After cleaning, proteins were extracted, digested using trypsin enzyme and then desalted on Sep-Pak C18 SPE cartridges. Peptide digests were analyzed by MALDI-TOF-MS before being separated and analyzed by nanoLC-ESI-MS/MS [3].

Results

In previous works [4], the protocol described above to characterize the proteins in mummy hair was performed on a single corpus of pre-Hispanic Andean mummies (San Miguel de Azapa Museum, Chile). We present here the results obtained on a second set of mummy hair from the "Musée des Confluences". The radiocarbon dating placed mummies at the end of the Archaic period (1600 years - 1485 years BP). No coating was detected. A new MS/MS data processing software after applying the Mascot search algorithm was used (Proline Zero/ProteinScape). The main ancient hair proteins were successfully identified. Although visually well preserved, indicators of degradation are observed on the molecular level. MALDI-MS spectra of enzymatic digest show significantly different profiles in the ancient samples compared to modern hair. Focusing on 6 keratins present in both modern and archaeological hair, the MS/MS data processing highlights in mummy hair keratins a much higher percentage of modified peptides and a larger contribution of PTMs induced by the ageing.

Conclusions

The results obtained make it possible to place the mummies in their historical context and to document the processes of mummification. The analytical strategy helps to understand the ageing behavior of capillary fiber in archaeological context. The implementation of parameters indicating the degradation state such as the percentage of modified peptides or of some specific PTMs could lead to a better conservation of heritage materials based on keratinized fibers.

Novel Aspect

Bottom-up proteomic strategy to characterize mummy hair at a molecular level. Use of PTMs to assess molecular preservation state.

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Keywords

Mummy hair, proteomics, PTMs

Authors

Charrié-Duhaut Armelle [1], Philipps Marie [2], Richardin Pascale [3], Leize-Wagner Emmanuelle [1]

1. Laboratoire de Spectrométrie de Masse des Interactions et des Systèmes (LSMIS), Université de Strasbourg, CNRS, CMC UMR 7140 (France)
2. Laboratoire de Spectrométrie de Masse des Interactions et des Systèmes (LSMIS), Université de Strasbourg, CNRS, CMC UMR 7140 (France)
3. Centre de Recherche et de Restauration des Musées de France / Préhistoire et Technologie (France)

Analysis of flavonoids in algae by mass spectrometry [P27]

Algae have become an attractive renewable bioresource in cosmetics because of their high concentrations of antioxidant and UV-absorbing compounds, such as e.g. flavonoids [2]. Despite the well-established bioactivity of algal extracts, studies using UV-detection or targeted MS^2 have not been sufficient to cover the structural diversity of active compounds [1,3,4]. To address this problem, we developed an untargeted screening approach based on the use of a HRAM Orbitrap mass spectrometry.

Twelve flavonoids were separated on a reverse phase C18 UPLC column and identified with a data-dependent MSn approach on an Orbitrap Fusion Lumos mass spectrometer operated in negative mode with data dependent MSn acquisition. MS^2/MS3 scans were triggered depending on a list of common fragments. Data obtained were processed with Compound Discoverer 2.1 software for the annotation of flavonoids using FISh strategies.

All the twelve flavonoids could be separated on the C18 column except the position isomers apigenin, genistein and naringenin which showed the same retention time. Preliminary targeted MS^2 experiments were carried out to determine characteristic common fragments and optimum collision energies to form them. The detection limit of flavonoids was around 0.5 ng/ml. Then, flavonoids were screened on the basis of their characteristic common fragments found in dd-MS^2 scans. Four common fragments were selected for the successful coverage of all the compounds. Moreover, position isomers were distinguished using their characteristic spectral MS^2/MS3 data. The data treatment workflow developed could rediscover and catalog all the twelve flavonoids after applying specific filters to exclude false positive among the 884 results.

Our untargeted screening approach based on Data-Dependent MSn Acquisition permitted to detect and identify successfully the twelve flavonoids using their fragmentation patterns. Compound Discoverer software 2.1 allowed efficient data extraction owing to its capability to collect structural information characteristic of a specific compound class.

The method will be used for the screening of algae samples and for the detection of potential new flavonoids.

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Keywords

LC-MSn, metabolomics, algae

Authors

Parailloux Maroussia [1], Godin Simon [1], Lobinski Ryszard [1]

1. Institut des sciences analytiques et de physico-chimie pour l'environnement et les materiaux (France)

Uncovering in vivo proxisome using a tagging proximity methodology coupled to LC-MSMS [P28]

Detection of protein-protein interaction (PPI) is often challenging, especially for weak and transient interactions or with membrane proteins. Over the last decade, developed proximity-tagging approaches allow the definition of an unbiased "proxisome" that is all of the proteins in direct interaction or in close vicinity of the protein of interest, in native conditions and without loss of subtle PPIs. In this study, we have adapted an assay based on the APEX2-dependent biotin ligation technology, initially developed in eukaryotic cells (Lam et al., 2015; Hung et al., 2016), to study bacterial complexes.

The Type VI secretion system (T6SS) is a nano-crossbow-like machinery of the enteroaggregative *Escherichia coli* that delivers effectors into both eukaryotic and bacterial cells. Assembly of its contractile sheath is coordinated by TssA, a multimeric protein that remains at the tip of the growing structure and incorporates new tube and sheath blocks.

To provide insights onto the late stages of T6SS biogenesis, TssA was fused to APEX2 to define the temporal contacts of TssA. This proteomic mapping approach revealed the proximity partners of TssA *in vivo*, among them a new member named TagA. This latter protein is a cytosolic protein tightly associated with the membrane. Analyses of sheath dynamics further demonstrate that TagA captures the distal end of the sheath to stop its polymerization and to maintain it under the extended conformation (Santin et al., 2018).

This approach is particularly powerful for dissecting the contacts of a dynamic protein that engages in different complexes during assembly of a multiprotein system or that is involved in different processes during the cell cycle.

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Authors

Lebrun Régine [1], Santin Yoann, Espinosa Leone, Doan Thierry, Cascales Eric

1. Plateforme Protéomique de l'Institut de Microbiologie de la Méditerranée (France)

Chemical depolymerization and analysis of synthetic and natural insoluble polymers by 1D and 2D high resolution FT-ICR mass spectrometry [P29]

Introduction

Polyolefins are nowadays present in a large number of materials and used in a wide variety of applications. They represent a class of molecules formed by the polymerization of one simple olefin such as butadiene or isoprene. The characterization of this group of organic macromolecules by mass spectrometry continues to be very challenging due to their high hydrophobicity and non-ionizable character. Olefin cross-metathesis is therefore used as a tolerant and efficient way to generate new carbon-carbon double bonds. Over the recent years, it has emerged as a powerful reaction for organic synthesis. It is based on a ruthenium metal catalyst, which shows high tolerance towards functional groups making this reaction an alternative attraction to other olefination methods.

Methods

Experiments were performed on a SolariX XR 9.4 T FT-ICR_MS from Bruker-Daltonics with a nanoESI ion source. In this work, a controlled olefin metathesis between a polyolefin and the cis-1,4-diacetoxy-2-butene in the presence of a ruthenium-based catalyst is used for the depolymerization, functionalization and characterization of unsaturated insoluble polymers. The depolymerization reactions of polybutadiene and polyisoprene were carried out in dichloromethane in the presence of the Hoveyda-Grubbs second generation catalyst and the cis-1,4-diacetoxy-2-butene as chain transfer agent at 40oC under nitrogen. Telechelic monomers, oligomers and polymers with different molecular weights were produced and analyzed by Fourier-Transform ion cyclotron resonance (FT-ICR) high resolution mass spectrometry. During the reaction, olefin isomerization is one of the side reactions that can significantly alter and decrease the yield of the desired product configuration. The addition of an electron-deficient reagent such as 2,6-dichloro-1,4-benzoquinone was required to prevent or suppress this isomerization process. For both cis/trans polyisoprene and polybutadiene, oligomers with the desired original configurations are obtained. When optimized, this method can be applied on a wide range of natural and synthetic polymers.

Results

Different reaction conditions were tested to optimize the depolymerization reaction. The success of the metathesis reaction and the formation of the desired products were first proven by 1H NMR analysis and then confirmed by high resolution mass spectrometry. A very complex mass spectrum with a high number of signals was obtained. The major family of depolymerized fragments was easily identified with a significant low mass error in the range of 10 ppb. However, much more signals appeared when narrowing the mass range of the spectrum. A special software based on a python code was used to identify the large number of obtained signals. This software determines the molecular formulas based on the number of CH2, oxygen and unsaturation in the molecule. We were able to attribute more than 2000 signals to their molecular formulas in which more than 9 different families of molecules were identified. We were also able to determine most of the phenomena that happened during the reaction such as oxidation, dehydrogenation and reticulation. We will present all the obtained mass spectra with a detailed explanation on how the software works and a representation of

all the molecular formulas and identified families. The precision in m/z, the isotopic fine structure, the differentiation between the different isotopes and the variation in the 13C/18O ratio with m/z will be discussed. We will also present the other analytical methods used to investigate additional attributes of the obtained structures such as stereochemistry using ion-mobility mass spectrometry for example. Results obtained from the application of this reaction on a range of insoluble polymers will be presented in addition. Finally, we will show and discuss all the drawn plots used to acquire additional information on the chemical composition of the obtained products such as Van Krevelen and DBE to carbon number pots.

Conclusions

The cross-metathesis reaction between the polymers and the chain transfer agent consists on breaking the double bonds of the polymer and forming new carbon-carbon double bond, which leads to end chain functionalization and the formation of new products containing monomers from the original depolymerized polymer. This reaction is mediated by the Hoveyda-Grubbs second-generation catalyst and will allow the formation of new polymeric fragments suitable for analysis by high resolution mass spectrometry coupled with innovative separation techniques in gas and liquid phase.

Novel Aspect

The application field of the methods developed in the project is very broad on analyses which are not feasible at this time: it includes biological studies of the biosynthesis of natural polymers, the characterization of chemically modified biopolymers, the understanding of synthetic polymer manufacturing processes and their environmental degradation. The analytical techniques which will be employed are state-of-the-art or currently under development. Depolymerization techniques using new generation organometallic catalyst has never been employed in analytical chemistry.

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Keywords

Depolymerization, organometallic catalyst, high resolution mass spectrometry.

Authors

Mahmoud Ziad [1], Mansour Sergui [1], Bray Fabrice [1], Chausset-Boissarie Laëtitia [1], Rolando Christian [1]

1. USR 3290 – MSAP – Miniaturisation pour la Synthèse, l'Analyse et la Protéomique, 59000 Lille, France. (France)

Screening transitions for the analysis of O,O-dialkyl alkylphosphonates at sub-ppm levels by gas chromatography - tandem mass spectrometry (GC-MS/MS) [P30]

O,O-dialkyl alkylphosphonates are listed in schedule 2B.4 of the Chemical Weapons Convention (CWC) because of their connections to organophosphorous nerve agents. O,O-dialkyl alkylphosphonates can be found as impurities in nerve agents, or used as precursors to synthesize them [1]. They can also be generated by nerve agents' decontamination using basic alcoholic solutions [1].

Due to its designation by the Organisation for the Prohibition of Chemical Warfare (OPCW) for the CWC related samples analysis, DGA CBRN Defense needs to develop and validate their own methods for the analysis of CWC chemicals and related compounds. Though GC-MS analysis allows performing full spectra screening of environmental samples, it is often limited by the concentration levels that can be detected with a single quadrupole mass spectrometer (50 ppb to 1 ppm in complex matrices). Use of a triple quadrupole mass spectrometer can lower the limit of detection and allow detecting below 10 ppb. However, its primary disadvantage is the necessity to know the compound to detect before the analysis in order to apply the adequate method/transitions.

In order to enlarge the laboratory's analysis capacity for CWC related compounds as trace levels (ppb range), it was chosen to identify 2 diagnostic transitions per family for the screening of O,O-dialkyl alkylphosphonates in environmental samples.

Three phosphonates' families were chosen: O,O-dialkyl methyl-, ethyl- and isopropyl-phosphonates. Optimization was realised in compliance with our quality management system.

In conclusion, at least three diagnostic transitions were selected for each tested compound and validated for most of them. Common diagnostic transitions for each three families (excluding O,O-dibenzyl alkylphosphonates) were identified and implemented in a screening GC-MS/MS method.

Further work will focus on estimating detection limits with this screening method and selecting diagnostic transitions for other CWC chemicals and related compounds in order to facilitate screening at sub-ppm concentration levels in complex matrices.

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Authors

Gaiao Lousa Marie [1], Spiandore Marie, Dissard Jacky

1. DGA (France)

Identification of Ricin by Immunocapture Extraction and LC-MS/MS or LC-MS/HRMS Analysis [P31]

Ricin is a naturally occurring toxin found in the seeds of the castor oil plant Ricinus communis whose high toxicity has been known for centuries. The inclusion of ricin in several weapon programs during World War II under the code name "compound W" and its potential use as a bioterrorism agent has led to its prohibition under both the Chemical and the Biological Weapons Conventions. As part of our Chemical Defence mission, we have developed a method to detect the presence of ricin in environmental and food samples which combines immunocapture-based ricin extraction and LC-MS/HRMS analysis.

Ricin is a water-soluble type 2 ribosome-inactivating protein composed of a catalytically active A-chain covalently linked to a sugar-binding B-chain via a single interchain disulphide bond. The sugar-binding property of the B-chain was used to selectively bind the ricin contained in aqueous samples to magnetic beads coated with in-house produced antibodies directed against the B-chain of ricin. The extracts obtained after samples clean-up and tryptic digestion were analysed by LC-MS/HRMS to detect ricin proteotypic peptides.

We were able to validate a method that allows detection and identification of ricin as well as discrimination between ricin and RCA 120, a much less toxic dimeric protein with high sequence similarity to ricin also found in castor beans, and abrin. However, once ricin has been identified in a sample it remains to be seen whether it is still biologically active. For that purpose, a second method was developed which combines a comparable immunocapture step with detection by LC-MS/MS of the adenine released during the depurination of a RNA substrate catalysed by the A-chain of ricin. Both methods were successfully applied to unknown liquid and solid samples during the second OPCW (Organisation for the Prohibition of Chemical Weapons) biotoxin exercise.

Authors

Mappa Charlotte [1], Enche Julien, Gordien Andréa, Favino Clotilde, Giral Christophe, Desoubries Charlotte, Dubois Cindy, Montauban Cécile, Morineaux-Hilaire Valérie, Bossee Anne

1. DGA (France)

Characterization of agro-food protein hydrolysates: challenge for short peptide identification (2 to 6 AAs). [P32]

Introduction

Agro-food industries generate significant amounts of protein rich co-products [1]. One means to valorize these co-products is through the generation of bioactive peptides using proteolysis [2]. Proteolysis results from nonspecific enzyme digestion or chemical hydrolysis leading to the generation of short peptides. The identification of peptides > 6 amino acid length within protein hydrolysates is a routinely achievable task.

However, identification of di-; tri-; tetra- and penta-peptides within complex protein hydrolysates is quite challenging when using conventional proteomic tools.

Methods

Within this study, we firstly illustrate the possibility of employing a modified C18 column (C18AQ), which is stable under 100% H2O elution conditions, to enhance the chromatographic retention of short peptides without the need for derivatization. Secondly, (**i**) the accuracy of *de novo* sequencing of short peptides using Peaks Studio software was evaluated and (**ii**) validation of *de novo* candidates with a retention time prediction model was assessed [3].

Results

The C18 chromatographic retention of 60 synthetic peptides was studied using 2 different reverse phase (RP) columns (C18 and C18AQ). As expected, the C18AQ enhanced the retention and recovery of short hydrophilic peptides (95% of peptides were retained) while the majority of these peptides were eluted during the first 5 min of the elution gradient with the C18 column.

The accuracy of Peaks Studio *de novo* sequencing and its scoring system, i.e. Average Local Confidence (ALC) score, was evaluated. The *de novo* identifications were impacted when a fixed ALC score was applied. A high ALC score (> 90%) led to the correct sequencing of 21/24 tetrapeptides. In contrast, a very low identification rate for di- and tri-peptides (1/22) was observed. Concomitantly, 7/10 pentapeptides were correctly sequenced while the remaining three pentapeptides false sequence options were proposed. Inversely, when the ALC score was reduced to 80%, the correct *de novo* sequencing of di- and tripeptides was enhanced but with an increased number of incorrect identifications.

Conclusions

The use of modified RP chromatography can help to improve the retention and recovery of short peptides. *De novo* sequencing seems to be a good alternative to more conventional proteomic algorithms for short peptide identification. However, *de novo* sequencing may lead, in certain instances, to incorrect sequencing and false candidates. For this reason, the use of retention time prediction models is recommended as a final validation step.

Authors

Belurier Eva [1], Theysgeur Sandy [1], Cudennec Benoit [1], Nongonierma Alice B [2], Ravallec Rozenn [1], Flahaut Christophe [1], Fitzgerald R.j. [2], Deracinois Barbara [1]|Institut Charles Viollette (ICV) - EA 7394 (France)

1. Department of Biological Sciences. University of Limerick, Castletroy, Limerick. (Irlande)

Development of a simple and fast method to quantify pyrethroids in meconium by an LC-MS / MS approach. [P33]

Introduction

The impact of endocrine disrupters, and especially pesticides, has been demonstrated in several studies in the occurrence of cancers, neurological disorders or disorders on reproduction or development. The impact of these compounds on the developing fetus, which is therefore very vulnerable, is now a major concern. Some pesticides, such as pyrethroids, are chemical contaminants used in industry, agriculture or household products. They are now present in all environmental compartments and thus contaminate the entire food chain to humans.

Numerous extraction methods and assays have been developed in order to target different families of pesticides without, however, having optimal conditions, particularly in terms of sensitivity and specificity for chemically different compounds (Berton et al., 2014). We have taken the bias of developing and optimizing a method of extraction and dosage to characterize the impregnation of the fetus specifically pyrethroids. This targeted pesticide research will be conducted in meconium to be closest to the actual exposure of the fetus during pregnancy. Indeed, the latter has been identified as the most sensitive matrix for detecting the accumulation of pesticide metabolites throughout pregnancy (Ostrea et al., 2008).

For this we relied on tandem mass spectrometry coupled with multiple Reaction Monitoring (MRM) to simultaneously measure the five most used pyrethroids (trans-permethrin, cis-permethrin, cypermethrin, cyfluthrin and deltamethrin).

Once the final protocol has been established, a validation step has been set up in order to control the

linearity, the matrix effect, but also to determine the limits of quantification, the limits of detections, the precision as well as the accuracy of each of the pyrethroids studied. The method thus developed made it possible to set up for the first time a quick and simple extraction and assay of all the targeted compounds with analytical performances adapted in terms of sensitivity and specificity.

Methods

The extraction was performed by liquid/liquid extraction. Chromatographic conditions for pyrethroids their metabolites were optimized using different analytical columns, mobile phases and way of elution.

The final method was validated for recovery, specificity, linearity, precision and accuracy before its

application to quantify samples of a toxicokinetic study in pregnant rats exposed daily to permethrin.

Results

Chromatographic separation was accomplished on a HSS T3 column with a gradient elution system. Two different mobile phases were used. For pyrethroids, the mobile phase consisted of ammonium acetate buffer and acetonitrile. For metabolites, acetonitrile and water containing 0.1% of formic acid were selected.

Relative recoveries were found to be in the range of 80 to 104% for pyrethroids and 73% to 82% for

metabolites. No interference from other components in the matrix were observed at the retention times of the compounds.

Matrix-matched calibration curves had satisfactory linearity up to 2000 ng/mL. The LOQ ranged from 1 to 250 ng/g for the compounds on the matrix. The intra-and inter-batch precision and accuracy were better than 15%.

Conclusions

A novel simple and rapid method was developed to quantify pyrethroids in meconium by an LC-MS / MS approach.

Novel Aspect

This is a new simple approach to detect and quantify pyrthroids in a complex mixture and to evaluate fetal contamination during pregnancy.

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Keywords

Meconium, Quantification, Pyrethroids

Authors

Baltora Elise [1], Nagorski Ingrid [1], Personne Stéphane [2], Chardon Karen [2], Marcelo Paulo [1]

1. Plateforme ICAP (France)
2. UMI01 INERIS - PERITOX (France)

Characterization of key aroma compounds in Burgundy truffle [P34]

Introduction

Truffles have an important economic value due to their gastronomic qualities appreciated in « grande cuisine ». While Perigord and White Alba truffles are well-valued, Burgundy truffle (*Tuber uncinatum*) is not well-characterized in its production area.

INRA is involved to help the producers to better characterize this truffle through different research axes, especially the influence of the ripeness and the geographical origin on aromatic composition [1]. For this purpose, we had first to define an analytical strategy to better characterize aroma compounds in this noble fungus.

Methods

Burgundy truffles were analyzed by Solid Phase MicroExtraction (SPME) [2] and Dynamic HeadSpace (DHS) [3] coupled with Gas Chromatography - Mass Spectrometry (GC-MS) for volatile organic compounds (VOC) identification. A new *in vitro* analytical method by Proton Transfer Reaction – Mass Spectrometry (PTR-MS) [4] was developed to obtain an aroma mass fingerprint of all the samples. In addition, sensory analyses were performed by a trained panel.

Results

Truffles are very aromatic, and 1 g of fungus is sufficient for GC-MS analyses. The DHS parameters were defined through a kinetic study: an incubation temperature of 36°C for 15 min was selected. The influence of the trapping and drying steps were studied to set the best parameters and to enable the identification of more than 70 compounds. The SPME technique allowed to better extract the most volatile compounds but does not allow to identify as many compounds.

Depletion of hydronium ions was observed by PTR-MS during the analysis of 25 mg of truffles. While it is not possible to weigh less without generating an error in the repeatability, a headspace dilution system with a humidified airflow has been developed to permit VOC analysis. After integrating the peak areas, the data were compiled to observe the evolution of the areas depending on time and incubation temperature. An analysis of variance (ANOVA) was performed to determine the significant differences between the different conditions and to propose the best methodology for fingerprint analysis.

Conclusions

This study enable the development of an analytical methodology to better characterize the Burgundy truffle. It is part of a broader project aimed at (i) Correlate the data from PTR-MS, GC-MS and sensory analyses to define what is a quality truffle (ii) Cross this results with genetic and microbiota investigations for a scientific contribution to the constitution of an IGP (Protected Geographical Indication) request.

Novel Aspect

This work leaded to methodological development for *in vitro* analysis of truffle by PTR-MS. It also allowed to create a new analytical protocol for DHS on our Gerstel equipment.

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Keywords

Truffle, PTR-MS, GC-MS

Authors

Lucchi Géraldine [1] [2], Reynaud Rémy [1], Gourrat Karine [1] [2], Andriot Isabelle [1] [2], Cordelle Sylvie [1] [2], Berdeaux Olivier [1] [2]

1. Centre des Sciences du Goût et de l'Alimentation [Dijon] (France)
2. Plateforme ChemoSens (France)

Optimization of polar antibiotics analysis by Liquid Chromatography – tandem Mass Spectrometry [P35]

Introduction

Aminoglycosides are used as veterinary antibiotics. In France, their consumption doubled in the last decade, owning 10% market share in animal medication in 2016 [1]. Because of antibiotic resistance risk, their analysis in water systems is necessary. Due to their high polarity (logP below -3), two main separation mechanisms are mainly used: Hydrophilic Interaction Liquid Chromatography (HILIC) and Ion-Pairing Chromatography (IPC). Heptafluorobutyric or trifluoroacetic acids are often used for IPC, although they are known to produce signal suppression in mass spectrometry.

Methods

Linear alkyl sulfonates (from C4 to C10) were used as IP reagents in the injection vial, adapted from Lehotay et al. [2] and LCGC [3]. Separation was performed with an XB-C18, on a 1200 HPLC system (Agilent) coupled to a 5500 Qtrap mass spectrometer (Sciex). Elution was based on acetonitrile (ACN), methanol (MeOH) and water, each with 0,1% formic acid. Electrospray ionization in positive mode and detection in Multiple Reaction Monitoring mode (MRM) were used.

Results

Three crucial parameters were optimized to obtain the best separation of seven aminoglycosides (AGs): Organic mobile phase, IP reagents and equilibrium study.

First, three organic mobile phases were tested: MeOH, ACN, and 1:1 MeOH: ACN. Standards vials were injected with each different IP reagent at a 50mM and one was left without IP. No retention occurred without IP. With IP, retention increased with the alkyl chain length, as hydrophobicity increased. The MeOH/ACN solution exhibited the best resolution.

Retention factors (k) were plotted against IP reagent chain length and only C6 and C7 gave k between 2 and 10. Mixtures of both, with different proportions were made (C6/C7). While both 50/50 and 25/75 mixtures resulted in a similar resolution, the former resulted in a faster separation, in 5 minutes.

Because IP salts were used, importance was given to column equilibration. After a cleaning of the column to remove previous salts, 6 injections of standards were realized consecutively. Retention equilibrium was achieved after 5 injections.

Conclusions

This method allows IP separation of AGs using low concentrations of IP reagents. Optimization of the method led to using a 1:1 ratio of C6 and C7 IP salts directly added in the injection vial. Both retention and peak shapes were improved with a 1:1 MeOH: ACN mixture as the organic eluent. As the time of equilibration is an important parameter when working with IP, it was also optimized. Instrumental limits of quantification obtained were between 0.5 and 15 µg/L.

Novel Aspect

Adding ion-pairing reagents in the vial instead of in the mobile phase results in less signal inhibition in the mass spectrometer. Optimized separation further improves sensitivity.

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Keywords

Aminoglycosides, Ion-Pairing, LC-MS/MS

Authors

Guironnet Alexandre, Sanchez-Cid-Torres Concepcion, Timothy Vogel, Wiest Laure, Vulliet Emmanuelle [1]

1. Institut des Sciences Analytiques, UMR 5280 CNRS - Université de Lyon 1, 5 rue de la Doua, 69100 Villeurbanne, France (France)

Analytical protocol development for the monitoring of pesticides in waterfowls' eggs [P36]

Introduction

In France, the surface of lakes and ponds is larger than that of rivers [1]. These structures play an important role in biodiversity preservation as waterfowls came to eat and to reproduce. However, they are often surrounded by fields and can be contaminated with pesticides [2]. These molecules can potentially accumulate in organisms and contaminate eggs which can compromise reproduction or development.

The aim of this study was to develop an analytical protocol for the characterization of the pesticide contamination of aquatic bird eggs.

Methods

A solid/liquid extraction and an LC-MS/MS method were developed to characterize 18 pesticides and 28 transformation products, dealing with wide range physicochemical properties (ex: logP from -1.4 to 7.4). Eggs from hens kept in battery were used as blank and as reference matrix when spiked with analytical standards. In anticipation of future sample management logistics, eggs were individually weighted, opened, mixed, lyophilized and sieved before extraction.

Results

Sample preparation was based on a solid/liquid extraction and optimized to remove interferences. 0.5g of eggs supplemented with internal standards, were first hydrated with 5 mL of water. Then, acidified acetonitrile (ACN) and heptane were mixed with the sample to perform extraction and remove lipids. The water/ACN mix was finally separated by centrifugation from heptane and solid fraction, and purified on MgSO4. Final extract was then diluted 1/10 in acidified water before injection.

The linearity was certified on 6 concentrations (from LOQ/2 to 10 LOQ) including blank. A double approach based on matrix match and internal standards quantification were undertaken in order to correct for recoveries and matrix effects. Blanks were clean for all compounds but fipronil, fipronil sulfone, s-metolachlor, prosulfocarb and terbutryn which have a nanogram range contamination (0.1 – 2.4 ng). Quantification limits were inferior to 10 ng/g for 34 pesticides and quantification yields superior to 75% for 33 ones, including the most polar ones.

Conclusions

This study provided both a reliable and robust method for the characterization of pesticides in eggs. The definitive protocol has quantification limits and a field of application compatible with traces analysis. Moreover, one of its strength is to be relatively simple to implement.

The next step of this study is to confirm the analytical performance on real samples, probably more complex than hens eggs.

Novel Aspect

This study is the first to focus on eggs for a large list of pesticides with a broad range of physicochemical proprerties and a special focus on polar transformation products.

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Keywords

Pesticides, eggs, LC-MS/MS

Authors

Dufour Vincent, Wiest Laure [1], Slaby Sylvain, Le Cors François, Auger Lucile, Cardoso Olivier, Curter Laurence, Pasquini Laure, Dauchy Xavier, Banas Damien, Vulliet Emmanuelle [2]

1. Institut des Sciences Analytiques (France)
2. Institut des Sciences Analytiques, UMR 5280 CNRS - Université de Lyon 1, 5 rue de la Doua, 69100 Villeurbanne, France (France)

Human Chorionic Gonadotropin glycoforms heterogeneity from two geographically distinct populations [P37]

Introduction

The hCG is an αβ heterodimeric glycoprotein that is glycosylated by up to 4 N-glycans and 4 O-glycans across 2 subunits. It is a hormone secreted by women in earliest weeks of pregnancy that acts on corpus luteum stabilization and progesterone secretion. hCG prenatal concentration is monitored as a biomarker for abnormal pregnancy, but its glycosylation is often overlooked despite a correlation with its biological and chemical properties, and patient's origin [1].

Thus, the characterization of hCG glycoproteoforms is crucial for relevant biotherapeutics design and diagnostic.

Methods

Aminoquinoline and coumaric acid were used as a one-pot MALDI N-glycan-derivation matrix [2], and sample preparation was optimized in order to compare N-glycosylation across different samples. Both N- and O-glycosylation were also assessed at the glycopeptide level using bottom-up glycoproteomics, introducing developments in sample proteolysis using pronase and mass spectrometry stepped-energy fragmentation [3].

Results

hCG glycosylation was explored by using two different approaches: glycans analysis by MALDI-MS and glycopeptides analysis by LC-ESI-MS. A MALDI derivation matrix protocol was optimized for sensitive characterization of released N-glycan that can be used for the high-throughput glycohormone analysis of large cohorts of samples. Moreover, we successfully used nonspecific proteolysis, stepped-energy fragmentation, and custom data processing methods to increase the confidence in glycopeptides identifications, especially for O-glycosylation sites that are closely packed on a 17 amino-acids region. This whole workflow was applied to hCGs from different populations and showed disparity between them: some GalNAc-containing N-glycans as well as trisialylated O-glycans might be examples of exclusive glycans, identified on one hCG only, that needs to be biologically tested.

Conclusions

In this study, the analysis of chorionic gonadotropins (CGs) glycoforms successfully allowed to discriminate samples according to their glycan composition using either MALDI N-glycan fingerprinting or shotgun glycoproteomics. Those developments improved chorionic gonadotropins glycosylation characterization in order to help future structure-function investigations.

Novel Aspect

Glycoprotein hormone structural characterization using pronase digestion combined to stepped-HCD and N-glycan MALDI fingerprinting with all-in-one derivation matrix

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Keywords

Glycosylation, Bottom-up proteomics, MALDI-TOF/TOF

Authors

Eskenazi Nicolas [1], Vinh Joëlle [1], Giangrande Chiara [1], Djimatey Ophélia [1]

1. ESPCI Paris, PSL Research University, Spectrométrie de Masse Biologique et Protéomique (SMBP), CNRS USR 3149 (France)

SPECIFIC DETECTION OF CYSTEINE SULPHENIC ACID BY COUPLING MASS SPECTROMETY WITH LASER INDUCED DISSOCIATION [P38]

In a context of population aging, discovery and validation of novel oxidative stress biomarkers for screening of neurodegenerative diseases is a key issue. One of the modification induced by reactive oxygen species (ROS) is the oxidation of protein cysteine (Cys) residues [1]. As this oxidative process is minor, the detection of these oxidized proteins at low concentrations is problematic due to the complexity and dynamic concentration range of the samples to be analyzed.

In order to improve the detection specificity for oxidized proteins, we use an experimental setup coupling mass spectrometry and laser induced dissociation (LID) in the visible range (473 nm) to add a stringent optical specificity to the mass selectivity [2]. Since peptides do not naturally absorb in the visible range, this new methodological approach relies on the proper chemical derivatization of oxidized Cys with a chromophore. In the presence of ROS, the Cys are oxidized in sulfenique acid SOH. These SOH groups are specifically grafted, via a cyclohexanedione group [3], with a Dabcyl chromophore which absorbs at 473 nm.

First tests were performed on a standard Cys containing peptide oxidized with oxygen peroxide (H2O2). Several factors influencing oxidation were highlighted: pH of the sample, reaction temperature, time as well as the amount of H2O2. Strongest quantity of H2O2 induced an over-oxidation of Cys into sulfonic acid SO3H and dimer formation. A 1:100 cys : H2O2 ratio is actually used in order to prevent this phenomenon and optimize the formation of mono-oxidized Cys with a yield of 1.4 %. Derivation with Dabcyl cyclohexanedione chromophore is carried out in parallel with Cys oxidation, at a 1:1 ratio, stabilizing the mono-oxide form. Increasing the chromophore amount (ratio > 1:5) only improves the derivation speed without impact on the quantity of grafted SOH. After reaction, the peptide with oxidized Cys and labelled with the chromophore is observed. This ion fragments in LID at 473 nm with a 99 % yield. b fragment ions allowing the peptide sequencing are produced, as well as a report ion arising from internal fragmentation of the Dabcyl chromophore.

The derivation is specific because only the mono-oxidized (SOH) peptide is derivatized. Non-oxidized or over-oxidized Cys peptides do not react with cyclohexadione and remain intact in LID, as they do not absorb the laser energy. This methodology is currently applied for a protein mixture. After enzymatic digest, only the oxidized Cys peptides derivatized with the chromophore will be specifically fragmented in LID.

We propose an innovative experimental setup based on mass spectrometry coupled with visible laser induced dissociation for specific detection of oxidized cysteines.

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Keywords

laser induced dissociation, cysteine oxidation, chromophore derivatization

Authors

Guillaubez Jean-Valery [1], Pitrat Delphine [2], Bretonniere Yann [2], Lemoine Jérôme [1], Girod Marion [1]

1. Institut des Sciences Analytiques (France)
2. Laboratoire de Chimie - UMR5182 (France)

Comparison of various fragmentation modes (CID, HCD, ETD, UVPD) for the characterization of intact proteins [P39]

Introduction

Top-down mass spectrometry offers important advantages for the characterization of intact proteins or proteoforms. Achieving comprehensive sequence coverage is required to characterize precisely primary sequences and map post-translational modifications. In this work, we sought to evaluate the benefit of the various fragmentation modes available on the Orbitrap Fusion Lumos, and in particular UVPD (Ultraviolet Photodissociation) for the characterization of mixtures of intact proteins.

Methods

LC-MS/MS experiments were performed using an Orbitap Fusion Lumos mass spectrometer. Two types of sample were tested: the Pierce Intact Protein Standard Mix and a lysate of *E. coli*. Intact proteins were separated using either a Proswift C4-RP-5H column (100 µm x 50 cm) or C4 in-house packed column (5 µm, 60 cm) with different gradients. The fragmentation modes tested were: CID, HCD, ETD, EThCD and UVPD. Data processing was performed using Xtract and Prosight Lite.

Results

The Pierce Intact Protein Standard Mix is an ideal sample for method development in top-down mass spectrometry, with proteins ranging from 9 kDa to 68 kDa. We set up various fragmentation modes to test their impact on all protein sequence coverage and identification score: CID (26%, 30%, 38%), HCD (10%, 14%, 18%), ETD (2 ms, 10 ms, 18 ms), UVPD (6 ms, 14 ms, 26 ms, 35 ms, 50 ms). Excellent sequence coverage and P-Score were obtained for proteins up to 30 kDa, the best results being for ETD 10 ms and UVPD 35 ms or 50 ms. For larger proteins, lower sequence coverage was achieved. However specific fragments were always obtained with UVPD and combining multiple fragmentation modes showed an added value in all cases. For the analysis of the *E. coli* lysate, we also compared the results of UVPD with other fragmentation modes in term of number of identified proteins/proteoforms and not only sequence coverage.

Conclusions

The multiple modes of dissociation available on the Orbitrap Fusion Lumos are very advantageous for intact protein analysis allowing high sequence coverage to be achieved. Our data indicate that UVPD alone provides unique fragments in many cases. Moreover, combining methods (up to 4) allows an increase in sequence coverage useful to characterize proteoform.

Novel Aspect

Thorough evaluation of multiple dissociation methods on the same instrument for the analysis of intact protein mixtures.

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Keywords

Fragmentation, intact proteins, Ultraviolet Photodissociation

Authors

Garcia Camille [1], Dupré Mathieu [1], Chamot-Rooke Julia [1]

1. Unité Spectrométrie de Masse pour la Biologie, Institut Pasteur, CNRS USR2000, Paris, France (France)

All Ion Laser Induced Dissociation in the C-Trap of a Q Exactive: Differential Mass Spectrometry as the Next Analytical Step with Photodissociation Methods [P40]

Introduction

Nowadays, one of the major struggle of bottom-up proteomics in data independent analysis (DIA) arises from the complexity of the fragmentation patterns, which induces interfering signal. The extreme specificity provided by laser induced dissociation (LID) in the visible range (473 nm), after chromophore derivatization, was used to streamline the detection of cysteine-containing proteins in DIA mode [1]. However, this approach requires to build large spectral libraries, as theoretical databases cannot be used for general searching.

Methods

The adopted laser ON-laser OFF approach consists in alternating full MS with and without laser irradiation. One can then build differential mass spectra from each pair of MS by subtracting one from another. Only the ions which intensity vary due to LID remain, while others, non-affected by the laser, disappear. The idea is to follow a DIA experimental procedure but fall back on a DDA analysis method, relieving the need for the initial constitution of a spectral library.

Results

First, instrument-wise, LID at 473 nm of derivatized Cys-proteins is now performed in the C-Trap rather than the HCD cell. Among multiple pros: (i) the duty cycle is improved since ion accumulation and LID are performed simultaneously during the orbitrap analysis of the previous ion bunch (ii) the collisional fragmentation due to transmission into the HCD cell (even at minimum energy) is suppressed, (iii) the trigger scheme becomes straightforward, (iv) the method can be applied to any generation of orbitrap.

Second, on the data-analysis side, a workflow is being worked on to give differential MS their full analytical power. Differential spectra spotlight as negative peaks the derivatized ions, which are specifically photo-fragmented, and as positive peaks their appearing LID fragments. Since the LID of dabcyl-tagged peptides produces classical b-/y- backbone fragments [2], the goal is to use differential MS to simultaneously identify the mass of the parent ion and list its fragments in order to submit a classical database search query.

Conclusions

The combination of LID in the C-Trap and differential analysis pushes photo-dissociation-based identification methods beyond the restriction of existing spectral libraries. The combination of DIA experiments with classical database search for protein sequencing may provide a best-of-both-worlds alternative in terms of identification potential. The differential MS workflow may be implemented on any mass spectrometer providing full MS capabilities.

Novel Aspect

LID in the C-Trap of orbitrap-type mass spectrometer. Automated database search from LID-differential MS.

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Keywords

Laser-induced dissociation (LID) ; Chromophore derivatization ; Identification from database search

Authors

Macaleese Luke [1], Dagany Xavier [1], Garcia Lény [2], Lemoine Jérôme [2], Dugourd Philippe [1], Girod Marion [2]

1. Institut Lumière Matière (France)
2. Institut des Sciences Analytiques (France)

The potential of charge transfer dissociation in structural biochemistry [P41]

Introduction

The generic fragmentation method used in most tandem MS experiments (low-energy collision-induced dissociation, LE-CID) shows clear limitations and is often insufficient to retrieve some essential structural information on carbohydrates. Our group has explored several alternative mass spectrometric methods towards more-complete structural characterization. This communication presents a setup that uses highly accelerated helium cations as a way to activate ions for tandem MS and shows considerable improvement over existing methods for the structural analysis of carbohydrates.

Methods

Charge transfer dissociation using kiloelectronvolt helium cations (He-CTD) [1] was achieved on a quadrupole ion trap mass spectrometer (amaZon SL, Bruker Daltonics, Bremen, Germany). High-energy dissociation was achieved within a total scan time of approximately 200 msec, including isolation/accumulation, activation, cooling, and scanning of fragments. These measurement times are compatible with the direct coupling with UHPLC.

Results

Originally introduced by the group at West Virginia University, He-CTD was successfully implemented on a second quadrupole ion trap at INRA in Nantes. The modified instrument was used to study carbohydrate products resulting from the enzymatic modification of a synthetic substrate. The use of carbohydrate-active enzymes to generate functional oligosaccharides is an attractive alternative to chemical pathways. In fact, enzymatic functionalization is specific, thus preventing undesired modifications of the polymer structure and maintaining labile modifications like sulfate groups.

Compared to CID, He-CTD produces a rich fragmentation of functionalized carbohydrates, with exhaustive structural information brought by many informative cross-ring fragments [2,3]. With the capability of He-CTD to be coupled to UHPLC, the setup led to the efficient characterization of isomers in mixture and revealed expected and non-expected products.

Conclusions

We demonstrated the feasibility and high performance of He-CTD in the field of structural glycosciences. The short acquisition times achieved with He-CTD and the affordable implementation of this technique in a conventional laboratory make it possible to envisage LC-MS/MS approaches through He-CTD activation for a variety of other biological applications that address high molecular and structural complexity.

Novel Aspect

High-energy activation in tandem MS is accessible with reasonable cost and ease of implementation, with performance making it compatible with UHPLC methods.

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Keywords

High-energy activation, online coupling with UHPLC, carbohydrate isomers.

Authors

Ropartz David [1], Mathieu Fanuel [2], Jackson Glen [3] [4], Rogniaux Hélène [2]

1. UR1268 Biopolymères Interactions Assemblages, INRA, F-44300 Nantes (France)
2. UR1268 Biopolymères Interactions Assemblages, INRA, F-44300 Nantes (France)
3. Department of Forensic and Investigative Science, West Virginia University, Morgantown, WV 26506-6121, USA. (États-Unis)
4. C. Eugene Bennett Department of Chemistry, West Virginia University, Morgantown, WV 26506, USA. (États-Unis)

Recherche Pharmaceutique

Expanding LC-MS beyond reverse-phase chromatography to characterize biologics [P42]

Traditionally for biologics, the chromatography, which is coupled to electrospray mass spectrometry, is reversed phased based since it is well suited for separation of peptides, proteins and antibodies. However, for some aspects this type of chromatography is not well adapted for the analysis, especially if differences are based on charge or size rather than hydrophobicity, or if the molecular entity analyzed is dissociated because of the denaturing conditions, (e.g. high organic solvent content, acidic solutions) employed in such analyses.

We have other the last years expanded the chromatographic space by including methods different form the traditional reverse phase chromatography and coupled to Electrospray:

Denaturing SEC-MS to differentiate half-antibodies from covalently linked bispecifics.

HILIC-FLR-MS/MS for released glycosylation identification and quantification.

CE-MS (charged based separation by ZipChip) for analysis of deamidation, iso-Asp, charge variants and large multimeric molecules.

Native SEC-MS to analyze non-covalent complexes.

These methods do nicely complete our standard LC-MS methods for the characterization of biologics, which are becoming more and more complex.

Examples illustrating the capabilities of these new methods in our characterization pipeline of biologics will be shown in this presentation and the advantages and disadvantages discussed.

Authors

Schindler Patrick [1]

1. Novartis Institutes for BioMedical Research (Suisse)

An innovative analytical strategy for biotransformation reactions investigation using the combination of MS and NMR data with chemometrics [P43]

Introduction

The ability of microorganisms such as filamentous fungi to mimic human metabolism or to generate new chemical entities plays an important role in the drug discovery process [1]. Indeed, microbial reactions can be used for metabolite structural confirmation and also, due to their regio- and enantioselectivity, to replace complex multistep synthesis. However, the biotransformation media are often very complex and it could be interesting to develop original analytical strategies to monitor and optimize the production of target metabolites.

Methods

To investigate and optimize phase I biotransformations of a potential non-imidazole histamine H3R antagonist [2], we have developed a MS, NMR and chemometrics integrated approach [3]. The analytical workflow includes: biotransformation protocols, sample preparation for MS and NMR, MS and NMR acquisitions and processing, multivariate analysis for MS and NMR data correlation.

Results

First, an automatic screening of eleven fungal strains was carried out by UHPLC-ESI-HRMS and principal component analysis (PCA) allowing the selection of the best candidates for phase I metabolites production. Then, the characterization of the metabolites contents was performed by MS/MS fingerprints and ion mobility separations (IMS). Subsequently, MS (tR, m/z) and NMR (1H and JRES) data were associated using a Consensus OPLS-DA multiblock [4] approach giving more precise information about biotransformation products formation and nutrients consumption. Finally, an experimental design based on the reexamination of MS data using a response surface methodology [5] (RSM) was set up to optimize the production of hydroxylated metabolites.

Conclusions

This study, demonstrated how MS and NMR acquisitions combined with statistics (PCA, Consensus OPLS-DA multiblock, RSM) offer a convenient tool to pharmaceutical industry for a better understanding and monitoring of complex biotransformation mixtures. We demonstrated the efficiency of our approach to refine the cultivation parameters (concentration, pH and kinetic) for phase I metabolites production.

Novel Aspect

Combination of MS and NMR data with chemometrics for fungi biotransformation reactions optimization.

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Keywords

Biotransformation, MS/NMR, Chemometrics

Authors

Palaric Cécile [1], Rigaud Sébastien [2], Mathiron David [2], Cailleu Dominique [2], Boccard Julien [3], Fontaine Jean-Xavier [4], Molinie Roland [4], Beaumal Jean-Yves [5], Gut Yoann [6], Renaud Tristan [1], Petit Alain [7], Pilard Serge [2]

1. Département Chimie Structurale (France)
2. Plateforme Analytique (France)
3. School of pharmaceutical Sciences, University of Geneva (Suisse)
4. BIOPI EA3900 (France)
5. Département Synthèse (France)
6. Département de Formulation Galénique (France)
7. Pôle d'Expertise Développement Pharmaceutique (France)

Insights from size exclusion chromatography with native mass spectrometry and ion mobility (SEC-native IM-MS) approaches for top- and middle- level characterization of anti-HIV trispecific antibody. [P44]

Introduction

Among all the MS-based techniques, native MS and its hyphenation to ion mobility (IM) have recently been successfully applied for the characterization of intact mAb-related product [1].

Here, we report the analytical characterization, using native MS approaches, of an empowered trispecific broadly antibody against HIV-1. We applied size exclusion chromatography (SEC) hyphenated to native MS and IM-MS for an in-depth characterization at the intact level and middle level after enzymatic digestion under non-denaturing conditions, especially to unravel conformer identification.

Methods

The LC system consists of the UHPLC Acquity H-Class (Waters) hyphenated to a Synapt G2 HDMS mass spectrometer (Waters). The SEC column used was an Acquity BEH SEC 200 Å, 4.6 x 300 mm, 1.7 µm) from Waters. The mobile phase was composed of 100 mM ammonium acetate pH 6.8. For middle level analysis, the enzymatic digestion was performed by incubating overnight at 37°C one unit of FabALACTICA (Genovis) per microgram of trispecific antibody.

Results

We first analyzed trispecific antibody by online SEC-native MS approach to perform intact mass measurement in a rapid and automated way [2]. Surprisingly, two SEC peaks were observed and were both identified by native MS as monomers. Conformational investigation through SEC-native IM-MS was performed, highlighting a difference of Collisional Cross Section (TWCCSN2) for the two conformers previously separated by SEC, probably explained by chemical interaction with SEC material. The conformers are originating to a mixture of isomeric forms of YPP motif located on Fab subunit. In order to confirm the YPP moiety is at the origin of the unexpected SEC behavior, we next performed SEC-native IM-MS experiment after FabALACTICA digestion, generating smaller sub-unit. Three main peaks were separated by SEC and identified by native MS as Fab1, coeluted Fc and Fab2 and another Fab1 subunit. Conformational characterization was also performed, where conformational differences in gas phase in terms of TWCCSN2 were observed for the two separated Fab1.

Conclusions

Altogether, our results demonstrate the ability to have comprehensive analytical characterization under non-denaturing conditions of new empowered trispecific antibody with SEC-native IM-MS methodology. SEC-native IM-MS at intact level revealed the presence of two conformers and experiment performed after enzymatic digestion allowed to localize the YPP moiety involved in the generation of conformers in Fab1 smaller sub-unit.

Novel Aspect

Characterization of new empowered trispecific antibody and two conformers originating by YPP residues with SEC-native IM-MS methodology at intact and middle level.

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Keywords

Size Exclusion Chromatography, Native Mass Spectrometry, Tripecific AntiBody

Authors

Ehkirch Anthony [1], Lechat Nelly [2], Martelet Armelle [2], Genet Bruno [2], Cianferani Sarah [1]

1. Laboratoire de Spectrométrie de Masse BioOrganique (France)
2. BioAnaltytics (BioA) department France (France)

Comprehensive proteomic profiling of erythrocytes following hypotonic dialysis-based drug encapsulation process [P45]

Introduction

Erythrocytes represent an attractive and unique approach as a carrier for various therapeutic agents. Encapsulation of therapeutic agents in red blood cells (RBC) offers several advantages compared to free-form compounds such as natural biocompatibility, broad distribution in the organism, increased circulatory half-life and improved safety profile as demonstrated in clinical trials. Using a proprietary osmotic-based platform, Erytech encapsulates various agents within RBCs. Eryaspase, L-asparaginase (ASNase) encapsulated inside RBC, is an investigational product currently under clinical development in several tumor types. Despite the benefits demonstrated by the encapsulation technology in both pre-clinical and clinical studies, little is known about the impact of the encapsulation process on RBC properties. The goal of this study was to evaluate the potential impact of hypotonic dialysis-based ASNase encapsulation on the RBC proteome.

Methods

Absolute proteome quantification was achieved by high-performance mass spectrometry at the 3P5 proteomic platform. The proteome of eryaspase was compared to processed RBC without ASNase (proRBC) and to corresponding non-processed packed RBC (pRBC). Mean corpuscular hemoglobin content was used as a standard for absolute protein quantification [1]. For all conditions, “ghost” RBC were also manufactured and analyzed to increase protein detection sensitivity. Hierarchical clustering, matrix volcano plot, as well as principal component analysis were performed.

Results

The combination of whole and ghost RBC analysis allowed the identification and quantification of 1957 proteins. A 20%-decrease in total RBC protein content was observed after hypotonic dialysis. The protein concentration inside RBC wasn’t affected due to a concomitant decrease in RBC volume. Pearson correlation coefficient showed no significant difference in protein content following hypotonic dialysis, with or without ASNase encapsulation. Similar profiles were obtained after hierarchical clustering analysis, reflecting the low variability of protein content between samples. As expected, only encapsulated ASNase content was significantly different in eryaspase compared to pRBC and proRBC samples.

Conclusions

These results indicate that hypotonic dialysis-based drug encapsulation has no major impact on RBC proteome, with or without addition of ASNase. Thus, processed RBC maintain the same proteomic landscape compared to non-processed RBC.

Novel Aspect

Labeling and spiking-free absolute quantification of an exogeneous therapeutic protein in its cell protein matrix.

References

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Keywords

global absolute quantification; therapeutic protein; red blood cells; hemoglobin

Authors

Robert Mélanie [1] [2] [3], Piedrahita Diana [2], Gautier Emilie-Fleur [4] [5], Salnot Virginie [4] [5], Joly Philippe [6] [7] [8] [9], Connes Philippe [10] [7] [8], Guillonneau François [4] [5], Mayeux Patrick [5] [11] [4], Scheer Alexander [2], Cibiel Agnes [2], Laperrousaz Bastien [2]

1. Laboratoire interuniversitaire de Biologie de la Motricité (LIBM) EA7424, Team « Vascular Biology and Red Blood Cell », Université Claude Bernard Lyon 1, (France)
2. Erytech Pharma (France)
3. Laboratoire d'excellence : Biogenèse et pathologies du globule rouge (France)
4. Institut Cochin (France)
5. Plateforme Protéomique Paris Descartes (France)
6. Centre de Biologie et de Pathologie Est - Biochimie des pathologies érythrocytaires (France)
7. Laboratoire Interuniversitaire de Biologie de la Motricité (LIBM) EA7424, Equipe "Biologie vasculaire et du globule rouge" (France)
8. Laboratoire d'Excellence du Globule Rouge (Labex GR-Ex), PRES Sorbonne, Paris, France. (France)
9. Laboratoire de Biochimie et de Biologie Moléculaire, UF de biochimie des pathologies érythrocytaires, Centre de Biologie et de Pathologie Est (France)
10. Institut Universitaire de France (France)
11. Laboratoire d'excellence : Biogenèse et pathologies du globule rouge (France)

Quantitation of Human Insulin-Like Growth Factor-1 and 2 in Serum by MRM-LC-MS/MS [P46]

Variety of mass spectrometry-based workflows have been developed to quantify human insulin-like growth factor-1 (IGF-1) and human insulin-like growth factor-2 (IGF-2) in clinical laboratories. In this work we developed a novel sample preparation workflow utilizing CHAPs detergent assisted IGF-1 and 2 dissociation and protein precipitation with acidic alcohol, followed by solid-phase extraction (SPE) cleanup and MRM-LC-MS/MS analysis. The results exhibited good linearity in the range of 2 to 1000 ng/mL for both intact IGF-1 and 2 in mouse serum.

Authors

Levasseur Claire [1]

1. Sciex (États-Unis)

Isotopically resolved Analysis of Protein Subunits using High Resolution Accurate Mass [P47]

The number and complexity of biotherapeutics are increasing as the pharmaceutical industry seeks to address novel disease areas and develop novel therapies with greater efficacy. An emerging approach for these studies is the use of assays which require limited sample preparation but offer data which enable rapid assessment of key quality attributes with greater sample throughput. Specifically, protein subunit analysis performed on reduced complexes or those subjected to limited proteolysis have emerged as strong candidates. Of benefit are limited sample preparation burden and the ability to generate isotopically resolved mass spectrometric data which enable high throughput and accurate assessment of targeted post-translational modifications.

Authors

Levasseur Claire [1]

1. Sciex (États-Unis)

Separation and analysis of monoclonal antibodies at intact and middle-up levels by sheathless capillary electrophoresis-mass spectrometry [P48]

Capillary electrophoresis-mass spectrometry coupling is a growing technique in biopharmaceutics characterization. Assessment of monoclonal antibodies (mAbs) is well known at the bottom-up level to obtain information about the sequence and post-translational modifications (PTMs). Intact and middle-up levels of analysis is an actual challenge to be closer to the real protein structure. Actual techniques are time consuming or cumbersome processes. Biopharmaceutical compagnies need rapid and accurate method to complete the characterization of their products.

A 15 minutes separation method has been developed to optimize characterization of partially digested and intact monoclonal antibodies. Thus, separation has been done on a positively charged coated capillary with optimized volatile background electrolyte and sample buffer. Three world-wide health authorities approved monoclonal antibodies have been used to set up a rapid and ease of use method.

Intact trastuzumab, rituximab and palivizumab isoforms have been partially separated with this method in less than 20 minutes under denaturing conditions. Another method has been developed to also separate F(ab)'2 and Fc fractions of these mAbs. For each monoclonal antibody, 2X-glycosylated and 1X-glycosylated structures have been identified and separated. Major glycoforms have been recovered, arising results earned with orthogonal methods. Concerning basic and acidic variants, potential aspartic acid isomerization modification and asparagine deamidation have been observed. Middle-up separation allowed to get separated peaks of F(ab)'2 and Fc and MS permitted to detect low mass differences of each part suggesting the presence of post translational modifications (PTMs). A reducing step allowed to split the F(ab)'2 fragment in Fd fragment and light chain (LC). Shaper focus on location and identity of PTMs has been established to get complementary characterization of mAbs.

Accurate mass determination for high-mass molecular species remains a challenge, but the progress in intact monoclonal antibodies separation appears very promising for biopharmaceutics characterization. Intermediate level gives an illustration of the repartition of PTMs on molecules and allowed to sharpen mAb characterization. CE-ESI-MS seems to be able to propose solutions at each analytical level, reinforcing its image and promote new alternatives.

Here, the developed methods allowed partial separation of isoforms with no method adjustment between samples and in a short time scale (< 12 minutes).

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Keywords

Capillary Electrophoresis, Mass Spectrometry, Monoclonal Antibodies

Authors

Giorgetti Jeremie [1], Lechner Antony, Del Nero Elise, Beck Alain, Francois Yannis-Nicolas [2], Leize-Wagner Emmanuelle [1]

1. Laboratoire de Spectrometrie de Masse des Interactions et des Systemes (France)
2. Laboratoire de Dynamique et Structure Moléculaire par Spectrométrie de Masse, CNRS-UMR 7177, University of Strasbourg (France)

Development and validation of an UHPLC-MS/MS method for quantification of ibrutinib and its dihydrodiol metabolite in plasma and cerebrospinal fluid [P49]

Introduction

Ibrutinib is an orally administered first-in-class irreversible Bruton's tyrosine kinase (BTK) covalent inhibitor for the treatment of patients with B-cell malignancies. Several isolated clinical observations reported its efficacy in central nervous system dissemination [1-2]. In this work we described the development and validation of an ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) procedure for the quantification of ibrutinib and its active metabolite PCI-45227 in human cerebrospinal fluid (CSF) and plasma.

Methods

The compounds were eluted on a Waters BEH C18 column (50.0 x 2.1 mm ; 1.7 µm) using a gradient elution with a mobile phase composed of ammonium formate buffer 5 mM pH 3.2 and acetonitrile + 0.1% formic acid with a flow rate of 400 µL.min-1. Two deuterated internal standards were used to obtain the most accurate quantification. The CSF samples were prepared by a simple and rapid dilution and the plasmatic samples were treated by protein precipitation.

Results

The method was validated according to current SFSTP requirements [3-4]. The validation results proved that the methods were suitable to quantify ibrutinib and PCI-45227 in real biological CSF samples from 0.50 (ibrutinib) or 1.00 (PCI-45227) to 30.00 ng.mL-1 and in plasma from 5.0 to 500.0 ng.mL-1. Lastly, for both matrices, accuracy profiles were plotted from the trueness and precision results using a 20% α-risk (β=80%) and the tolerance intervals were comprised within the acceptance limits fixed at ±25% for the LLOQ and ±15% for the other concentrations.

Conclusions

These methods were successfully applied to quantify ibrutinib and PCI-45227 in real human CSF and plasmatic samples.

Novel Aspect

This is the first complete validated method for quantification of ibrutinib and PCI-45227 in CSF.

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Keywords

Ibrutinib, metabolite, cerebrospinal fluid

Authors

Danel Cécile [1], Beauvais David [2], Genay Stéphanie [1], Odou Pascal [1], Goossens Jean-François [1]

1. Groupe de Recherche sur les formes Injectables et les Technologies Associées (GRITA)-EA 7365 (France)
2. CHRU hématologie (France)

Plant Biology and Food Science

Linking cocoa polyphenol composition to chocolate quality with Average-Mass-Spectra fingerprints [P50]

Introduction

Chocolate quality is largely due to the presence of polyphenols, and especially of flavan-3-ols (condensed tannins and their derivatives) that are present in large amounts in cocoa beans and contribute bitterness and astringency to the final product.

Prediction of chocolate quality from cocoa composition would be of great help for chocolate makers who currently base their sourcing on a time consuming and costly procedure involving sensory analysis of chocolates made using a standardized process.

The aim of the present work was to investigate the potential of polyphenol-fingerprints for discrimination of cocoa samples and their attribution to chocolate sensory groups.

Methods and Results

Sixty cocoa samples have been processed into chocolates cocoa samples and attributed to four different sensory groups by sensory analysis. Cocoa polyphenol extracts have been prepared from the ground and defatted cocoa beans and analyzed by UHPLC-ESI-IT-MS. Averaging the mass spectrum recorded on the whole UHPLC profile provided cocoa polyphenol fingerprints, which were combined into a matrix and processed with chemometrics (PCA, PLS-DA) to select the most meaningful molecules for discrimination of the chocolate sensory groups.

Sixteen samples representative of the four sensory groups were used for selection of the most discriminant variables. Non supervised PCA analysis of the average mass spectra showed that IT-MS is sensitive and quantitative enough to discriminate the sensory groups without prior selection of target compounds, *i.e* polyphenolic bio-markers. A supervised chemometrics treatment, PLS-DA, was applied to this data to select the most relevant molecules for the discrimination. A larger set of 44 cocoa samples was used to validate the results.

Conclusions and Novel Aspects

The fingerprinting method proved to be quick and efficient, and the chemometrics highlighted 29 mass signals of known and unknown molecules that were finally targeted [1], enabling sensory-group discrimination. Most of these signals were attributed to flavan-3-ols, including 2 newly described ethyl-bridged flavan-3-ols [2].

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Keywords

Cocoa Polyphenols, Chocolate Quality, Polyphenol Fingerprints

Authors

Fayeulle Noémie [1], Meudec Emmanuelle [2], Verbaere Arnaud [2], Boulet Jean-Claude [3], Hue Clotilde, Boulanger Renaud, Cheynier Véronique [2], Sommerer Nicolas [2] [4]

1. Plateforme Polyphénols, UMR SPO, INRA, Montpellier Supagro, Université de Montpellier (France)
2. INRA - UMR1083 SPO - Plateforme d'analyse des polyphénols (France)
3. INRA, UMR Sciences pour l'Oenologie (France)
4. Université de Montpellier (France)

Interactomics reveals novel regulatory mechanisms of plant aquaporins [P51]

The absorption of soil water by roots is crucial for plants to maintain their water status, and relies on water transport through an apoplastic (cell wall) and through a cell-to-cell pathway. The former one can be affected at the endodermis by initial formation of a Casparian strip and further deposition of suberin lamellas [1] ; the latter and major one is regulated by the function of aquaporins [2]. Proteins that molecularly interact with two majors root aquaporins in *Arabidopsis* (PIP1;2 and PIP2;1) were searched to get new insights into regulatory mechanisms of root water transport using a immuno-purification strategy coupled to protein identification and quantification by mass spectrometry. Such interactome revealed PIPs to behave as a platform for recruitment of a wide range of transport activities and provided novel insights into regulation of PIP cellular trafficking by osmotic and oxidative treatments [3]. Surprisingly, 4 Casparian strip membrane domain protein-like (CASPL) also co-purified with PIP2;1. Two of them, CASPL1B1 and CASPL1D2, directly interact with PIP2;1 and are exclusively expressed in suberized endodermal cells, suggesting a cell-specific role in suberization and/or water transport regulation. Whereas none of the *caspl1b* and *caspl1d* mutants showed root hydraulic conductivity phenotype, a negative role for CASPL1D isoforms in suberization under control or salt stress conditions was observed. Surprisingly, reduced activity of aquaporins in the *pip2;1xpip2;2* double mutant, caused a significant increase in endodermal suberin deposition [4], suggesting a putative relationship between CASPL1D and aquaporins to modulate endodermal suberization. Finally, we showed an activation of aquaporin PIP2;1 by CASPL1B1, that required an additional aquaporin phosphorylation involving a putative third partner [5]. Thus, this work reveals an unexpected relationship between apoplastic and cell-to-cell root water transport pathways and opens novel perspectives in understanding PIP regulatory mechanisms and their role in adjustment of plant water status.

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Keywords

Aquaporin, interactomics, suberin

Authors

Champeyroux Chloé, Bellati Jorge, Barberon Marie, Rofidal Valérie, Maurel Christophe, Santoni Véronique [1]

1. Lab. Biochimie et Physiologie Moléculaire des Plantes. INRA-CNRS-SupAgro-UM (France)

Analysis of Heterocyclic Aromatic Amines in beef samples prepared and cooked according the French usual methods using QuEChERS extraction and UHPLC-APCI-MS/MS. [P52]

Introduction

Heterocyclic Aromatic Amines (HAs) are neo-formed compounds generated during the cooking of meats and suspected to be mutagenic and carcinogenic for rodents and man.

We present an original and rapid method using QuEChERS extraction followed by UHPLC-APCI-MS/MS for the analysis of 16 HAs in cooked beef meat. The aim of this study was to determine HAs level in different types of beef samples representative of the usual cooking practices in France (household cooking, restaurant, collective catering) to establish a database of HA levels in most representative French beef samples.

Methods

A methodology was developed and validated for quantification of 16 HAs in 1g lyophilized cooked meat samples. The assay combines the use of QuEChERS extraction, and a mixed mode SPE purification with UHPLC-APCI-MS/MS analysis in positive ionization, in MRM mode on a triple quadrupole instrument, based on 2 specific transitions and using internal standard. 60 samples were analyzed representing 10 types of beef pieces, with various formulation and cooking methods.

Results

A simple and fast method was developed to overcome the traditional extraction method of HAs (long, complex, solvent consuming) and to adapt it to the particular physicochemical properties of HAs and the fat nature of the beef matrix. The classical QuEChERS method was modified to minimize matrix effects and signal extinction by using an additional solid phase purification in mixed mode combining a polymer adsorbent and a cation exchange. This method benefits from both the rapidity of QuEChERS and the specificity and sensitivity of UHPLC-APCI-MS/MS. The performances of the validated method are presented in terms of selectivity, sensitivity, linearity, carry-over, recovery, matrix effect, repeatability, accuracy and intermediate precision in doped beef meat extracts.

In beef samples, 9 HAs were absent or below the quantification limits. The 7 others were found at levels similar to those depicted in literature, depending mainly on the cooking conditions. These results allowed to draw a panorama of French exposure to HAs via beef consumption.

Conclusion

This original methodology enables to quickly and easily assess 16 HAs in complex beef meat matrices by UHPLC-APCI-MS/MS. After complete validation, this method was applied to assess HA levels in beef samples representative of French consumption and practices (household, restaurant and canteen) to point out the most problematic ones and provide

possible recommendations to minimize HA formation during meat cooking in order to reduce consumer exposure towards HAs.

Novel Aspect

Easy, rapid and repeatable extraction and purification method combined with an UHPLC-APCI-MS/MS method suitable for complex and fatty meat matrices.

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Keywords

Heterocyclic Aromatic Amines, UHPLC-APCI-MS/MS, beef-meat

Authors

Chevolleau Sylvie [1] [2], Meurillon Maîa [3], Bouville Alyssa, Angenieux Magaly, Mercier Frederic, Scislowski Valerie, Debrauwer Laurent

1. Axiom Platform, UMR 1331 TOXALIM, MetaToul-MetaboHUB, National Infrastructure of Metabolomics and Fluxomics, F-31027 Toulouse (France)
2. Toxalim, Université de Toulouse, INRA, ENVT, INP-Purpan, Université de Toulouse 3 Paul Sabatier, F-31027 Toulouse (France)
3. QuaPA, UR370, INRA, F-63122 Saint-Genès-Champanelle (France)

Research of new defence mechanisms deployed by Glycine Max (soybean) roots and border cells by shot gun proteomic analyses [P53]

As part of the process of reducing pesticides, biocontrol agents are currently developed. They consist mainly of natural molecules or beneficial organisms allowing to improve plant defence mechanisms. These mechanisms are rather well studied in aerial parts of plants, but very under-studied in roots. The work presented here deals with the characterization of root defence, and more particularly a better understanding of the root extracellular trap (RET). The RET is a complex network consisting of border cells (BCs), glycomolecules and extracellular DNA. Notwithstanding their importance as regards the RET, no high throughput proteomic study on border cells is available yet.

In this work, we compare *Glycine max* roots and border cells by shot gun proteomic analysis exposed to a MAMP (PEP13) versus control conditions. In the first comparison, results of BCs study allowed the identification of nearly 1000 proteins, from which some tens presented differential abundances in the defence situation. Aside, the study of root samples resulted in approximately 1200 identified proteins and the quantitative analysis highlighted around 100 proteins. Interestingly, when comparing all the different samples, from a total of 1500 identified proteins, more than 300 proteins were specific of BCs whereas more than 500 specific of roots. As a first piece of conclusion, these results revealed a specific protein profile of BCs compared to roots, arguing a functional role of these particular cells. In addition, although the roots appear to recruit more proteins in the immune defence mechanisms, BCs also mobilize some important proteins in these conditions. The annotation of functional pathways associated to these regulations will be further investigated.

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Authors

Chambard Marie [1], Ben Mlouka Mohamed Amine [2], Lefranc Benjamin [3], Leprince Jérome [3], Driouich Azeddine [1], Vicré Maïté [1], Follet-Gueye Marie-Laure [1], Hardouin Julie [2], Cosette Pascal [2], Boulogne Isabelle [1]

1. Glyco-MEV (France)|Plateforme PISSARO (France)
2. Plateforme PRIMACEN (France)

TCP14 phosphorylation by MPK8 as a potential new regulation pathway of seed germination in Arabidopsis thaliana [P54]

Introduction

Dormancy release is a critical physiological mechanism allowing seed germination when favourable conditions are met [1]. Evidence suggest the involvement of mitogen-activated protein kinase (MAPK/MPK) pathways in regulating dormancy release [2,3]. In this study, we investigated the role of the protein MPK8 for dormancy release in *Arabidopsis thaliana*.

Methods

BiFC and co-immuno-precipitation experiments were performed for *in vivo* interaction studies.

TiO2 enrichment and nanoLC-MS/MS performed on a timsTOF-Pro mass spectrometer (Bruker) for phosphorylation characterization.

Results

Specifically, we identified the transcription factor TCP14, an important regulator of seed germination, as a new interaction partner of MPK8 *in vivo*. Using BiFC and co-immuno-precipitation experiments, we evidenced that MPK8 interacted with TCP14 in the nucleus. We characterized *in vitro* phosphorylation of TCP14 by MPK8 at the position S102 with nanoLC-MS/MS. This site does not correspond to the canonical [S/T-P] MPK motif and may represent a new motif targeted specifically by class-D plant MPKs [4].

Conclusions

In conclusion, we demonstrated the interplay between MPK8 and TCP14, which helps understanding their role in the regulation of seed germination

Novel Aspect

Our work provides new evidence of the interaction between MPK8 and TCP14 and of TCP14 to act as a substrate of MPK8.

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Keywords

Phosphorylation, MAP Kinase 8, Teosinte Branched 1/Cycloidea/Proliferating cell factor 14

Authors

Zhang Wei [1], Matheron Lucrèce [2], Pionneau Cédric [3], Puyaubert Juliette [1], Baudouin Emmanuel [1]

1. Sorbonne Université, CNRS, Laboratoire de Biologie du Développement, Institut de Biologie Paris Seine (LBD-IBPS), Paris 75005 (France)
2. Sorbonne Université, Institut de Biologie Paris Seine, Plateforme de spectrométrie de masse et protéomique, Paris 75005, France (France)
3. Sorbonne Université, INSERM, UMS 37 PASS, Plateforme Post-génomique de la Pitié-Salpêtrière (P3S), F-75013 Paris, France (France)

Ionisation and Fragmentation Methods

Development of supercritical fluid chromatography hyphenated with electron ionization mass spectrometry [P55]

Interest in natural extracts among cosmetologists, perfumers and consumers has increased and now an ever-growing number of company wish to create products using only rich, complex and evocative natural ingredients. A major goal in natural product discovery programs is to rapidly dereplicate known molecules from complex extracts. Dereplication procedures are currently emerging such as nuclear magnetic resonance (NMR), high resolution mass spectrometry (HRMS), tandem mass spectrometry (MS/MS). In that context, electron ionization is significantly underused mainly due to the limited range of compounds accessible by gas chromatography whereas EI MS sectra are highly informative. The challenge is thus the hyphenation of electron ionization with modern chromatography such as supercritical fluid chromatography keeping robustness and sensitivity.

SFC experiments were performed with an Agilent 1260 Infinity Analytical System consisted of an SFC binary pump, a degasser, an SFC autosampler with 1.2 µL loop, an Aurora SFC Fusion ™ A5 module, a thermostatted column compartment. Detection was performed using full scan analysis with an Agilent Mass spectrometer 5977 simple quadrupole equipped with an electron ionization source. The column was an Hypercarb ™ (100 × 2.1 mm, 3 µm; Thermo Fisher Scientific). Isoctaric mobile phase consisting of CO2 (A) with 5% or without ethanol (B) was used at a flow rate of 0.7 mL/min. The column temperature was kept at 40 °C and the back-pressure was fixed at 130 bar. The injection volume was set as 1 µL.

Monoterpenes, as standard molecules were injected (e.g. α-pinene, limonene) as well complex matrices such as essential oils (citrus family). Mass spectra was recorded and compared to commercial libraries (Wiley and NIST17) leading to qualifying scores closed to 75 even if known annotations were not on the top of the list. One of the main reason is the need to ignore fragments below *m/z* 44 due to the presence of CO2 radical ion. It must be noticed that mass spectra are different with and without ethanol as co-solvent. When adding ethanol, MS spectra are representative of mixed EI and chemical ionization processes. Further fundamental studies on ionization process is ongoing.

The proof of concept of SFC-EI-MS has been demonstrated. Optimization in order to gain in terms of sensitivity and robustness is continuing.

Keywords

Supercritical fluid chromatography, Electron ionization

Authors

Santerre Cyrille [1], Vallet Nadine [1], Delannay Eldra, Touboul David [2]

1. ISIPCA (France)
2. ICSN (France)

Investigation of the formation of c and z ions in the Penetratin peptide sequence using high energy CID (MALDI-TOF/TOF) [P56]

Introduction

Penetratin (RQIKIWFQNRRMKWKK-NH2) is a membrane-active peptide that we characterised using tandem MS experiments by MALDI-TOF/TOF or ESI-LTQ-Orbitrap. CID fragmentation of peptides mainly leads to b and y fragment ions. In our case, c and z ions corresponding to the fragmentation between Q8 and N9 are observed in high energy CID MS/MS spectra only. Few papers report the production of c ions in the litterature [1,2]. The aim of this study is to understand how c and z ions, which are the base peaks of the MALDI-TOF/TOF spectra,can be produced.

Methods

We synthesized shorter analogues of Penetratin (deca-, hexa- and pentapeptides). We varied the nature of the residues on both sides of the QN sequence. alpha-cyano 4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid MALDI matrices were tested. Peptides were fragmented using low (ESI-LTQ-Orbitrap, collision energy varying from 15 to 60 %) and high energy CID (MALDI-TOF/TOF). Molecular dynamics of the Penetratin *in vaccum* was performed (Amber forcefield ff14SB).

Results

We show that this specific fragmentation is independant of the nature of the matrix since c and z ions are obtained with both CHCA) and 2,5-DHB which is described as a reducing matrix leading to the production of radical hydrogens (H°) which could favor the production of c and z ions [3].

The presence of these c and z ions seems to be related to the amount of energy given to the ions to fragment during the tandem MS experiment. The c and z ions are the base peaks of the MALDI-TOF/TOF spectra whereas low energy CID fragmentation gives only b and y ions. MS/MS fragmentation of the shorter analogues shows that the length of the peptide plays an important role in the fragmentation of the QN peptide bond since the relative intensity of the c fragment ion is proportional to the length of the peptide. Interestingly, molecular dynamics of the Penetratin, performed *in vaccum,* shows that the QN region has a propension to organise in an alpha-helix, whereas other residues of the sequence do not spontaneously structure.

Conclusions

This structure/fragmentation study coupled to molecular dynamics will need further investigation. Experiments are underway to test the use of different activation times and techniques (ECD, ETD, HCD), different levels of activation energies (collisions with xenon), in order to better understand the mechanisms leading to the production of these c and z complementary ions at the QN position of the Penetratin.

Novel Aspect

Better knowledge of gas-phase peptide fragmentation will help improving search engines used for de novo sequencing and for protein identification/characterization.

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Keywords

c and z fragment ions, MALDI-TOF/TOF, peptides fragmentation

Authors

Bechtella Leïla [1], Leprévost Samy [2], Matheron Lucrèce [3], Clodic Gilles [3], Sagan Sandrine [2], Bolbach Gerard [4], Walrant Astrid [2], Sachon Emmanuelle [3] [5]

1. Laboratoire des biomolécules (LBM) (France)
2. Laboratoire des Biomolécules (LBM) (France)
3. Plateforme de spectrométrie de masse et protéomique IBPS (France)
4. Laboratoire des Biomolécules (LBM) (France)
5. Laboratoire des Biomolécules (LBM) (France)

A combination of Field Asymmetric waveform Ion Mobility Spectrometry (FAIMS) with tandem Mass Spectrometry to study synthetic polymers activated by Synchrotron UV Radiation [P57]

Introduction

The physicochemical properties of synthetic polymers are closely dependent on their structures. Tandem mass spectrometry (MS/MS) is necessary in order to obtain detailed structural information. The main technique used to perform MS/MS analysis is collision-induced dissociation (CID) [1]. Others activation techniques have been developed more recently such as electron-transfer dissociation [2,3] and UV irradiation [4,5]. The goal of this work was to study the effect of wavelength-tunable UV synchrotron irradiation of polymer ions, combined or not with FAIMS.

Methods

Precursor ions produced by ESI (positive mode) from different model polymers have been stored in an ion trap mass spectrometer coupled to the DISCO beamline (52-221 nm), and then activated by UV synchrotron irradiation during a variable time period and over a range of wavelengths. For the highest mass polymers, UV activation was combined with field asymmetric waveform ion mobility spectrometry (FAIMS) allowing the selection of multiply charged precursor ions.

Results

The effect of the deposited energy amount on photo-fragmentation pathways (or on the photoionization) of the polymer were studied as a function of the polymer. In the case of two polydimethylsiloxanes, the photoactivation allows to obtain structural information through backbone fragmentations contrary to CID. Under the same conditions (photon energy, irradiation time) UV activation of polydimethylsiloxanes gave different fragmentations after UV irradiation depending on the end group of the polymer. Moreover, from others polymers, FAIMS module permits to separate and study ions that can be difficult to isolate and particularly in the case of isobaric ions or for high mass polymer by CID and UV irradiation.

Conclusion

The results demonstrate the added-value of wavelength-tunable UV photoactivation versus commercial dissociation methods in the framework of various structural challenges in polymer analysis. Combined or not with FAIMS, new opportunities for the characterization of polymers by tandem MS experiments were created by employing a synchrotron UV beam.

Novel Aspect

The combination of FAIMS with MS/MS to study synthetic polymers activated by Synchrotron UV Radiation. The study of UV dissociation pathways as a function of polymer structure.

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Keywords

synthetic polymers, UV photo-activation, field asymmetric waveform ion mobility

Authors

Aloui Inès [1], Legros Véronique [1], Giuliani Alexandre [2] [3], Buchmann William [1]

1. Laboratoire Analyse et Modélisation pour la Biologie et l'Environnement (LAMBE) (France)
2. Synchrotron SOLEIL (France)
3. INRA (France)

Ion Mobility and Ion Spectroscopy

An Improved Calibration Approach for Travelling Wave Ion Mobility Spectrometry: Robust, High-precision Collision Cross Sections [P58]

Introduction

Travelling Wave Ion Mobility (TWIM), has developed into a ubiquitous separation tool, impacting global efforts in areas ranging from food analyses to drug discovery. The relationship between the average ion velocity achieved and the TWIM operating parameters remains incompletely understood, so TWIM instruments are typically calibrated to obtain accurate collision cross section (CCS) values. Here, we introduce a dramatically improved calibration methodology, yielding robust calibrations over a significantly improved range of ions and conditions.

Methods

This calibration related average ion velocity to mobility and mass-to-charge ratio, and utilised six free parameters. Using a new analytical approximation we derive a calibration requiring only two free parameters. Using an enhanced SIMION simulation of the device, we identify an additional effect related to off-axis ion distribution. We accommodate this by adding one further parameter to the calibration. Experimental data for a variety of small molecules, peptides and proteins were acquired using Q-IMS-ToF instruments.

Results

Our efforts to develop a deeper understanding of ion transport during TWIM have directly resulted in a new six-parameter calibration expression that takes velocity relaxation effects into account to produce calibrated TWIM CCS values of enhanced accuracy and precision. Furthermore, we have developed a simplified two-parameter expression which retains the ability to account for ion velocity relaxation. Our preliminary data evaluates the effectiveness of these new expressions in comparison to power-law calibrations preformed under the same experimental conditions using a wide range of wave amplitudes and velocities. We have evaluated calibration performance in different TWIM pressure regimes, and using a large number of analytes, ranging from metabolites and small peptides (e.g. 151 Å2) to multiprotein complexes (e.g. 13,400Å2).

Conclusions

Our results demonstrate that these new calibration functions produce CCSs of significantly improved precision and accuracy across a much wider range of TWIM conditions than has previously been possible. Our new expressions provide uniformly improved CCS precisions over any ion mixture tested, and are much more robust to changes in TWIM parameters. In particular, we routinely achieve relative precisions that are a factor of 5 improved over typical power-law calibrated TWIM data.

Novel Aspect

Novel calibration methods for TWIM instruments, and improved experimental designs for high-accuracy CCS measurements for a wide range of ions.

Keywords

Ion mobility, IMS, TWIM, Cyclic IMS, Calibration

Authors

Hybois Mickael [1], Richardson Keith, Langridge David, Giles Kevin

1. Waters Corporation (France)

Influence of proton location on C-ON bond cleavage upon CID of oligo(alkoxyamine amide)s [P59]

Introduction

Binary information can be stored in sequence-defined oligo(alkoxyamine amide)s, where a 0/1 coding system was implemented based on the mass of two amide synthons linked to a nitroxide moiety [1]. Binary messages were readily retrieved by MS/MS sequencing since dissociation of these polymers mainly occurred via homolysis of all C−ON bonds between the coding unit and the nitroxide moiety. However, complementary fragments expected from such a charge-remote reaction were not all observed, suggesting that the adducted proton strongly influenced this homolytic cleavage [2].

Methods

Oligo(alkoxyamine amide) trimers used as models were ionized in positive mode electrospray and subjected to tandem mass spectrometry and ion mobility spectrometry (IMS) experiments using a Synapt G2 HDMS instrument. Geometry optimization was done after DFT calculations using M06-2X functional with the 6-31G(d) basis set implemented in the Gaussian-16 program. The MOBCAL software was used to convert Cartesian coordinates in collision cross section (CCS) values.

Results

Theoretical calculations showed that protonation most favorably occurred at nitroxide moieties or at the central amide group compared to terminal amides. This explained why the fragment at the left-hand side of the first C−ON bond was always released as a radical and hence never detected. Also consistent with calculations, protonation of nitroxide nitrogen was shown to substantially raise C−ON bond dissociation energy [3], hence preventing their homolysis and so accounting for the lack of the fragment at the right-hand side of the last C−ON bond. IMS separation of some of these protomers offered a unique opportunity to experimentally support these assumptions. However, individual MS/MS spectra recorded after IMS separation were, at first glance, contradictory because they showed formation of fragments upon homolysis of protonated alkoxyamine bond. However, these results could be rationalized by DFT-optimized structures that permitted to envisage a proton transfer onto the nearby amide oxygen has occurred prior to C−ON bond homolysis [4].

Conclusions

Combining theoretical calculations with MS/MS data recorded for IMS-resolved protomers of tri(alkoxyamine amide)s permitted a better understanding of the dissociation behavior of these sequence-coded oligomers.

Novel Aspect

Protomer-specific conformations and their influence on dissociation of sequence-controlled oligomers

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Keywords

Protomers, dissociation mechanism, TWIMS-MS(/MS)

Authors

Chendo Christophe [1], Poyer Salomé [2], Lutz Jean-François [3], Siri Didier [2], Charles Laurence [2]

1. Fédération des Sciences Chimiques - Spectropole (France)
2. Aix-Marseille Université - Institut de Chimie Radicalaire (France)
3. Institut Charles Sadron (France)

Collision cross sections of phosphoric acid cluster anions in helium measured by drift tube ion mobility mass spectrometry [P60]

Introduction

In the last years, ion mobility mass spectrometry (IMS-MS) has improved structural analysis and compounds identification by giving access to collision cross sections (CCS). Due to the great significance of CCS in different analytical fields, the scientific world is moving towards an increasingly wide and accurate database of well-characterized compounds. In this study, we present 75 CCS values in helium for phosphoric acid cluster anions $[(H_3PO_4)_n - zH]^{z-}$ with charge state (z) up to 4-.

Methods

The clusters were obtained with an electrospray source operated in the negative ion mode from a 5 mM phosphoric acid solution in water/acetonitrile (50/50 v/v). Uniform electric field drift tube ion mobility mass spectrometry (DTIMS-MS) measurements were performed in helium over an interval of eight months, on an Agilent 6560 IMS qTOF (Santa Clara, CA) and CCS values (noted $^{DT}CCS_{He}$) were determined applying a classic multi field approach.

Results

As phosphoric acid clusters are maintained by hydrogen bonds, their clusters are fragile structures that allow to evaluate the effect of different experimental conditions on the retention of weak bonds and its effect on CCS values. We probed harsh and soft voltage gradients in the electrospray (ESI) source before the IMS and two different voltage gradients in the post-IMS region. The greatest variations in the ion mobility and mass spectra consisted in a change of the distribution of the cluster anions size (n) and charge (z), with a higher amount of multiply charged species for soft ESI conditions (soft pre IMS voltage gradients) and lower proportion of dissociation after the IMS device, for very soft post-IMS voltage gradients. However, the CCS values did not change with experimental conditions for a given cluster, provided that it kept intact from the IMS to the mass analyser.

Conclusions

A total of 75 $^{DT}CCS_{He}$ values of clusters anions of charge states 1-, 2-, 3- and 4- were found to be in good agreement among 3 to 10 replicated values, with a relative standard deviation between 0.1-1. 4%. CCS of H_3PO_4 cluster anions were found well correlated to the aggregation number.

Novel Aspect

CCS of H_3PO_4 cluster anions with charge states 1-, 2-, 3- and 4- independent of pre-IMS and post-IMS conditions and well correlated to the aggregation number.

Keywords

ion mobility, collision cross sections, clusters

Authors

Calabrese Valentina [1], Lavanant Hélène [1], Rosu Frédéric [2], Gabelica Valérie [3], Afonso Carlos [1]

1. Normandie Univ, UNIROUEN, INSA Rouen, CNRS, COBRA (France)
2. CNRS UMS3033 (France)
3. Régulations Naturelles et Artificielles (France)

Multi-omics

What cellular immunology and proteomics reveal about host-tick-pathogen interactions in Heartwater. [P61]

Introduction

Amblyomma variegatum, the tropical bont tick, is a species of veterinary importance being considered as one of major pest of ruminants in Africa, Indian Ocean islands and in the Caribbean where it transmits the intracellular rickettsiale *Ehrlichia ruminantium*, the agent of Heartwater, or is associated with reactivation of latent dermatophilosis. The aim of an integrated analysis by cellular immunology and proteomics was to better understand the complementary roles of *A. variegatum* saliva and *E. ruminantium* virulence factors in their modulation of the host's immune system.

Methods

We developed *in vitro* cell culture models both to support *E. ruminantium* growth and to decipher the modulation of immune cells response by tick saliva. We used quantitative proteomics to characterise *A. variegatum* sialomes, serological profiles of infected ruminants, endothelial cells and bacterium profiles during *E. ruminantium* infection kinetics, and mass spectrometry-based assays to study protein phosphorylation and glycosylation in the bacterium.

Results

A. variegatum crude saliva induced a significant decrease in host mitogen-stimulated lymphoproliferation, linked to a significant depolarisation of host macrophages. Interestingly, these cellular effects appeared to be independent of the presence of *E. ruminantium*, whereas the comparison of the saliva of *E. ruminantium*-infected ticks versus uninfected ones evidenced differences in the expression of the sialomes.

The role of ticks in the transmission of *E. ruminantium* was also highlighted by the modulation of host serological biomarkers, depending on whether the bacterium was injected mechanically by a syringe or naturally by tick bite. *E. ruminantium* induced important changes in key protein networks of infected endothelial cells, with a down regulation at the host cell invasion and before lysis, and the up regulation of host proteins during the bacterium replicative phase. We also identified for the first time bacterial proteins and key effectors, together with post-translational modifications affecting bacterial pathogenesis.

Conclusions

We highlighted the cellular and molecular determinants driving the *A. variegatum* saliva-mediated immunomodulation promoting *E. ruminantium* transmission, and reconstructed for the first time the bacterium-host molecular interactions. Such analyses provide a basis to identify key host proteins associated with bacterial replication that could be targeted to interfere with the infectious process and consequent induced pathophysiology of Heartwater.

Novel Aspect

The pioneering results on proteomes presented provide a useful resource for further exploration of host-tick-pathogen protein expressions and interactions in Heartwater.

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Keywords

Heartwater, tick sialome, host-tick-pathogen molecular interactions

Authors

Fernandez Bernard [1]

1. CIRAD UMR ASTRE (France)

Cellular structures as pivot for the survival of the resurrection plant Craterostigma plantagineum. [P62]

Description

Water shortage and the occurrence of drought periods are predicted to increase in the next decades. Targets for biotechnological programs aimed at improving drought tolerance of crops depend on the identification of the mechanisms by which plant withstand drought. In this perspective a group of plants, collectively called resurrection plants, is studied for their capacity to lose up to 98% of their water content and re-establish growth after rehydration.

Drought exposure, resulting in water loss and the concomitant loss of turgor, is linked to to the physical destruction of cell wall and plasma membrane structures, leading to cell leakage and ultimately cell death. The central hypothesis of this project is that changes in the cell wall proteome and its polysaccharide composition enable *Craterostigma plantagineum* to tolerate the mechanical tension caused by a loss of more than 95% of its relative water content and allow folding/unfolding during dehydration/rehydration cycle. Similarly changes in the plasma membrane proteome and its lipid composition are thought to contribute to its preservation.

Changes in the cell wall and membrane proteome during the complete dehydration/rehydration cycle have been studied using gel-based and gel-free approaches. The subsequent integration of these data with RNAseq transcriptomics and GC- and LC-MS metabolite data is ongoing. This is linked to observed changes in the chemical composition of these cellular structures, here an up-to-date view on the data, their integration and their biological interpretation will be given.

Keywords

resurrection plant, polysaccharides, desiccation, cell wall, -omics

Authors

Leclercq Céline [1]

1. Luxembourg Institute of Science and Technology (Luxembourg)

OneOmics™ Project – Proteomics Processing Pipeline in the Cloud [P63]

Easy to use cloud processing pipeline developed for the speed and scale requirements of today's industrialized proteomics labs Identify proteins, perform iTRAQ® Reagent experiments and build spectral ion libraries with the ID core Process SWATH® acquisition data, normalize and compute protein fold change difference across sample set with the QUANT core

Visualize protein expression data, perform cluster/trend analysis to find significant protein changes, compare datasets, and obtain ontology information using the VISUALIZATION core.

Authors

Dojahn Joerg [1]

1. Sciex (Allemagne)

Mass Spectrometry Imaging

MASS SPECTROMETRY IMAGING TO STUDY OXALIPLATIN PENETRATION IN OVARY OF PATIENTS TREATED FOR A PERITONEAL CARCINOMATOSIS WITH HIPEC [P64]

Peritoneal surface malignancies are common evolution of tumours from the gastrointestinal or gynaecological organs that spread throughout the abdominal cavity. For about the past ten years, a platinum-based Hyperthermic Intraperitoneal Chemotherapy (HIPEC) has been developed to be more aggressive toward these tumours. Indeed it allows a direct contact of the drug with the tissues. During the HIPEC procedure, the metallodrug circulates into the peritoneal cavity and can affect other organs such as ovaries. The moderate risk of gonadotoxicty of oxaliplatin intravenously injected has alrealy been demonstrated. However the evaluation of the diffusion of the oxaliplatin into the ovary due to the HIPEC bath was never investigated. Knowledge of penetration and distribution of this drug within the ovaries will help to better understand the effects of the drug on ovary functionality, especially for young patients with procreation project.

Direct imaging of metal-containing compounds using MALDI-MSI is challenging due to poor ionization efficiency and signal suppression from biological matrix effects. Therefore MALDI-MSI is typically used in conjunction with LA-ICP MS on sequential tissues sections and images are correlated. We have shown the complementarity of these two techniques with the localization of platinum drug into samples of peritoneal carcinomatosis nodules of colorectal origin.

This communication will present MALDI-TOF MS and LA-ICP MS imaging technologies to localize and quantify the amount of, respectively, complexed and metallic platinum present in the ovaries of patients before and after they underwent a HIPEC therapy for the treatment of a peritoneal carcinomatosis of colorectal origin. The developed quantitative methodology for LA-ICP MS analysis will be presented as well.

Keywords

LA-ICP MS, MALDI-TOF MS, ovaries

Authors

Larroque Marion [1], Arnaudguilhem Carine [2], Sgarbura Olivia [3], Quenet François [3], Carrère Sebastien [3], Bouyssiere Brice [2], Larroque Christian [4], Mounicou Sandra [2]

1. ICM/ Unité de Recherche Translationnelle, Campus Val d'Aurelle (France)
2. CNRS/ UNIV PAU & PAYS ADOUR, INSTITUT DES SCIENCES ANALYTIQUES ET DE PHYSICO-CHIMIE POUR L'ENVIRONNEMENT ET LES MATERIAUX, UMR 5254, 64000, PAU, France (France)
3. ICM/ Service Chirurgie A2, Campus Val d'Aurelle (France)
4. IRCM/INSERM U896, ICM Val d'Aurelle (France)

Accessible and reproducible mass spectrometry imaging data analysis in Galaxy [P65]

Introduction

Mass spectrometry imaging (MSI) is increasingly used in biological and translational research as it has the ability to determine the spatial distribution of hundreds of analytes in a sample. Being at the interface of proteomics/metabolomics and imaging, the acquired data sets are large and complex and often analyzed with proprietary software or in-house scripts, which hinder reproducibility. Open source software solutions that enable reproducible data analysis often require programming skills and are therefore not accessible to many MSI researchers.

Results

We have integrated 18 dedicated mass spectrometry imaging tools into the Galaxy framework to allow accessible, reproducible, and transparent data analysis. Our tools are based on the open source software Cardinal [1], MALDIquant [2], and scikit-image [3]. They enable all major MSI analysis steps such as quality control, visualization, preprocessing, statistical analysis, and image co-registration. Further, we created hands-on training material for use cases in proteomics and metabolomics. To demonstrate the utility of our tools, we re-analyzed a publicly available N-linked glycan imaging dataset. By providing the entire analysis history online, we highlight how the Galaxy framework fosters transparent and reproducible research.

Conclusion

The Galaxy framework has emerged as a powerful analysis platform for the analysis of MSI data with ease of use and access together with high levels of reproducibility and transparency.

Novel Aspect

This work presents the first scalable, collaborative and reproducible work bench for mass spectrometry imaging data.

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Keywords

mass spectrometry imaging, data analysis, reproducibility

Authors

Föll Melanie [1], Moritz Lennart [1], Wollmann Thomas [2], Stillger Maren [3], Vockert Niklas [2], Werner Martin [1], Bronsert Peter [1], Rohr Karl [2], Grüning Björn [4], Schilling Oliver [1]

1. Institute of Surgical Pathology, Medical Center – University of Freiburg (Allemagne)
2. Biomedical Computer Vision Group, BioQuant, IPMB, Heidelberg University (Allemagne)
3. Institute of Molecular Medicine and Cell Research, Faculty of Medicine, University of Freiburg (Allemagne)
4. Department of Computer Science, University of Freiburg (Allemagne)

A MALDI mass spectrometry study of the segregation of lignocellulosic structures in several maize stems with contrasted degradability [P66]

Introduction

To offset the exhaustion of fossil fuels without competing with food productions, second-generation biofuels deriving from the lignocellulosic biomass are under development. Maize stems are good candidates for a future industrial production, but the yield of their enzymatic conversion remains low. The factors involved are not fully understood and need to be hierarchized [1]. This work maps the distribution of the hemicelluloses and hydroxycinnamic acids (HCA), and explores the molecular structure of the hemicelluloses - in connection with stem maturity and tissue degradability.

Methods

Different enzymes and treatments against lignocellulosic compounds were applied on cross sections of maize stems. Four genotypes of contrasted degradability were studied at five developmental stages. To get at the same time spatial and chemical information, stems were analysed by enzymatically assisted MALDI mass spectrometry imaging (MALDI MSI) [2, 3].

Results

The obtained results highlighted that the chemical structure of hemicelluloses varies according to the stem cross-section region: arabinoxylans (AX) were observed in the center of the stem, while methylglucuronoxylans (MGX) were more peripheral. Variations according to development stages and genotypes were also investigated, and a relationship could be established with the degradability of the tissues. The second lead was the localization of hydroxycinnamic acids (HCA) linked to hemicellulose and/or to lignins. These HCA, namely ferulic acid (FA) and *para*-coumaric acid (pCA), are key compounds of the lignocellulosic network and were shown to influence tissue degradation. FA and pCA were detected by LDI, after an *in-solution* degradation of the maize stems by alkaline hydrolysis. The next step will be to adapt the protocols, to make them compatible with LDI mass spectrometry imaging.

Conclusions

MALDI-MSI results show a correlation between the presence of lignin (determined by FASGA coloration) and the structural characteristics of hemicelluloses. A lignified region of stems contains more MGX enriched hemicelluloses, what could be related to a lower degradability. For the HCA study, the protocols need now to be developed in order to be compatible with LDI mass spectrometry imaging and allow the localization of the compounds.

Novel Aspect

MALDI MSI showed the correlation of some structural characteristics of hemicelluloses with lignification of stem tissues.

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Keywords

MALDI MSI, lignocelluloses, maize stems

Authors

Arnaud Bastien [1], Durand Sylvie [1], Fanuel Mathieu [1], Guillon Fabienne [1], Méchin Valérie [2], Rogniaux Hélène [1]

1. UR1268 Biopolymères Interactions Assemblages, INRA, F-44300 Nantes (France)
2. UMR 1318, Institut Jean-Pierre Bourgin, INRA-AgroParisTech, CNRS, Université Paris-Saclay, Versailles, France (France)

Identification of the tissular targets of Chlordecone in the normal and cancerous rat prostate by MALDI imaging [P67]

Introduction

Chlordecone (CLD) is an organochlorine pesticide extensively used in the French West Indies to fight weevils in banana plantations from 1973 to 1993. This has led to a persistent pollution of the environment and to the contamination of the local population for several decades with effects demonstrated on human health. Indeed, CLD was positively associated with an increased risk of prostate cancer [1]. The aim of this project was to localize and quantify CLD in the normal and cancerous prostate in the rats by quantitative MALDI imaging.

Methods

Sprague-Dawley and TRAP (transgenic rat model for adenocarcinoma of prostate) rats were exposed weekly to CLD by gavage at a dose of 5 mg/kg/bw for 1, 15 or 20 weeks. MALDI imaging experiments were performed as described previously [2]: standard solutions of CLD were spotted on a blank prostate section placed next to exposed sections for calibration. The slide was then coated with the matrix solution spiked with an internal standard and analyzed with a MALDI-FT-ICR.

Results

Tissular targets of CLD in the normal prostate and in the prostate cancer were identified by superposing MALDI images of CLD with histological images. In the normal prostate, CLD was detected in the epithelium and in the secretions of prostate acini. In the prostate cancer, CLD was located in tumor foci but not detected in the stroma. Profiles of CLD accumulation obtained for both types of prostatic tissues showed that the quantity of CLD strongly increased from 1 to 15 weeks but reached a plateau at 20 weeks. Our results suggest that CLD accumulates similarly in the normal prostate and in the prostate cancer. Immunohistochemical experiments were performed with antibodies directed against proliferation and basal cells markers to investigate the link between CLD exposure and tumor progression in the TRAP rats. It revealed that prostate cancer was already very advanced at the moment of the first gavage with CLD. With this TRAP model, similar tumor progression was observed for animals exposed or not to CLD and for all time of exposure.

Conclusions

CLD is an agonist of ERα and an antagonist of ERß. In the normal prostate, ERα is oncogenic and expressed in the stroma whereas ERß is anti-oncogenic and mainly expressed in the epithelium. The detection of CLD in the prostate epithelium suggests that it could decrease the protective effect of ERß. A similar accumulation of CLD was observed in normal prostate and in prostate cancer. However, no link was found between CLD exposure and tumor progression with the TRAP model.

Novel Aspect

Chlordecone, which is an antagonist of ERß, seems to be located in the prostate epithelium where this protective receptor is highly expressed.

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Keywords

MALDI imaging, environmental toxicant, distribution studies

Authors

Lagarrigue Mélanie [1], Sourice Justine [1], Rondel Karine [1], Lavigne Régis [1], Guével Blandine [1], Pineau Charles [1]

1. Protim (France)

In-situ exploration of the lipid remodeling during sperm maturation in the rat epididymis by MALDI imaging mass spectrometry [P68]

Introduction

Spermatozoa acquire their fertilizing capacity during a maturation process that occurs in the epididymis. This process involves a substantial remodeling of the proteins and lipids located at the surface of the gamete. Epididymis can be divided into 3 regions (caput, corpus and cauda) or into 19 intra-regional segments based on histology [1]. Most studies carried out on the evolution of the lipid composition during epididymal maturation were performed on sperm or tissue extracts. Here, we used MALDI imaging to study the distribution of lipids directly in the rat epididymis tissue.

Methods

An epididymis was excised from a 90 day-old Sprague-Dawley rat, embedded in 2% CMC then frozen in isopentane. Epididymis sections (20 µm thick) were prepared at -15°C. For 3D MALDI imaging, sections were collected every 40 µm in the head of epididymis. Sections coated with DHB were analyzed on a MALDI-FTICR with a lateral resolution of 50 µm. SCILS Lab was used for spatial segmentation, ROC analysis and 3D reconstruction. Data were uploaded onto METASPACE [2] for annotation.

Results

The spatial segmentation of molecular information provided by MALDI imaging revealed that the rat epididymis could be divided into 21 molecular clusters different from the 19 intra-regional segments. The discriminative m/z values that contributed the most to each molecular cluster were determined by ROC analysis and annotated with METASPACE. Although phosphatidylcholines, triacylglycerols and phosphatidylethanolamines were predominantly annotated in the caput, an important increase in the number of plasmalogens, phosphosphingomyelins and lysophosphatidylcholines detected in the cauda was observed. MALDI images revealed that molecules belonging to the same family can have very different localizations within the epididymis. Molecular annotation was confirmed for some lipids by on-tissue MS/MS experiments. A 3D-model of the epididymis head was reconstructed from 61 sections analyzed with a lateral resolution of 50 µm to obtain information on the localization of a given analyte in the whole volume of the tissue.

Conclusions

We defined a new segmentation of rat epididymis based on molecular features. Computational methods enabled the determination of discriminative m/z values contributing the most to a given region and the proposition of molecular annotations, confirmed by on-tissue MS/MS for some. Our results are very complementary to those previously obtained on sperm or tissue extracts [3,4] and confirm the substantial lipid remodeling that occurs during the epididymal maturation.

Novel Aspect

MALDI imaging and spatial segmentation confirm the substantial remodeling of lipid composition during the transit of spermatozoa through the rat epididymis.

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Keywords

MALDI imaging, lipids, gamete maturation

Authors

Lagarrigue Mélanie [1], Lavigne Régis [1], Guével Blandine [1], Pineau Charles [1]

1. Protim (France)

Investigation of bacteriophage T5 using multi-scale mass spectrometry [P69]

Introduction

Bacteriophages, or bacterial viruses, are the most abundant biological entity on earth. Their study led to the development of modern molecular biology and biotechnologies. Bacteriophages assembly has so far been studied using biochemical and structural biology approaches, but only a few mass spectrometry (MS)-based investigations have been reported. In the present work, we investigated the composition of bacteriophage T5 capsid and tail using multi-scale MS: Nanoelectromechanical-MS of capsids, MALDI-MS of tail subunits, and proteomic analysis.

Method

Since T5 capsid and tail cover a large range of masses, we used 3 different types of mass spectrometry. NEMS-MS can analyze large biological particles such as intact viruses, handling masses up to 150 MDa. MALDI-MS analyzes biomolecules in the kDa range, providing information about intact subunits and their modifications. MS-based proteomics allows the identification and quantification of T5 capsid and tail subunits.

Results

Empty or filled T5 capsids were electrosprayed and detected by NEMS-MS. Measured masses were close to expected values (26 MDa & 105 MDa). Proteomic analyses of decorated capsids identified and provided semi-quantitative data on all capsid proteins. Sequence coverage confirmed cleavage of the major capsid protein scaffolding domain, and processing of the portal and protease. A plot of the abundance vs. copy number suggested that the copy number of the protease and portal protein could be similar, contrary to previous assumptions. Proteomic and MALDI-MS analyses of T5 tails revealed the 11 expected proteins from the tail complex. We also identified two previously uncharacterized T5 proteins. In our experiments, a 20 aa portion in the C-ter region of the tape-measure protein pb2 was detected. This was unexpected as the last ~71 aa of pb2 are known to be cleaved during the assembly process. Comparing proteomics and MALDI-MS data, we were able to learn more about the processing of tail proteins.

Conclusion

NEMS is one of the rare MS technologies operating in MDa-GDa range. It allowed analysis of intact bacteriophage T5 capsids above 100 MDa. Proteomics provided information about the capsid protease and confirmed the tail composition, while detecting two previously uncharacterized T5 proteins. MALDI-MS could detect all tail proteins and determine posttranslational modifications. This study confirmed the potential of multi-scale MS for studying viral systems.

Novel aspects

1. Suggesting exact copy number of protease in T5 phage

2. Factors regulating protease activity.

3. Know more about processing of proteins present in the tail.

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Keywords

Bacteriophage T5, Nano mechanical resonators, MALDI-MS

Authors

Clement Kavya [1], Epalle Nathan [2], Novion Ducassou Julia [1], Dominguez Medina Sergio [1], Signor Luca [2], Coute Yohann [1], Hentz Sébastien [3], Breyton Cécile [2], Boulanger Pascale [4], Boeri Erba Elisabetta [2], Masselon Christophe [1]

1. CEA Grenoble - IRIG/BGE/EDYP (France)|Institut de Biologie Structurale (France)
2. LETI DCOS (France)
3. Institut de Biologie Intégrative de la Cellule (France)

3D and high sensitivity micrometric mass spectrometry imaging [P70]

Introduction

TOF-SIMS is recognized for its high spatial resolution imaging capability. However, it is relatively less popular in biological application compared to MALDI MSI, due to severe molecular fragmentation and lack of MS/MS capability in most commercial instruments. This thesis is aimed to get a better understanding of ion desorption/ionization under cluster impacts, to achieve in situ structural characterization using the parallel imaging MS/MS spectrometer, and to investigate cellular distribution and biosynthesis of wood metabolites with high resolution SIMS imaging [1].

Methods

The study of ion production under cluster impacts was performed on a TOF-SIMS VI instrument equipped with Bismuth and Argon cluster ion beams. The same instrument was used to map the wood metabolites in 2D and 3D. In situ structural characterization was achieved using the parallel imaging MS/MS Spectrometer (PHI nanoTOF II). Wood samples corresponding to differnt developping stages were analyzed to study the biosynthesis of two bioactive metabolites.

Results

Internal energy distributions of secondary ions were measured using survival yield method which involves the analyses of a series of benzylpyridinium ions. Investigation of various impacting conditions (energy, velocity, cluster size) suggested that velocity of the clusters play a major role in internal energy distribution and molecular fragmentation in the low energy per atom regime ($E/n < 10$ eV) [2].

The evaluation of the MS/MS fragmentation and parallel imaging capabilities of the recently developed PHI nanoTOF II spectrometer showed successful in situ MS/MS mapping of bioactive metabolites rubrynolide and rubrenolide in Amazonia wood species *Sextonia rubra* despite their low abundance in the wood sample [3]. In situ identification of related precursor metabolites of rubrynolide and rubrenolide in the same tree species was subequently achieved. Finally, 2D and 3D TOF-SIMS imaging revealed that the ray parenchyma cells were responsible of the biosynthesis of these two bioactive metabolites in the wood [4].

Conclusions

Soft ionization analysis can be realized in SIMS with controlled beam velocity. In addition, the small disappearance cross section measured for argon clusters suggest they induce minimum sample damage during the analysis. High resolution 2D/3D TOF-SIMS imaging and in situ structural characterization with parallel imaging MS/MS technique lead to the proposal of a possible biosynthesis pathway of two bioactive metabolites in *S. rubra* tree species.

Novel Aspect

High resolution 2D/3D TOF-ISMS imaging and in situ structural characterization allow to visualize metabolites in complex biological systems.

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Keywords

TOF-SIMS, internal energy, wood metabolites

Authors

Fu Tingting [1] [2]

1. Institut des Sciences Analytiques (France)
2. Institut de chimie des substances naturelles et Institut de Physique Nucléaire d'Orsay (France)

MALDI Mass Spectrometry Imaging Applied to Tuberculosis [P71]

Introduction

Mycobacterium tuberculosis (*Mtb*), the etiological agent of tuberculosis, establishes a durable lung infection in complex lesions where it is found intracellularly in various immune cell types and extracellularly in the central necrotic core of these lesions. Our current efforts are focused on increasing spatial resolution by MALDI mass spectrometry imaging to visualize and identify the structures of molecules of interest at the cellular and subcellular level, *i.e.* lifting a scientific barrier.

Methods

The AP-SMALDI-Orbitrap (TransMIT, ThermoFisher) is one of the most competitive instrument dedicated to mass spectrometry imaging. This instrument combines a high spatial resolution down to 5 µm with the high specificities of the Orbitrap. This approach will be applied to a tissue section of tuberculosis infected mice who will receive anti-TB antibiotics.

Results

The methodological developments have shown that lipid biomarkers related to tuberculosis were co-localized with the infectious agent or different types of cell population. All of these biomolecules have been identified and characterized. Moreover, thank to the high spatial resolution provided by the AP-MALDI ion source, the penetration of anti-tuberculosis drugs has been investigated into the granuloma, and we have observed the accumulation of clofazamine in macrophages.

Conclusions

The ability to visualize bacterial drugs, metabolites and biomarkers with high resolution MALDI imaging allows the direct co-localization of drugs with specific bacterial target populations. Future applications of bacterial biomarker imaging might include the therapeutic monitoring of drugs at the site of individual lesions by visualizing the metabolic response of mycobacteria.

Novel Aspect

MSI has the potential to unearth important spatial and temporal relationships in biological systems.

Keywords

MALDI-Mass Spectrometry Imaging, Tuberculosis, Antibiotics.

Authors

Blanc Landry [1], Dartois Véronique [2], Desbenoit Nicolas [1]

1. CBMN, Université de Bordeaux (France)

2. Center for Discovery and Innovation, Hackensack Meridian Health (États-Unis)

Lipidomics / Metabolomics

Metabolic exploration of the mutualist microorganism community associated to Guiana Termite workers. [P72]

Introduction

Associations of social insects and microorganisms constitute an ecological and chemical niche of interest. Those interactions are well described within Apocrita (ant, bee, wasp) [1]. Excluding trophiobiosis, little work has been done on termites/microorganism interaction though [2]. Following a previous investigation demonstrating the production of antimicrobial compounds by one of the isolated strain [3], we expand our investigation to the 130 strains associated with termite workers.

Methods

All strains were cultivated and extracted by ethyl acetate. The crude extracts were then solubilized in methanol and separated by reverse phase HPLC on C18 column. Hence, metabolites were detected by data dependent acquisition method (DDA) with a Q-TOF instrument (6540 Agilent Technologies). Within a 1.2 second cycle, the 5 most intense ions (one per 200 milliseconds) from a first high resolution MS spectrum are fragmented. Data were finally clustered by molecular networking using MetGem [4] software.

Results

The biological activity of our crude extracts on three human pathogens -methicillin resistant *Staphylococcus aureus*, *Candida albicans* and *Trichophyton rubrum* were mapped over the molecular network. This additional layer highlighted undescribed and potentially active molecules. These molecules will be isolated and their structures will be resolved using NMR, circular dichroism and crystallography.

Conclusions

Tandem mass spectrometry combined to molecular networking and activity tests helped us to accelerate the identification of putative new antimicrobial compound. These approaches will speed up the molecular characterization of the original ecological niche from termites/microorganism association.

Novel Aspect

This work combines recent dereplication tool driven by tandem mass spectrometry data, *i.e.* molecular networking, with the exploration of novel ecological niche from termites/microorganism association.

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Keywords

Natural products, molecular network, mutualism

Authors

Hébra Téo, Touboul David [1], Eparvier Véronique [1]

1. Institut de Chimie des Substances Naturelles (France)

Cartography of secondary metabolites from yeast strains by LC-MS/MS [P73]

Yeasts are unicellular fungi. Their fermentation ability and some other biochemical processes can lead to the synthesis of secondary metabolites. These could be bio-active compounds and potentially be interesting for pharmaceutical or agro food industry. Our study focuses on the cartography and identification of secondary metabolites from new exotic yeast strains. These strains have been recently characterized and so are still unexplored for what they produce.

The analytical method is an untargeted approach using liquid chromatography coupled with high resolution mass spectrometry (UHPLC-MS/MS). The UHPLC instrument is coupled to a hybrid mass spectrometer, a Quadrupole-Time of Flight analyzer with an Electro Spray Ionization source. Secondary metabolites are extracted from the yeast cultures by organic solvents. Then the analysis is performed in Data Dependent Scan, fragmenting the most intense ions at each scan.

The interpretation method is based on molecular networking. The GNPS platform (Global Natural Products Social molecular networking (Wang et al., 2016)) uses the MS/MS data to build networks as structurally similar compounds have similar fragmentation patterns. MS/MS spectra are also compared to public libraries in order to annotate some compounds within the samples. In order to expand the metabolite coverage, the APCI and APPI ionization sources are considered.

This innovative bioinformatics tool and the associated workflow allows obtaining a cartography of known and unknown metabolites. Our methodology was validated on Saccharomyces cerevisiae then applied to exotic strains.

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Keywords

secondary metabolites; molecular networking; unexplored yeasts.

Authors

Perruchon Olivier [1], Schmitz-Afonso Isabelle [1], Grondin Cécile [2], Casaregola Serge [2], Afonso Carlos [1], Elomri Abdelhakim [1]

1. Chimie Organique et Bioorganique : Reactivité et Analyse (France)
2. MICrobiologie de l'ALImentation au Service de la Santé humaine (France)

Analysis of Lipid Signaling Class Analytes Using a Travelling Wave Cyclic Ion Mobility Separator [P74]

Introduction

Lipid class separation is readily achieved using chromatographic and MS techniques; however, the analysis remains challenging due to the chemical structure diversity and isobaric nature of these compounds. The addition of IMS to discovery workflows enhances system peak capacity and improves isomer resolution. IM separation was achieved using a multi-pass travelling-wave cyclic IM (cIM)-device. MS and CID fragmentation data were obtained on precursor IM separated analytes followed by ToF mass measurement.

Methods

Unsaturated free fatty acid (FA) standards, differing in chain length and number of cis/trans configurations, steroid hormones (androgens), isomers differing in the position of a functional group, and isobaric lipid mediators (prostaglandins) were chosen to determine the degree of IM separation required to separate isomers and isobars. Data were collected on a cyclic ion mobility-enabled quadrupole time-of-flight (Q-cIM-oaToF) mass spectrometer.

Results

The required IM resolution ($\Omega/\Delta\Omega$) values typically ranged from 100 to 350. Unsaturated FAs with two or more double bonds, separated by two mid-chain carbons, could not be distinguished. Shorter, structurally more rigid and compact FAs were discriminated at reduced resolution, as could longer chain mono-unsaturated FAs as a result of partial chain back-folding. Following IM separation, isomeric FAs were successfully CID fragmented and identified. The analysis of 17-hydroxyprogesterone and 21-hydroxyprogesterone, an isobaric adrenal steroid pair, indicated that an IM resolution ($\Omega/\Delta\Omega$) of at least 200 was required to baseline separate these analytes. Following IM separation, nearly identical, but individual product ion spectra were readily detected, arguing the need for the separation of these types of compounds.

A mixture of 11-deoxycortisol, 21-deoxycortisol, and corticosterone was partially resolved. Here, after 12 passes through the cIM device, 21-deoxycortisol could be separated from the two other steroids, and following 20 passes, 11-deoxycortisol but with the two remaining steroids now partially resolved. The separation of other lipid signaling class analytes, such as prostaglandins and other lipid mediators, by cIM will also be investigated and presented.

Novel Aspect

A Q-cIM-oaToF research platform has been characterized and applied to the IM separation of isomeric lipid signaling analytes.

Keywords

Ion mobility, Cyclic IMS, TWIM, Lipids, Isomer resolution

Authors

Hybois Mickael [1], Mcgullagh Mike, Palmer Martin, Marsden-Edwards Emma, Langridge James, Vissers Johannes Pc

1. Waters Corporation (France)

Study of the toxic effect of diphenyl phosphate on the liver metabolism of mice by HPLC-HRMS [P75]

Introduction

Diphenyl phosphate (DPP) is one of the main metabolites of triphenyl phosphate (TPP), organophosphorus additive widely used as flame retardant in substitution of polybrominated diphenylethers and polybrominated biphenyls. In term of exposure, the migration of organophosphate flame retardants constitutes a serious concern for population health [1]. As few reports, mainly carried during *in vitro* experiments, deal with the impact of DPP on human health [2, 3], a metabolomics *in vivo* study on rodent model was performed by HPLC-HRMS using two different and complementary stationary phases, C18 and HILIC.

Methods

Mice of 3 weeks were fed with three concentrations of DPP in water (0.1, 1, 10 mg/mL) during seven weeks, doses chosen through their relevance in term of human exposure. After lyophilization and grinding of the livers, a mass of 20 mg was extracted by solid-liquid extraction. The organic phase was split in two aliquots that were evaporated under nitrogen to be analyzed in parallel by HPLC-HRMS based on the reverse phase C18 and HILIC separations. The extractions were performed by two operators and from eight independent samples for each concentration to obtain a suitable statistical analysis. The analysis were carry out with an UHPLC system (U3000 Thermo®) coupled to a QToF mass spectrometer (MaxisPlus, Bruker®). The data were processed using MetaboScape 4.0 and were statistically treated using univariate and supervised statistical tools.

Results

The principal discriminant compounds were lipid transporter (carnitine type molecules) and lipid or lipid precursors (for example oleic acid, linoleic acid). The results indicated clearly a disruption of the control pathways of lipid metabolism.

Conclusions

To the best of our knowledge, this is the first time that the effect of DPP is studied *in vivo* experiments. This work confirms the hypothesis of previous studies where other OPEs disturbed lipid metabolism and transportation.

Novel Aspect

Transcriptomic and immunohistochemical analysis are ongoing; the first results are coherent with those obtained with this metabolomic approach. The successful combination of analytical and biological methodologies shows the huge potential of these techniques to evaluate the effect of these and other kind of compounds in the body functioning.

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Keywords

DPP, LC-HRMS, Metabolomics, liver

Authors

Fildier Aurélie, Buleté Audrey, Vulliet Emmanuelle [1]

1. Institut des Sciences Analytiques, UMR 5280 CNRS - Université de Lyon 1, 5 rue de la Doua, 69100 Villeurbanne, France (France)

Study of iodinated contrast agents impact on two aquatic model organisms by metabolomics and metallomics approaches (ACTIONS' project) [P76]

Introduction

Iodinated contrast media (ICMs) are widely used to artificially increase contrast for a better visualization of organs, tissues or tumors for diagnostic purposes. These compounds are ubiquitously present in water in contents up to tens of µg/L even some mg/L. Thus, a continuous exposition to ICMs could lead to adverse impacts on aquatic organisms, not only on fish that play a major role in the structure and functioning of aquatic food webs, but also on filtering organisms, whose filtering nature makes them good bioaccumulators.

Methods

After studying the distribution of iodine in the different parts of both organisms, fish and bivalve, by elementary imaging, the determination of iodine bioaccumulation and its potential interactions with biomolecules will be performed by a metallomic approach combining HPLC-ICPMS and HPLC-ESI-MS/MS. In parallel, a study of the ICMs effects on the metabolism of two sentinel organisms will be performed by a metabolomic approach in LC-HRMS.

Results

The ACTIONS' project - funded by PNREST Anses, 2018/1/222 - will acquire data on the exposure of two - vertebrate and invertebrate - model organisms of our rivers to two ICMs with different osmolality, both substances being present ubiquitously and at high doses in the aquatic environment.

After an acute exposure to ICM doses, in order to ensure the compounds entering into the organisms, the iodine repartition will be first studied in both organisms. Then a chronic exposure at environmental doses will be experimented, and metallomic and metabolomic studies will be performed on the organs/tissues selected.

The complementarity of the analytical techniques proposed here will enable the study of both the distribution and the speciation of iodized compounds in two model aquatic organisms and to search for potential metabolites / markers of exposure and effects of ICMs. Data collected from these two organisms will predict the potential risks of these substances toward aquatic ecosystems.

Conclusions

ICMs occurrence in water is widely documented but few studies deal with their bioaccumulation or effects on aquatic organisms. The bioaccumulation and overall ICMs effects on two model organisms' physiology will be studied. ACTIONS will get essential information on the relevance of studying some physiological pathways of interest for future targeted multi-biomarker analyzes.

Novel Aspect

Complementarity of analytical techniques to study both the distribution and speciation of ICMs in model aquatic organisms and to search for potential metabolites/markers of exposure and effect.

Keywords

Metabolomics, metallomics, aquatic organisms, iodinated-contrast agents

Authors

Daniele Gaëlle, Mounicou Sandra [1], Arnaudguilhem Carine [2], Geffard Alain [3], Geffard-Dedourge Odile, Bado-Nilles Anne [4], Porcher Jean-Marc [5], Lebeau Emmanuelle [6], Fildier Aurélie, Bonnefoy Christelle, Vulliet Emmanuelle [7]

1. CNRS/UPPA, UMR 5254, LCABIE (France)
2. Laboratoire de Chimie Analytique Bio-Inorganique et Environnement (France)
3. Université de Reims Champagne-Ardenne (France)
4. Stress Environnementaux et BIOsurveillance des milieux aquatiques (France)
5. Institut National de l'Environnement Industriel et des Risques (France)
6. Université de Reims Champagne-Ardenne (France)
7. Institut des Sciences Analytiques, UMR 5280 CNRS - Université de Lyon 1, 5 rue de la Doua, 69100 Villeurbanne, France (France)

A real time metabolomics profiling approach using Rapid Evaporative Ionization Mass Spectrometry (REIMS) to classify meat samples [P77]

Introduction

The frequent occurrence of food scandals in the world has led to the implementation of strategies to up-grade the food supply system to a high food safety standard. Such strategy relies on sophisticated sample preparation procedures and long analysis time. Thus, to face the increasing demand, fast and accurate analytical platforms are required for screening purposes. To that end, Ambient Mass Spectrometry (AMS) and one of its declinations Rapid Evaporative Ionization Mass spectrometry (REIMS), offer a promising approach as it needs few to no sample preparation.

Methods

The iKnife hand-held sampling device (Waters, Wilmslow, UK) was used to apply a localized high frequency electric current to the surface of each meat sample. The meat samples included control muscle samples of pork and samples from animals treated with the beta-agonist compound ractopamine. Mass spectrometric analysis was carried out on a Synapt G2-Si instrument. Replicate burns of a QC sample (muscle/liver) were collected between every 10 samples.

Results

On tissue samples, the REIMS generated mass spectrometric profiles are dominated by lipids and that specificity is used to discriminate samples by their lipidic fingerprint. The combination of REIMS with untargeted metabolomics workflow was investigated to identify carcasses from pig treated animals on the basis of a modification of indirect metabolites profile due to the use of ractopamine, a β-agonist substance that may be used in some parts of the world as growth promoter in livestock, although forbidden in a number of countries. The strategy was found successful when tested on different muscle types (loin, shoulder and ham). Multivariate statistical software package LiveIDTM (Waters) was used as a model builder and direct recognition tool. Classification performances were 0% false negative and 10 % false positive, which fully answers requirements of a screening strategy.

Conclusions

REIMS implemented in an untargeted-metabolomics workflow can be considered as a high-throughput and powerful strategy for real-time classification in relation to ractopamine exposure in pig. This AMS approach still need some performances assessment (reprod./sensitivity/selectivity) and ease of use to fulfil all industrial and legislatives requirements before being implemented as rapid screening test, at the slaughterhouse or at boarder inspection points.

Novel Aspect

We herein report the novel application of REIMS in a metabolomics approach to instantaneously detect the administration of prohibited compounds to livestock animals.

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Keywords

Ambient mass spectrometry, REIMS, meat authenticity

Authors

Gaiffe Gabriel [1], Pouponneau Karinne [1], Guitton Yann [1], Monteau Fabrice [1], Stead Sara [2], Le Bizec Bruno [1], Dervilly-Pinel Gaud [1]

1. Laboratoire d'étude des Résidus et Contaminants dans les Aliments (France)
2. Waters Corporation UK (Royaume-Uni)

A complete workflow for improved untargeted metabolome annotation and identification using ultra high-resolution accurate mass and LC-MSn Orbitrap-based mass spectrometry [P78]

Introduction

The annotation of hundreds of unknown metabolites from human plasma is one of the most difficult challenges faced by metabolomics scientists. Automated annotation must incorporate accurate mass, isotope pattern and isotopic fine structure to obtain accurate elemental formula for searching appropriate metabolite databases. The MS2 and MS3 spectra of unknowns should be searched for identity and similarity matches against a high-quality MSn mass spectral library and the level of identification reported for each metabolite should be based on the consensus of the available analytical measures.

Methods

SRM1950 (NIST) human pooled plasma was precipitated and separated on a Thermo ScientificTM HypersilTM Gold C18 column (2.1x150mm, 1.9µm). LC-MS analyses were performed on a Thermo Scientific™ Orbitrap ID-X™ Tribrid™ mass spectrometer. The AcquireX DeepScan data acquisition strategy was used to automatically generate background exclusion and compound inclusion lists that were updated iteratively for replicate injections. Raw data were analyzed using Thermo Scientific™ Compound Discoverer software to annotate compounds present in the plasma extracts.

Results and Conclusions

LC-MS data were acquired for SRM1950 plasma extracts and for extracts spiked with metabolite standards. The AcquireX Deepscan intelligent acquisition method was used to prioritize molecular ions for data dependent MS/MS. The AcquireX algorithm generated an exclusion list from a blank injection, to minimize fragmentation of background ions. An inclusion list was generated from LC-MS of the plasma sample, by performing feature detection and adduct/isotope grouping. These automatically generated lists were updated between LC-MS/MS injections, to reduce redundant data collection and to select more unique metabolites for fragmentation. Data were analyzed with Compound Discoverer software based on a formula search of selected ChemSpider databases and fragmentation search against the MSn mzCloud library.

Preliminary results using the AcquireX acquisition strategy show that more than 2800 compounds were detected in the positive ion reversed-phase analysis with 90% of MS2 spectra being acquired on the preferred protonated ions. Using the elemental compositions of molecular species determined from ultra-high resolution MS data more than 2500 database hits were found. The MS2 product ions were searched against the mzCloud MSn library (>16,000 compounds and 3.9 million spectra) providing more than 1900 similarity matches and 350 identity matches to known metabolites. Database hits without an mzCloud match were prioritized using the mzLogic algorithm that ranks annotations by mapping potential structures to known fragment ion structures in mzCloud. This automated workflow for global analysis and data processing resulted in improved metabolome coverage and highly confident annotations as illustrated using metabolite standards spiked into plasma.

Novel Aspect

Improved metabolome coverage using automated acquisition and data processing to maximize metabolite annotation and validate confident identification of relevant metabolites

Keywords

Orbitrap ID-X, metabolomics, untargeted profiling, AcquireX

Authors

Dauly Claire [1]

1. Thermofisher Scientific (France)

Characterization of proteins involved in plant defense responses by an LC-MS/MS approach for phytohormone quantification [P79]

Plants are sensitive to different bioagressors and have to adapt their defense mechanisms accordingly. We use the model plant *Arabidopsis thaliana* to study the interaction with the downy mildew oomycete pathogen, *Hyaloperonospora arabidopsidis (Hpa*). Defense responses against this pathogen are governed by cellular signaling pathways that are coordinated by the phytohormone salicylic acid (SA). In its unconjugated form, SA is the active defense molecule, but it becomes cytotoxic when it accumulates to high levels. UDP-glucosyltransferases (UGTs) catalyze the transfer of a glucose residue to SA thus forming SA-glucoside (SAG) or SA-glucose ester (SGE), which both are inactive forms destined for vacuolar storage. Arabidopsis mutants for the glucosytranferase UGT76B1 are more resistant to *Hpa* infection, whereas plants that overexpress the enzyme are more susceptible. *Ugt76b1* mutants accelerate and enhance the onset of SA-dependant defenses, when compared to wild-type plants. A comparative transcriptome analysis between the *ugt76b1* mutant and wild-type plants revealed EXTRACELLULAR LIPASE 4 (EXL4) as a potential regulatory protein in UGT76B1-mediated defense responses.

In order to validate and explain the functions of both UGT76B1 and EXL4 in the SA-dependent defense pathway, the Analytical Biochemistry Platform (Sophia Agrobiotech Institute) set up a metabolomics approach to quantify free and storage forms of SA in mutant and wild-type Arabidopsis by LC-MS (microTOFQII, Bruker – PlantBIOs facilities *https://www6.paca.inra.fr/institut-sophia-agrobiotech/Infrastructure-PlantBIOs*). Our results partially validate a hypothetical model and lead us to propose other potential hypotheses about the function of both enzymes.

Keywords

Small molecules, Mass spectrometry, Plant Health

Authors

Seassau Aurélie [1], Allasia Valérie [1], Magliano Marc [1], Keller Harald [1]

1. Institut Sophia Agrobiotech [Sophia Antipolis] (France)

Characterization of microorganisms by proteins and lipids MALDI-TOF fingerprints: case studies [P140]

Introduction

MALDI-TOF MS is an emerging technique for microbial identification, characterization and typing that has been successfully used in the clinical field. Though, studies related to the characterization of remain scarce. In order to fill this gap, protein profiles of various microorganisms have been acquired and classified. Moreover lipid profiles have been obtained and are expected to be complementary to protein profiles for rapid classification.

Methods

The lipids of each strain are extracted after maceration in an MTBE / MeOH mixture and then recovery of the organic phase and evaporation of the solvent. These lipid extracts are solubilized in a CHCl3 / MeOH mixture (2/1) and then analyzed by MALDI TOF in positive mode (DHB matrix at 20 mg/mL in THF) and negative (9-AA matrix at 10 mg/mL in MeOH). Protein extraction: (CH3CN / H2O / HCOOH: 50/15/35) then MALDI-TOF analysis (α-CHCA matrix at 10 mg/mL)

Results

We were able to confirm that the spectral fingerprint of a protein extract is dependent on the species of environmental microorganism, whether for a bacterium or a fungus. For example, 16S sequencing of strains belonging to genera such as Bacillus is not sufficiently discriminating for identification at the species level. On the other hand, clusters within the same genus appear well defined when observing the spectral imprints of these same strains. Unlike protein extracts, lipid fingerprints do not seem to depend on the species but only on the genus in most cases. Only data on fungi of the genus Fusarium allow discrimination at the species level. The study related to lipid fingerprints has shown that strain classification can be performed even though genetic sequencing has not been performed or if the protein fingerprint is too specific to be related to a known species.

Conclusion

In our study we observed a perfect concordance of "clustering" between genetics and spectral fingerprints. We have therefore been able to provide a proof of concept concerning the possible identification of the different environmental microorganisms by comparison of protein and / or lipid fingerprints and to show the important potential of MALDI-TOF mass spectrometry for the identification of these environmental microorganisms.

Novel Aspect

New MALDI-TOF methodology for the identification of environmental strains (phytopathogens, mutualist microorganisms, endophytes, etc.)

Keywords

MALDI-TOF, proteins, microorganisms

Authors

Vincent Guérineau, Morgane Barthélemy, Marceau Levasseur, Téo Hébra, Véronique Eparvier, David Touboul.

1. Institut de Chimie des Substances Naturelles, UPR 2301, Université Paris Saclay, CNRS, Gif-sur-Yvette

Proteomics Sciences

An In-solution digestion method to improve identification Extracellular Matrix and Membrane Proteome coverage in small sample amounts [P80]

Introduction

The extracellular matrix (ECM) is a complex meshwork of cross-linked proteins providing both biophysical and biochemical cues that are important regulators of cell proliferation, survival, differentiation, and migration. ECM is a highly dynamic structure that is present in all tissues and continuously undergoes controlled remodeling [1]. Quantitative proteomic analyzes of ECM proteins without pre-fractionation or enrichment beforehand are usually already difficult because of the different biochemical nature of its components. It becomes a real challenge on tiny tissues.

Methods

We test different protocols of extraction and digestion murine pial arteries and brain microvessels. The peptides were separated by nanoLC before to be analysed by Qex HF mass spectrometer. Raw mass spectral files were searched with MaxQuant against the SwissProt *mus musculus* database protein. Evaluation was performed according to the number of identified proteins, quantified proteins and the number of ECM proteins, with reproducibility and repeatability.

Results

Six protocols of extraction/digestion have been tested with minimal manipulation for robust label free quantification, which requires high reproducibility and repeatability. The proteolysis was optimized so that hydrophobic proteins such as membrane proteins could be identified together with small soluble proteins: up to 40% of membrane proteins and up to 12% of ECM proteins. The method was tested over a period of 3 years (stability of number of protein ids and retention times) which enabled the comparison over a long period of analysis.

The processing workflow for the normalization of extracted ion chromatogram was optimized, taking into account the missing values, to keep a maximum information with a minimum alteration. The bioinformatic processing was carried out by a homemade R script to implement the selected workflow which was not directly available. This new algorithm performs adapted imputations to discover new proteins. With the best method, we could identify more than 3000 proteins and until 100 ECM proteins in single one shot.

Conclusions

We developed a workflow to reproducibly analyze very tiny tissue samples like murine small vessels, and to identify ECM proteins without enrichment. We validated it on a murine model with a known and documented small vessel disease. The structural and physicochemical diversity of ECM proteins makes their analysis particularly complex, but identifying these proteins is very important to understand the development of some pathologies such as cancer or fibrosis.

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Keywords

Proteomics, matrisome, label free quantitative analysis

Authors

Haddad Iman [1], Joutel Anne [2] [3], Vinh Joëlle [4]

1. Laboratoire de Spectrométrie de Masse Biologique et Protéomique (France)
2. Genetics and Pathogenesis of Cerebrovascular Diseases (France)
3. Hôpital Lariboisière (APHP) (France)
4. Spectrométrie de Masse Biologique et Protéomique (France)

Study of Tetracycline-induced inhibition of protein synthesis in bacteria by isotopic labelling-MS approach [P81]

Introduction

The global spread of antibiotic resistance among pathogenic bacteria is recognized as one of the biggest concerns in public health and a research priority in microbiology. Tetracycline (Tc) is a bacteriostatic antibiotic that binds to ribosomes and blocks protein synthesis. Live-cell microscopy experiments led to the observation that in presence of inhibitory concentration of tetracycline, protein synthesis was not completely inhibited in wild-type *E. coli* strain (WT) whereas they are not produced for a mutant. The maintenance of residual translation activity depends on the role of AcrAB-TolC efflux pump, which transports the drug outside the cells. The objective is to evaluate the level of protein synthesis in *wt* and AcrAB-TolC mutant strains, in the presence and in the absence of tetracycline.

Methods

With TMT quantitative proteomics approach on a Q Exactive HF mass spectrometer, we compared the protein composition of a total extract before (T0) and after incubation for 3H with 10 mg/mL of tetracycline (T3) in wt and acrA, acrB and tolC mutants.

Results

We found that Tc treatment results in the underrepresentation of 28.5% of significantly quantified proteins (*P-Value* < 0.05) in *wt* cells. This percentage increases to 33.9%, 41.8% and 47% in *acrA, acrB* and *tolC* mutants, respectively, corroborating that AcrAB-TolC complex acts to maintain protein synthesis in the presence of Tc. Unexpectedly, Tc treatment also induces the overrepresentation of a same set of proteins in all strains. These overrepresented proteins are all associated with the cell outer membrane, among which OmpA, OmpC and OmpX, TonB-dependent transporters (FhuA, FecA and BtuB), ion transporters (Tsx), proteins involved in membrane lipid composition (FadL) and in murein synthesis (MipA). It is probable that these proteins are specifically regulated in response to Tc-induced stresses.

Reference

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Keywords

Bacteria, drug resistance, quantitative proteomics

Authors

Page Adeline [1], Delolme Frédéric [1], Nolivos Sophie [2], Cayron Julien [2], Dedieu Annick [2], Lesterlin Christian [2]

1. SFR Biosciences (France)
2. Molecular Microbiology and Structural Biochemistry (France)

Impact of quantitative proteomics in better understanding Ehrlichia ruminantium infectious process and better defining an inactivated vaccine against Heartwater. [P82]

Introduction

Ehrlichia ruminantium, the causative agent of Heartwater in ruminants, is an obligate intracellular rickettsiale transmitted by ticks of the genus *Amblyomma*, resulting in disseminated vascular endothelial cell infection with deleterious vascular leakage. A better understanding of both the expression of bacterial proteins and the regulation of host proteins during the infectious kinetics of *E. ruminantium* in endothelial cells is crucial for the development of improved control strategies such as multivalent inactivated vaccines.

Methods

We performed a quantitative proteomic profiling of both infectious kinetics of cultured endothelial cells and sera from experimentally infected ruminants, in order to identify proteins and biochemical pathways affected by *E. ruminantium* infection. Quantitative proteomics allowed us also to qualify the batches of inactivated vaccines prepared from bacteria purified after lysis of synchronised endothelial cells infections.

Results

We identified for the first time bacterial proteins and key effectors, and demonstrated that proteins from the infectious kinetics of cultured endothelial cells are first down regulated during the host cell invasion by the bacterium, then the replicative phase of *E. ruminantium* is associated with the induction of numerous host proteins and finally a second down regulation host proteins occur prior to cell lysis.

The role of ticks in the transmission of *E. ruminantium* was also highlighted by the modulation of serological biomarkers in the ruminant host, depending on whether the bacterium was injected mechanically by a syringe or naturally by tick bite.

The quantitative proteomic profiling of *E. ruminantium* purified after host cell lysis highlighted the persistence of about 35% of host proteins in vaccine batches. Moreover, reproducibility between batches allowed a ranking of proteins of interest to define a standard pattern from effective historical vaccine batches to qualify new production batches without animal testing.

Conclusions

Quantitative proteomics is a method of choice to dissect pathogen-host interactions, to determine the weight of the vector in modulating the host response, as well as to define new methods of qualifying batches of multivalent inactivated vaccines. Such integrated analyses pave the way towards improved strategies to interfere with the infectious processes in the corresponding tick-borne disease Heartwater.

Novel Aspect

Quantitative proteomics profiling constitutes a pilot for control quality of multivalent inactivated vaccines against Heartwater.

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Keywords

Ehrlichia ruminantium, tick-bacterium-host molecular interactions, vaccine quality control.

Authors

Holzmuller Philippe [1] [2]

1. CIRAD UMR ASTRE (France)
2. ASTRE, Univ Montpellier, CIRAD, INRA, Montpellier, France (France)

Use of ion mobility on a TimsTOF Pro instrument to improve proteomics analysis performances [P83]

Introduction

The use of Trapped ion mobility spectrometry (Tims) in the TimsTOF Pro allows decreasing the complexity of spectra by the addition of a new separation dimension. This technology is recent and the data generated are not yet fully exploited. In this context, two versions of the MaxQuant software [1] were compared for their identification and quantification performances. The main interest of this work was to evaluate the impact of latest MaxQuant version which has been optimzed for the processing of 4-dimensional PASEF data [2] for protein identification and quantification.

Methods

HeLa cells proteins and mixtures of UPS1 standard proteins in a yeast background were reduced, alkylated and digested with trypsin. Peptides were analysed by nLC-IMS-MS/MS (NanoElute coupled with a TimsTOF Pro, Bruker) using a PASEF scan mode [3]. MaxQuant ver. 1.6.2.10 and ver. 1.6.6.0 were used to analyse data both qualitatively and quantitatively. Classical FDR filters were applied on PSM and protein levels for peptide/protein identification validations.

Results

Comparisons were done on the numbers of identified and quantified proteins after analysis with both MaxQuant versions. Overall, numbers of identified and quantified proteins were higher with MaxQuant ver. 1.6.6.0 for all treated datasets from 10 to 200ng of injected HeLa digest. The identification of UPS1 standard proteins spiked in different quantities in a complex background is improved. All 48 UPS1 proteins were identified in the 5 and 2.5 fmol-spiked points in 200 ng of background, while up to 25 UPS1 proteins were identified in the 25 amol-spiked point. An improvement is also seen in quantification. For quantification performance evaluation, additional filters were applied to keep only proteins quantified with no missing value for an injection triplicate and to keep only proteins quantified with a CV on the LFQ lower than 20%. With those filters applied, the latest MaxQuant version allowed improved numbers of quantified proteins all over the spiked concentration range.

Conclusions

The use of Tims data in MaxQuant ver. 1.6.6.0 for the treatment of TimsTOF Pro analyses improves the number of identified and quantified proteins with various amount of material, or for spiked-in standards in a complex background. The use of Tims information for data treatment is only at its beginning and more improvements are awaited in the future. Investigation of a new denoising algorithm in the acquisition software is currently under progress.

Novel Aspect

This work illustrates the impact of using tims generated data as a fourth dimension for the identification and quantification of proteins with MaxQuant.

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Keywords

nLC-IMS-MS/MS, MaxQuant

Authors

Moritz Chloé [1], Strub Jean-Marc [1], Carapito Christine [1], Schaeffer-Reiss Christine [1]

1. Laboratoire de Spectrométrie de Masse Bio-Organique (LSMBO) (France)

In vitro and in cellulo Mass Spectrometry Study of TEAD palmitoylation [P84]

Introduction

The TEAD proteins consist of 4 transcription factors which modulate gene expression in response to the Hippo pathway by interacting with co-activator proteins such as YAP and TAZ. The hippo pathway plays an important role in cancer progression [1]. Recent studies have shown that TEAD contain a hydrophobic central pocket (CP), occupied by a palmitate moiety. Activity and stability of TEAD are regulated by this modification [2]. As, the TEAD CP can be targeted to inhibit YAP-TEAD dependent transcription, it is crucial to fully understand the palmitoylation status of TEAD.

Methods

LC/MS/MS-based assays were developed on recombinant TEAD1. Intact protein and digests were analyzed on a PepSwift Monolithic column coupled to a Q Exactive HF. Targeted MS/MS assays were developed using the modified peptides identified from the recombinant protein. Endogenous TEAD1 was then immunoprecipitated from H2052 cells and digestion was performed on IP beads using the iST Sample Preparation Kit (Preomics). Targeted MS assays were applied on those digests.

Results

Intact mass measurement allowed us to rapidly characterize the protein modification status of the recombinant protein. Peptide mapping permitted not only to confirm the expected site of palmitoylation (cysteine 359 in the CP) but also to detect palmitoylation and mirystoylation on the lysine residue 336 located at the entry of TEAD1 CP. These modified peptide signatures, obtained on recombinant protein, were used to search for the modifications of endogenous TEAD1 in H2052 cells after enrichment of the protein by immunoprecipitation. We showed that these hydrophobic peptides could not be recovered after in-gel digestion but that digestion performed directly on IP beads allowed us to recover those peptides and thus to prove the existence of the C-palmitoylated peptide SPMC359EYMINFIHK and of the peptides QVVEK336VETEYAR with K being myristoylated & palmitoylated.

Conclusions

In this study, we developed a MS-based strategy to characterize post translational modifications directly on TEAD1 protein expressed in eukaryote H2052 cells. This strategy could be applied to assess target occupancy by covalent inhibitors.

Novel Aspect

Those modifications have never been identified directly on endogenous TEAD in cellulo.

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Keywords

TEAD, Palmitoylation, Targeted MS/MS

Authors

Buzy Armelle [1], Guichard Olivier [1], Zachayus Jean-Luc [1], Venier Olivier [1], Courtin Olivier [1], Valtingojer Iris [1], Mikol Vincent [1], Guillemot Jean-Claude [1]

1. SANOFI Recherche (France)

Quantitative proteome and phosphoproteome for the characterization of the serine/threonine kinase PrkC of Clostridium difficile and identification of kinase substrates [P85]

Introduction

Clostridium difficile is the leading cause of intestinal nosocomial post-antibiotic infections in adults. During infection, the bacterium must rapidly respond and adapt to the host environment by using survival strategies. Phosphorylation is a reversible post-translational modification employed for signal transduction and regulation. Bacterial serine/threonine kinases and their associated phosphatase play an essential role in the regulation of many different processes in bacteria, such as cell division, cell wall biosynthesis, antibiotic tolerance and virulence.

Methods

A TiO2 based enrichment method was used to characterize the STK PrkC and identify its kinase substrates. These phosphoproteomic approaches are now well established for eukaryote cells but still remain very challenging when it comes to analyze PTMs of bacteria due to the very low stoichiometry, limited dynamic range, and quantitative difficulties. Optimized TiO2 based enrichment was applied for the analysis of the phosphoproteome of WT strain and Δ*prkC* mutant.

Results

Our fully optimized protocol has allowed the identification of more than 1200 phosphosites, which is the highest number of phosphosites identified in this bacterium so far. By comparing the phosphoproteome of the WT strain and the Δ*prkC* mutant, we identified substrates of PrkC corresponding to proteins that participate in different cell processes affected in the Δ*prkC* mutant. This result suggests that PrkC in *CD* has a pleiotropic effect by targeting multiple pathways. Our identified phosphosites have been validated using complementary approaches. Among PrkC identified targets, IreB is a small protein of unknown function that is well conserved in Firmicutes. IreB has been identified as a negative regulator of cephalosporin resistance in *Enterococcus faecalis* [2]. We demonstrated *in vivo* and *in vitro* that IreB was specifically phosphorylated by PrkC at Threonine 8. Interestingly, using size-exclusion chromatography we observed that phosphorylation of IreB promotes its oligomerization. The others candidates are currently studied.

Conclusions

We implemented a method to enrich on bacterial phosphopeptides and obtained a comprehensive phosphoproteomics data on CD. These data represent a resource to decipher the signaling mechanisms contributing to resistance eventually leading to alternative strategies to combat antimicrobial resistance.

Novel Aspect

Implementation of a bacterial phosphoproteomics

References

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Keywords

Phosphoproteomics, Bacteria, *Clostridium difficile (CD)*

Authors

Matondo Mariette [1]

1. Institut Pasteur (France)

Proteomic approach identifies differential protein expression in cultured primary fibroblasts with fetal bovine serum at different passage and under stimulation with TGF-β1 [P86]

Introduction

Fibroblasts (Fb) are key effectors cells in systemic sclerosis (SSc) [1]. Fb stimulation with TGF-β1 is considered as the positive control in studies assessing the fibrogenesis [2]. The lack of standardization of TGF-β1 stimulation might be responsible for discrepancies in experiments performed in different conditions. Depending on the culture medium, the origin of the Fb and the ageing, cells express different protein profiles [3,4]. The aim of this study is to evaluate culture conditions and stimulation in order to standardize our approach and allow inter-data comparisons.

Methods

Primary dermal normal human Fb (ATCC ®, PCS-201-012™) were cultured at passage P3, P5 and P7 with and without Fetal Bovine Serum (FBS). At fifth passage, Fb were stimulated or not with different concentrations of TGF-β1 (0.04, 1 and 5ng/mL) (R&D Systems; 240-B-002) during 24, 48 and 72 hours. Proteins were analyzed using an eFASP LC-MS/MS (Thermo Scientific; Q Exactive +). Proteins quantitation was performed by Maxquant and statistical analysis by Perseus using ANOVA.

Results

Comparison of the passage and the conditions of culture with and without FBS: A total of 2625 were identified, of which 583 showed differential expression using ANOVA analysis. As expected, principal component analysis (PCA) revealed several clusters of differential proteins expression conditioned by the passage and the culture medium.

Comparison of Fb P5 stimulated or not with TGF-β-1 at different times: A total of 3267 proteins were identified, of which 1921 showed differential expression using ANOVA analysis. PCA revealed several clusters of differential proteins expression. There were clear clusters of protein expression related to (i) unstimulated and stimulated conditions, (ii) between the three different times of stimulation and (iii) TGF-β1 concentrations used. Although, the expression of proteins in Fb exposed to 0.04 and 1ng/mL of TGF-β1 during 72h were rather close, there was a unique proteins profile related to the condition with 5ng/mL of TGF-β1 during 72h.

Conclusions

This study highlights a variation of proteins expression depending on: (i) the passage and the conditions of culture and (ii) both stimulation time and TGF-β1 concentrations in primary Fb. The identification of protein differentially expressed will provide insights in the impact of TGF-β1 on Fb physiology with stimulation. These data underline the need of standardization of culture conditions to allow inter-data comparisons using in sensitive « omic » approaches.

Novel Aspect

In SSc, this study raises the importance of standardization of conditions (medium, cell passage) and controls (TGF-β1 concentrations) in interpretation inter-data results.

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Keywords

Fibroblasts, LC-MS/MS, TGF-β1

Authors

Vivier Solange [1], Chepy Aurelien [1] [2] [3], Bray Fabrice [4], Guerrier Thomas [1] [5], Balden Maité [1] [5], Rolando Christian [4], Hachulla Eric [1] [2] [3], Launay David [1] [2] [3], Dubucquoi Sylvain [1] [2] [5], Sobanski Vincent [1] [2] [3]

1. Univ. Lille, Inserm, CHU Lille, U995 - LIRIC - Lille Inflammation Research International Center, F-59000 Lille, France (France)
2. Département de Médecine Interne et Immunologie Clinique, CHU de Lille (France)
3. Centre national de référence maladies systémiques et auto-immunes rares (sclérodermie systémique), CHU Lille (France)
4. Miniaturisation pour la Synthèse, l'Analyse et la Protéomique - USR 3290 (France)
5. Institut d'Immunologie, Centre de Biologie Pathologie, CHU Lille, F-59000 Lille, France (France)

A candidate reference method for PCT quantification in human serum using ID-LC MS/MS [P87]

Introduction

Procalcitonin (PCT) is a promising biomarker for the early and accurate diagnosis of sepsis and a potential means to guide antibiotic stewardship, reducing inappropriate antibiotic use [1,2]. For many years, routine assays of PCT have been performed using immunoassays. Recently, a study showed that these immunoassay methods might not be used interchangeably for patient monitoring because of a significant difference of results [3]. An higher order reference method and certified reference materials are urgently needed, which might help to harmonize and standardize these assays.

Methods

To ensure metrological traceability of clinical PCT measurements to SI, a candidate reference measurement procedure based on ID-LC-MS/MS has been developed using a peptide-related PCT as primary reference calibrator. Therefore, the peptide primary reference calibrator was SI traceably quantified using amino acid analysis and its impurity profiling was assessed by intact mass spectrometry.

Results

LC-Orbitrap MS based method in the PRM mode is demonstrated as the best method instead of DDA mode (TopN) for targeted quantification of proteins very low abundant in a complex biological matrix.

An analytical method for PCT quantification in human serum using peptide-based calibration approach was validated. The assay proved to be linear and acceptable, with a regression coefficient above 0.997. The intra-assay and inter-assay accuracy were closed to 100% for three concentration levels of quality control and the intra-assay and inter-assay imprecisions were below 6.8%. LLOQ value was evaluated at 2.3 ng/mL using standard-flow LC. Downscaling our analysis using in nano-flow LC was demonstrated to be 30 times more sensitive than standard-flow LC using the same sample preparation.

Conclusions

The method developed aims at assessing accuracy of routine methods and certifying concentration of secondary calibrators that could be used to recalibrate immunoassays if desired. The calibration range with an LLOQ above 2 ng/mL using standard-flow LC, did not encompass the low clinical cut-offs but remains adapted for the patient monitoring over time. Nano-flow LC seems to be adapted for the quantification of very-low abundant proteins in complex matrices.

Novel Aspect

The analytical performance of peptide-based calibration method should be challenged to the protein-based calibration method using stable isotope labeled protein.

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Keywords

Procalcitonin, sepsis, reference method

Authors

Huynh Huu Hien, Boeuf Amandine, Lalere Béatrice [1], Vaslin-Reimann Sophie [1], Delatour Vincent [1], Vinh Joelle [2]

1. LNE - Département « Biomédical et chimie organique » (France)
2. Laboratory of Biological Mass Spectrometry and Proteomics, CNRS USR3149, ESPCI Paris, PSL Research University (France)

Label-free quantitative proteomics of human muscle cells treated with the serum from brown bears [P88]

Introduction

Muscle atrophy is a main adverse outcome of, e.g., ageing and physical inactivity. Studies using rodent/human models allow basic knowledge on muscle atrophy mechanisms to progress. However, there is still no effective therapeutic strategies. Hibernating animals exhibit a limited loss of muscle mass and strength despite prolonged fasting and physical inactivity. Recently, we have demonstrated that the hibernating bear serum inhibits proteolysis in human muscle cells [1]. Here we explore the systematic effects of the winter bear serum on the proteome from human muscle cells.

Methods

Human muscle cells from different donors were incubated with the serum from bears collected during hibernation and the active period. The proteome of human muscle cells was analyzed on a NanoAcquity (Waters) – Q-Exactive Plus (Thermo Scientific) system in two parallel label-free quantitative proteomics experiments (n=8 each). Protein identification (1% FDR) and quantification were performed with MaxQuant software.

Results

Robustness of our system was monitored thanks to iRT peptides showing low retention time shifts and reproducible intensity measurements (CV <20%). Thanks to Andromeda analysis, 3242 and 3605 proteins were identified in the first and second experiment, respectively. 72% of proteins were commonly identified in both experiments. 3034 and 3304 proteins were quantified (only one missing value accepted per group) in the first and second experiment, respectively. Student t-tests highlighted 48 and 42 differential proteins in the first and second experiment respectively (p-value < 0.05). Functional categorization of proteins, from GO term extraction, revealed that carbohydrate, lipid, and protein metabolisms were among the main affected pathways in human cells exposed to the hibernating bear serum. 6 proteins were commonly identified as differentially expressed in the two experiments, with similar fold-changes, namely P4H2A, OAT, FBLN1, THBS2, HMGCS1 and ARFGAP1.

Conclusions

Robustness of label-free quantitative proteomics highlighted that the serum of hibernating bears regulates mainly fuel/energy metabolism in human muscle cells. This is in perfect line with the in vivo situation [2], suggesting that the bear serum is inducing a hibernation-like state in human cells. The 6 proteins regulated similarly whatever cell donors and/or bear sera will help to recognize active serum fractions/compounds in the near future.

Novel Aspect

Label-free quantitative proteomics identified cross-species effects of circulating bear compounds, which remain to be identified, on human muscle cells.

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Keywords

Human skeletal muscle, label-free quantitative proteomics, bear serum, metabolism

Authors

Chazarin Blandine [1] [2], Chanon Stéphanie [3], Gauquelin-Koch Guillemette [1], Blanc Stéphane [4], Lefai Etienne [5], Bertile Fabrice [2]

1. Centre National d'Études Spatiales (France)
2. IPHC-DSA, LSMBO (France)
3. CarMen laboratory (France)
4. IPHC, DEPE (France)
5. Univ. Auvergne,INRA (France)

Large-scale quantitative proteomics revealed the nature and cause of the different metabolic features of weak and strong antibiotic producers Streptomyces species [P89]

Introduction

The *Streptomyces* genus is well known for its ability to produce numerous and diverse bio-active molecules useful to human health, including life-saving antibiotics. The biosynthesis of these specialized metabolites usually occurs at stationary stages of growth and is triggered by nutritional limitations, especially in phosphate [1]. Despite numerous important scientific contributions over the past 40 years, a systemic understanding of the biosynthesis of these bio-active metabolites and the function for the producing bacteria remains incomplete.

Methods

To address these questions, a high-throughput label-free proteomic analysis of two closely related model species *S. coelicolor* and *S. lividans* was carried out in an Orbitrap FusionTM LumosTM TribridTM in DDA mode. These strains possess identical biosynthetic pathways directing the synthesis of three well characterized secondary metabolites (CDA, RED and ACT) but only *S. coelicolor* produces them at high levels. The bioinformatic pipeline was performed using open-source and free software (X!Tandem Pipeline/MassChroQ/MCQR) developed at PAPPSO facilities [2,3,4].

Results

This study represents the largest proteome dataset of the *Streptomyces* genus with 4372 different proteins identified. The main outcome of this work was the significantly lower abundance, in *S. coelicolor* compared to *S. lividans*, of the two-component system PhoR/PhoP which is known to control positively the expression of proteins involved in phosphate (P) scavenging and uptake and negatively proteins involved in nitrogen (N) assimilation. This resulted into low P and high N availability in *S. coelicolor* that supported the activation of the Krebs cycle and thus the oxidative metabolism of this strain. However, ATP synthesis linked to an active oxidative metabolism requires phosphate whereas *S. coelicolor* is severely limited in phosphate. The latter induced the production of the specialized metabolite ACT. Since this process is known to correlate with an abrupt drop in the intracellular ATP concentration [1] we propose that ACT would capture the electrons of the respiratory chain in order to reduce ATP synthesis and thus adjust ATP synthesis to low P availability.

Conclusions

This work is pioneering in the elucidation of the basis of the metabolic differences underlying the drastically different abilities of *S. coelicolor* and *S. lividans* to produce antibiotics and their origin. This new knowledge is expected to lead to the conception of novel strategies to access the vast metabolic diversity encoded in the numerous silent biosynthetic pathways present in the *Streptomyces* genomes.

Novel Aspect

A novel view of the the role played by the antibiotics in the regulation of the energetic metabolism of the producing bacteria in conditions where phosphate is scarce was described.

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Keywords

label-free proteomics, antibiotics, oxidative metabolism.

Authors

Millan Oropeza Aaron [1], Henry Céline [1], Lejeune Clara [2], David Michelle [2], Virolle Marie-Jöelle [2]

1. INRA - MICALIS - PAPPSO (France)
2. Institut de Biologie Intégrative de la Cellule (France)

Proteomic analysis of podocytes in nephrotic syndrome [P90]

Introduction

Focal Segmental Glomerulosclerosis (FSGS), a form of idiopathic nephrotic syndrome, is a rare kidney disease defined by edema, hypoalbuminemia and massive proteinuria. Podocytes are kidney cells that contribute in maintaining the structural integrity of the glomerular filtration barrier as its injury results in proteinuria [1].

The molecular mechanisms in the podocytes during the disease remain poorly understood.

Using mass spectrometry, we aim to identify total and rafts proteome changes to better understand the molecular mechanisms of podocytes dysfunction in FSGS.

Methods

A human podocyte cell line was stimulated with 8 plasma exchange of control (n=4) and FSGS (n=4) patients. Proteins (4h and 24h stimulation) were digested with S-TrapTM and peptides were fractionated at high pH. For the raft proteome, a 3-step discontinuous density gradient was made with OptiprepTM [2]. Peptides were analysed with a Q Exactive PLUS using a 2h-gradient. Label-free quantification and statistical analysis were performed with Maxquant and Perseus.

Results

Stimulation of podocytes with plasma was performed for 30min, 4h or 24h. Plasma stimulation resulted in total proteome changes mostly at 4h. Little changes were observed at 24h. 5200 proteins were quantified for total proteome analysis at 4h and 24h. Forty-six proteins were found differentially expressed at 4h between the two groups. Among these proteins, one protein involved in vesicle exocytosis is upregulated. At 30min, 2500 proteins from the enriched fraction of rafts were quantified. 52 proteins are differentially recruited in the raft fraction between the two groups. Among these proteins, components of the mTOR signaling pathway are upregulated among podocytes treated with plasma of FSGS patients. Interestingly, seven mitochondrial proteins are downregulated in the FSGS group.

Conclusions

Our studies reveal that plasma exchange from FSGS patients have an effect on a protein, highly specific of vesicle exocytosis at 4h. Furthermore, our results suggest a dysfunction of mitochondrial functions and dysregulation of the mTOR signaling in this disease. Validation of these proteins by Western-Blot in the human podocyte cell line and in glomeruli from FSGS patients are ongoing.

Novel Aspect

This study sheds new lights into the molecular mechanisms of podocytes dysfunction in FSGS.

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Keywords

Kidney, podocytes, label-free

Authors

Chhuon Cérina [1], Jung Vincent [1], Lipecka Joanna [1], Sahali Djillali [2], Ollero Mario [2], Chiara Guerrera [1]

1. Plateforme Protéomique 3p5-Necker, Université Paris Descartes-Structure Fédérative de Recherche Necker, Inserm US24/CNRS UMS3633 (France)
2. Institut Mondor de Recherche Biomédicale (France)

Towards turnkey targeted proteomics solution using internal standard triggered acquisition on modified Orbitrap mass spectrometers [P91]

Introduction

The advances in HRAM mass spectrometry instrumentation have enabled new approaches for targeted quantitative proteomics, including a prototype implementation of an internal standard triggered-PRM (IS-PRM) to dynamically control the acquisition process and maximize its efficiency. Here, the acquisition scheme of the approach has been revisited, while keeping the same rationale, to enable a more generic implementation on latest generation Orbitrap-based instruments.

Methods

The analyses were performed on Thermo Scientific Orbitrap Exploris 480 and Orbitrap Eclipse Tribrid mass spectrometers. New acquisition schemes were implemented in the instrument control software. Different set of peptide standards were used to develop and evaluate the modified IS-PRM method, including commercially available kits and custom made SIL peptide panels. Analyses were applied to cancer line models, tissue and plasma samples.

Results

In comparison with conventional PRM, the initial implementation of IS-PRM significantly enhanced the acquisition efficiency (typically 5-fold higher) and expanded the scale and data quality of targeted experiments. In spite of these clear benefits, the technique has been weakly embraced across the community, mainly due to its limited access (requiring special API license), and the need for specific informatics tools to support method preparation. Therefore, in order to foster a broader adoption of the approach, its generic implementation in the native instrument control software of latest generation Orbitrap-based instruments has been conducted, while improving method usability. This required alternative data collection/evaluation strategies to be explored in “watch mode”, including the interrogation of MS1 data. One adapted user-friendly version of IS-PRM method has ultimately been retained, yielding similar acquisition efficiency to the original version (>80%), and superior robustness against chromatographic variations.

Conclusions

The analytical performance of the method applied to the analyses of various types of samples in various formats (30-800 IS) has been further evaluated, confirming its capability to confidently quantify endogenous peptides in the low amol range at all scales. The ability to embed in instrument software pre-set (optimized) methods associated with predefined kits of IS represents a decisive step towards the provision of turnkey targeted proteomics solution.

Novel Aspect

Implementation of an internal standard triggered targeted quantitation workflow in the native instrument control software of new Orbitrap mass spectrometers.

Keywords

SureQuant, IS-PRM, targeted proteomics

Authors

Dauly Claire [1]

1. Thermofisher Scientific (France)

Complementary use of mass spectrometry and cryo-electron microscopy to assess the maturity of live attenuated dengue vaccine viruses [P92]

Dengue virus (DENV) infection is a global health threat with the potential to affect at least 3.6 billion people living in areas of risk. No specific curative treatments against dengue disease are available and vaccines are currently the only way to prevent the disease. The tetravalent dengue vaccine developed by Sanofi-Pasteur has demonstrated significant efficacy in phase III studies and is now licensed in several countries for the prevention of disease in dengue-seropositives over 9 years of age. The vaccine is composed of four recombinant, live, attenuated vaccines (CYD 1–4) based on a yellow fever vaccine 17D (YFV 17D) backbone, each expressing the pre-membrane (prM) and envelope (E) genes of one of the four DENV serotypes. Virus maturity could impact the biological activity of the vaccine viruses.

To address this question, the maturity of the four vaccine viruses used in phase III clinical studies was assessed by two complementary techniques: mass spectrometry (MS) and cryo-electron microscopy (cryoEM). MS assessed viral maturity at the molecular level by quantifying specifically the prM, and M proteins. CryoEM provided information at the particle level, allowing visualizing the different phenotypes of viral particles: spiky (immature), smooth/bumpy (mature), and mixed (partially mature).

Results of the two assays used in this study show that all four CYD dengue vaccine viruses present in lots used in phase III efficacy trials, present in the majority a mature phenotype.

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Keywords

dengue vaccine, virus maturity, mass spectrometry

Authors

Trauchessec Mathieu [1], Lambert Olivier [2], Bonnafous Pierre [3], Berard Yves [4], Barriere Fabienne [4], Huillet Celine [4], Marco Sergio [4], Sirohi Devika [5], Hedrick Victoria [5], Kuhn Richard [5], Guy Bruno [4], Ronzon Frederic [4], Manin Catherine [4]

1. ANAQUANT (France)
2. Institute of Chemistry & Biology of Membranes & Nano-objects (France)
3. Université de Bordeaux, IECB, Inserm U1212, CNRS 5320 (France)
4. Sanofi Pasteur [Marcy-l'Étoile, France] (France)
5. Purdue University [West Lafayette] (États-Unis)

Non-invasive repetitive trans-spinal magnetic stimulation (rTSMS) restores lost functions by glial scar-modulation after spinal cord injury [P93]

Spinal cord injury (SCI) disrupts communication within the nervous system, leading to a permanent loss of sensitive and motor functions below the injury level. The cellular and molecular response of the spinal cord is complex and characterized by acute and chronic response. Despite progress in the understanding of injury mechanisms, no clinical trial has demonstrated the efficacy of a repair strategy for improving functional recovery. Here, we report a non-invasive treatment based on repetitive trans-spinal magnetic stimulations (rTSMS) following SCI. Our hypothesis is that magnetic fields exert neuroprotective and remodelling effects in our mice model.

Functionnal analyses demonstrated that rTSMS enhanced tissue repair and functional recovery in acute or chronic context and in juvenile or aged mice. In order to get molecular insights associated to these stimulations, a differential proteomics analysis was performed. This “label free” approach compared proteome of the injury area between SCI mice treated with rTSMS or not. Quantitative analysis showed 148 upregulated proteins in SCI mice with rTSMS and 8 down-regulated proteins. Among upregulated proteins, we highlighted proteins implicated in axon regeneration, myelinisation, cell proliferation and neurotransmission. Together, these results demonstrated that rTSMS decreased demyelination and increased neuronal survival, axonal regrowth and proliferation of spinal cord stem cells. Finally, this rTSMS-induced therapeutic effect in a pre-clinical rodent model, have paved the route for a possible translation to clinical application in humans.

Keywords

Spinal cord injury; Repetitive magnetic stimulation; Quantitative proteomic

Authors

Chalfouh Chaima [1], Hardouin Julie [2], Guillou Clément [2], Duclos Célia [1], Xiaofei Li [3], Delarue Quentin [1], Schapman Damien [4], Cosette Pascal [2], Marie Jean-Paul [1], Guérout Nicolas [1]

1. EA3830 GRHV, IRIB (France)
2. Pissaro Proteomic Facilitiy, IRIB (France)
3. Department of Neurobiology, Care sciences and Society, Sweden (Suède)
4. SFR IRIB, PRIMACEN (France)

A modified Orbitrap™ Tribrid mass spectrometer with real-time search and advanced spectral processing enhances multiplexed proteome coverage and quantification accuracy. [P94]

Introduction

Isobaric tagging strategies using Tandem Mass Tags™ (TMT™) are powerful tools for studying how proteins interact and function in biological systems. Up to 11 samples can be multiplexing in a single high-resolution LC/MS experiment to enable state-of-the-art quantitative analysis of peptide and protein abundance. However, co-isolated ion interference can suppress ratio quantification and thereby mask true differences in protein abundance. Multiple methods and hardware solutions help alleviate the negative influence of interfering ions (SPS, FAIMS Pro, statistical analysis). However, all of these solutions come with draw backs. Here we evaluate the benefits of a modified Orbitrap™ Tribrid™ mass spectrometer including real time search capabilities, advanced spectral processing algorithms, and modified hardware to enhance TMT quantification accuracy and proteome coverage.

Methods

To assess the accuracy, precision, and sensitivity of the modifed Orbitrap Tribrid mass spectrometer for TMT based quantitation, we utilized the Thermo Scientific™ Pierce™ TMT11plex yeast digest standard. This standardized sample provides users with a tool to measure the accuracy, precision, and proteome depth of TMT methods across different instrumentation. For liquid chromatography (LC), we used an EASY-nLC™ 1200 HPLC in combination with an EASY-Spray™ C18 50cm column and an EASY-Spray ion source with a column heater set to 45°C. We collected data both with and without a FAIMS Pro™ interface. Samples were analyzed on a modified Orbitrap Tribrid mass spectrometer. Raw data files were processed using Thermo Scientific™ Proteome Discoverer™ 2.3 software using the SEQUEST® HT search engine.

Preliminary data

Synchronous precursor selection (SPS) based methods provided higher accuracy compared to MS2 methods for TMT quantitation. However, depending on precursor isolation specificity and which fragments are selected for MS3 fragmentation, quantitative accuracy can still be distorted. To improve upon existing SPS methods, we implemented a Real Time Search (RTS) filter between the MS2 and MS3 scans. This feature benefits TMT SPS-MS3 methods in two distinct ways. First, MS3 scans are only triggered if a peptide-spectrum match (PSM) is identified from the preceding MS2. This increased the number of peptides identified with SPS-RTS-MS3 by 30%. Secondly, RTS identifies precursors for MS3 on-line that are generated from the identified peptide. Thus, TMT SPS-RTS-MS3 quantitation can be improved to be 95% isolation interference free. Next, we evaluated a new feature call TurboTMT, powered by the ΦSDM algorithm. ΦSDM is an advanced spectra processing algorithm that increases resolution within a range of the spectrum without requiring a longer transient. Applying ΦSDM specifically to the TMT reporter ions increased the resolution sufficient to baseline resolve TMT isotopologues even when using transients that produce a 30,000 or 15,000 resolving power MS2 scan. ΦSDM increased both the spectral acquisition rate for TMT11plex experiments and the number of identifications for SPS-MS3. Additionally, the modified Orbitrap Tribrid mass spectrometer has an optimized quadrupole

that improves ion transmission. It is possible to use narrower isolation widths to improve TMT quantitation accuracy. Overall, the modified Orbitrap Tribrid mass spectrometer includes unique features such as RTS for TMT SPS-MS3 based quantitation, theoretical precursor isotopic envelope fitting, and TurboTMT, which together allow for intelligent acquisition methods that improve quantitation accuracy, precision, and proteome coverage.

Novel aspect

We present a new hardware and software features that enhances proteome coverage and quantification accuracy for Tandem Mass Tags™ (TMT™).

Authors

Ndiaye Sega [1], Robitaille Aaron M., Huguet Romain, Bailey Derek, Mcallister Graeme, Kreutzmann Arne, Mourad Daniel, Lopez-Ferrer Daniel, Huhmer Andreas, Zabrouskov Vlad

1. Thermo Fisher Scientific (France)

Scanning SWATH ® Acquisition Method for Improved Compound Screening [P95]

Ideally, a generic screening method would work for any compound. Targeted methods are useful, but they must be maintained: new compounds must be added to them, and retention times need adjustment occasionally. SWATH ® acquisition is close to ideal; it acquires MS/MS that represent every precursor mass of interest at every time point. However, there are some challenges. The deconvolution of MS/MS (for library searching), or calculation of ion ratios from fragments, requires that fragments from different compounds have a different chromatographic profile or elution time. Internal standards often have similar fragmentation and retention time with the compound they are based on, which can make identification difficult. A new acquisition technique, Scanning SWATH acquisition enables measurement of both precursor mass and fragmentation for all precursors during an LC run.

Authors

Dojahn Joerg [1]

1. Sciex (Allemagne)

Static in vitro digestion tests to assess allergenic risk of novel proteins – use of a peptidomics workflow based on free softwares [P96]

Introduction

The resistance of proteins to digestion may play a role in determining their allergenic potential. In order to evaluate the digestibility of proteins, *in vitro* protocols seem appropriate when ethical constraints hinder *in vivo* studies. In our study, the 'pepsin resistance test', currently used by the European Food Safety Authority to assess allergenicity of proteins is compared to more physiologically relevant *in vitro* digestion protocols. A peptidomics workflow based on free softwares is used to identify, quantify and perform statistical analysis on the peptides produced.

Methods

Comparison of an infant, early phase adult, and late phase adult model is being applied on a panel of allergen/non-allergen proteins from animal and vegetal origin. The proteolysis rate in gastric and intestinal compartment is determined in order to detect persistent intact protein (SDS-PAGE) and hydrolysis products larger 9 amino acids (LC-MS).

All peptidomics data (NanoLC fitted to Quadrupole-Orbitrap MS) were processed with free softwares: from data conversion to peptide identification, quantification and statistical analysis.

Results

More than two thousands NanoLC-MS/MS runs were acquired during the course of this project and the number of peptides identified ranged from one to five hundred per proteins depending on their resistance to enzymatic hydrolysis. All peptides identified (X!tandempipeline software [1]) were automatically quantified (MassChroQ [2]) in all samples, regardless the fact that it was identified at this particular time of digestion. Peptide quantification results processed in the R software environment allowed the clustering of peptides based on their behavior regarding the digestion protocol used and the digestion time (0,5 to 60min). Proteins that are not pepsin resistant show slower kinetics of proteolysis for the infant model of digestion, highlighting the fact that, for this population, they may still cause undesired immune responses via sensitization in the duodenum.

Conclusions

Differences in the kinetics of proteolysis have been found across models for proteins that are not pepsin resistant. This project highlights the importance of a multi-test protocol to assess protein digestibility.

Novel Aspect

Using a full pipeline of free softwares for peptidomics data treatment [3], we were able to point out peptide clusters which are only abundant when more physiologically relevant models of digestion were used.

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Keywords

allergenicity, *in vitro* digestion, peptidomics

Authors

Jardin Julien [1], Briard-Bion Valérie [1], Torcello Gomez Amélia [2], Dupont Didier [1], Mackie Alan [2]

1. Science et Technologie du Lait et de l'Oeuf (France)
2. School of Food Science and Nutrition. University of Leeds (Royaume-Uni)

The proteom of neutrophils in sickle cell disease reveals an unexpected activation of Interferon alpha signaling pathway [P97]

Sickle cell disease (SCD) is an autosomal recessive genetic hemoglobinopathy originating from a unique mutation in the globin gene and characterized by chronic hemolytic anemia, painful vaso-occlusive crisis (VOC), progressive organ failure and premature death. Although this disease is reported as a red cell disorder, many cell types, including endothelial cells and polymorphonuclear neutrophils (PMNs), contribute to its pathophysiology. In particular, activated PMNs have been implicated to play an important role in the initiation and propagation of vaso-occlusive events in SCD. Activated PMNs engage in a complex process of abnormal interactions with activated endothelial cells, platelets and circulating erythrocytes contributing to endothelial injury and decreased blood flow. In the present study, global proteome analysis of PMNs was performed using label-free mass spectrometry analysis of 4 SCD patients (SS) in steady state versus 4 control subjects (AA).

Purified PMNs were incubated at 4°C for 5 minutes in 1 mL PBS containing 2mM Diisopropylfluorophosphate (Sigma) to avoid excessive protein degradation during sample preparation. After three washes in cold PBS, 2 millions of neutrophils were lysed. 50µg of proteins were reduced, alkylated and digested with 1 µg trypsin using the FASP protocol. Peptide mixtures were fractionated by strong cationic exchange (SCX) StageTips in 5 fractions and analyzed using 3 hours gradients on an Orbitrap Fusion mass spectrometer. Raw data were processed using Maxquant and Perseus software.

We identified a total of 4,634 proteins both in AA and SS PMNs with 3,069 of these proteins identified in at least three samples for each condition. To identify biological pathway modified in the proteome of neutrophils from SS patients, we determine the significantly differentially expressed proteins compared to AA neutrophils. We identified 95 proteins differentially expressed with SS/AA ratio >1.3 or <0.7. 53 proteins are overexpressed and 42 proteins are down regulated in the neutrophils of the SS group compared to the AA group. A fisher exact test performed using proteins down regulated in SS neutrophils did not show any biological pathway, in contrast, analyzing upregulated proteins revealed a strong involvement of the alpha interferon response pathway. In agreement with proteomic data, western blot analyses showed the significant overexpression of the main ISP proteins MX1, ISG15 and IFIT1 as well as the STAT1 and STAT2 proteins, in the SS patients compared to controls.

In conclusion, quantitative proteomics analyses of purified neutrophils showed an immune and inflammatory signature in SCD.

Our findings provide the first evidence of a dysfunction of the IFN-alpha signaling pathway that could play an important role in the pathogenesis of SCD.

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Keywords

Sickle cell disease, LFQ, interferon

Authors

Hermand Patricia [1] [2], Azouzi Slim [1] [2], Gautier Emilie-Fleur [3], Guillonneau François [3], Bondet Vincent [4], Duffy Darragh [4], Salnot Virginie [3], Mayeux Patrick [3], Le Van Kim Caroline [1] [5], Koehl Bérengère [5] [1]

1. Institut National de la Transfusion Sanguine (France)
2. Laboratoire d'Excellence GR-Ex (France)
3. Plateforme de Protéomique Paris Descartes (3P5) (France)
4. Immunobiology of Dendritic Cells (France)
5. Laboratoire d'Excellence GR-Ex (France)

Label-free quantitative comparison of stacking gel, tube-gel, FASP, S-TRAP, SPE and liquid digestion preparation methods [P98]

High-throughput shotgun proteomics is challenged by detergent incompatibility with MS analysis and sample preparation has a strong influence on the accuracy and robustness of the analysis. Current classical approaches use stacking-gel (SG) Solid Phase Extraction (SPE) or liquid digestion (LD), but due to the repetitive processing of samples, their recovery and overall yield, they are time-consuming and have led to the development of new methods. In recent years, filter-based strategies such as filter-aided sample preparation (FASP) based on a molecular weight cut-off (MWCO) and its new alternative, the suspension traps (S-TRAP) confining particulate protein suspensions with the subsequent depletion of interfering substances, have reduced the total processing time.

The objective of this work was to compare for the first time all these preparation methods, i.e. FASP, S-TRAP, SPE, SG, TG and LD before subjecting the samples to a label-free semi-quantitative proteomic analysis (shotgun proteomics). The analyses were performed using high-performance liquid chromatography coupled with tandem mass spectrometry (nHPLC - LTQ Velos OrbiTrap, ThermoFisher Scientific).

A soluble fraction of muscle proteins (100 µg), spiked with 1.5% of casein, was used to assess sample preparation and 10 replicates were prepared for each method. Taking into consideration all methods, a total of 526 proteins were identified. The largest number of identified proteins was obtained by FASP (293) and S-TRAP (273) followed by SG (249) method. Quantitative analysis of the results by Venn diagram, principal-component analysis, hierarchical clustering and the abundance ranking of quantitative proteins highlight significant differences in the soluble fraction of muscle proteins according to sample preparation procedures. Therefore, these statistical results and the qualitative analyses of significant proteins indicate that S-TRAP method outperforms FASP method. Moreover, although the SG method sometimes gives slightly better results, the faster and easier S-TRAP method turns out to be the best alternative to replace it, resulting in an ultrafast sample-preparation approach for shotgun proteomics. In order to verify that our observations from muscle proteins can be generalized regardless of the nature of the protein samples, other matrices will be tested.

Authors

Viala Didier [1], Maillet Robin, Chambon Christophe, Hebraud Michel

1. INRA (France)

Two-dimensional Mass Spectrometry for Quantitative Proteomics [P99]

Introduction

Two-dimensional mass spectrometry (2D MS) is a method for tandem mass spectrometry without ion isolation before fragmentation [1]. 2D mass spectra show the fragmentation patterns of all analytes. Dissociation lines for the loss of a given mass and charge can be extracted from 2D mass spectra [2].

In TMT-labelled quantitative proteomics, distinguishing co-eluting isobaric peptides is both time-and sample-consuming [3]. In this study, we show the potential that extracting dissociation lines from 2D mass spectra offers for quantitative TMT-labelled proteomics.

Methods

A tryptic digest of BSA was tagged with TMT0, TMT126/127N/128C (Thermo Fisher Scientific) and was analyzed by direct infusion MS, MS/MS and 2D MS using a 12 T solariX FT-ICR MS (Bruker Daltonics) with infrared multiphoton dissociation (IRMPD) and electron capture dissociation (ECD) as fragmentation methods. Data processing using SPIKE [4] was used to create MASCOT-compatible data files and to assign peptides using TMT cleavages.

Results

IRMPD MS/MS spectra of TMT-tagged peptides showed the presence of the reporter ion at a signal-to-noise (S/N) ratio over 10, and complementary fragments at S/N ratio < 10. ECD MS/MS spectra did not show reporter ions, but show the complementary fragment at S/N ratio > 10. Both fragmentation methods yielded complete cleavage coverage for peptide sequencing.

Both IRMPD and ECD 2D MS yielded about 70% sequence coverage from the peptide mass fingerprint of the precursor ions within 5 ppm in a MASCOT database search. The fragmentation patterns of all precursor ions yielded 45% cleavage coverage of BSA in a MASCOT database search.

Dissociation lines were extracted from the 2D mass spectra for the loss of the reporter ion from 2+, 3+, and 4+ precursors. The resolving power for peaks on the dissociation lines was 40,000 at *m/z* 400. Manual peak assignment on the dissociation lines yielded a 24% sequence coverage with IRMPD and 57% sequence coverage with ECD. Relative intensities in the isotopic distributions were consistent over all precursors in the sample.

Conclusions

2D MS of TMT-labelled BSA yields peptides mass fingerprints, peptide sequencing, and TMT cleavage for quantitative proteomics. For relative quantification, 2D MS offers high resolution precursor-fragment correlation by *m/z* ratio and charge state. The next step in this study is coupling 2D MS with offline liquid chromatography to compare LC-2D MS results for quantitative proteomics to LC MS/MS and LC MS/MS/MS.

Novel Aspect

This study establishes the proof-of-concept that 2D MS can be used for quantitative proteomics.

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Keywords

TMT-labelled proteomics, two-dimensional mass spectrometry, Fourier transform ion cyclotron resonance mass spectrometry

Authors

Van Agthoven Maria [1], Charlton Georgina [1], Wootton Christopher [1], Morgan Tomos [1], Lam Yuko [1], Delsuc Marc-André [2], Barrow Mark [1], Jones Alexandra [3], O'connor Peter [1]

1. Department of Chemistry, University of Warwick, Gibbet Hill Road, CV4 7AL Coventry (Royaume-Uni)
2. Institut de Génétique et de Biologie Moléculaire et Cellulaire (France)
3. School of Life Sciences, University of Warwick, Gibbet Hill Road, CV4 7AL Coventry (Royaume-Uni)

A dual strategy for mapping the Bacillus cereus thiol proteome [P100]

Bacillus cereus is a foodborne pathogen able to grow under various oxygenation conditions, such as those encountered in foods and the human intestine. Under anaerobiosis, *B. cereus* growth is coupled with a decrease in extracellular redox potential [1]. We hypothesized that this redox potential decrease is linked to the redox state of the thiol proteome, i.e. the set of proteins with reversibly modified thiols [2].The analysis of thiol proteome is a challenge. Here, we present a dual strategy for the differential trapping of *B. cereus* thiols.

Cells were collected at early, mid- and late exponential growth phase from regulated batch cultures and treated with TCA to quench thiol-disulfide exchange reactions. Cysteines were labelled using two strategies before detection using shotgun proteomics [3]. In the first one, thiols were alkylated with IAM, then reversible thiol modifications were reduced with DTT, and alkylated with NEM. In the second strategy, the order of alkylating agents has been reversed.

At the protein level, our results identified nearly 1000 proteins in each strategy. We analyzed the modified cysteines detected during each growth phase. In early exponential growth phase, the first and the second methods detected 167 and 366 unique cysteine containing peptides corresponding to 121 and 256 proteins, respectively. The second growth phase revealed in the first labelling 161 unique cysteine peptides representing 121 modified proteins while the second labelling detected a number of 260 cysteine peptides corresponding to 170 proteins. During the third phase of growth, the two strategies identified around 200 unique cysteine peptides corresponding to 150 proteins.

Our data showed that, despite differences in the target number detected, each strategy allowed to obtain a large number of proteins. Moreover, we obtained a set of 100 proteins by overlapping data between the two approaches throughout the growth of *B. cereus*. This set was considered as a robust data set.

We have developed a dual cysteine labeling approach to determine the redox state of *B. cereus* protein thiols during anoxic growth. Applicability and robustness of this technique were demonstrated despite the low number of Cys residues in B. *cereus* proteome.

This work is the first to specifically target the thiol proteome of *B. cereus*. In addition, we used a novel model of the broadly applied differential cysteine labeling approach.

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Keywords

B. cereus, redox, thiol

Authors

Hamitouche Fella [1], Schmitt Philippe, Duport Catherine, Dedieu Luc

1. UMR408 SQPOV Sécurité et Qualité des Produits d'Origine Végétale, INRA, Université d'Avignon, F-84000 Avignon, France. (France)

Evolution of histones during late erythropoiesis : total quantity does not change, although linker histones increase. [P101]

Introduction

During mammalian late erythropoiesis there is a dramatic condensation of the chromatin followed by nucleus removal (enucleation). Controversies concerning total quantity of histones before the enucleation recently arose: Some groups reported depletion of histones [1-2], while others [3-4] proposed that a large proportion of histones remain in the nucleus.

Yet independantly from their divergence of conclusions, one could notice that 3 of these groups didn't use quantitative methods. Here we chosed a superSILAC quantitative proteomic analysis to study evolution of the total quantity of histones on the first hand, and the evolution of each histone isoforms independently on the second hand. Finally, we studied the stoichiometric distribution of linker histones that are known to be involved in chromatin architecture.

Methods

We used MEDEP cells as a murine model of erythropoiesis. At each time of differentiation, we mixed five millions of cells with a heavy SILAC reference. Then we isolated the nucleus using a small concentration of NP40 and purified histones with H_2SO_4. Light to heavy intensities of shared peptides inside H2A, H2B and H3 families allowed us to follow the total quantity of histones. To follow the quantity of histones isoforms we looked at the light to heavy intensity of their unique peptides. Finally, We used an algorithm (HiQUANT) proposed by Slavov[5] to estimate the stoichiometry of linker histones isoforms.

Results

The study of shared peptides revealed that the quantity of the different core histones (H2A, H2B, H3 and H4) have the same pattern during late erythropoiesis. This pattern follows DNA mean quantity. This probably reflects the decrease of proliferation that happens during late erythropoiesis and it does not involve a significant decrease of core histones from cells in G0/G1 phases. In addition, some isoforms such as macro-H2A, H2A.Z and H2A.J decrease more dramatically. Nevertheless, this decrease does not affect the evolution of total H2A quantity. This suggests that macro-H2A, H2A.Z and H2A.J represent a very slight portion of H2A histones.

Most of histone linkers present a stable quantity during late erythropoiesis while some of them increase H1.2. The Hiquant stoichiometric analysis revealed that these isoforms are one of the most expressed in our model. So, the increase of H1.2 has an impact on the total quantity of linker histones.

Conclusions

Our results show that the total quantity of core histones does not change during late erythropoiesis although macro-H2A, H2A.Z and H2A.J decrease. On the contrary, linker histones increase slightly probably because of the H1.2 change.

Novel Aspect

The stoichiometric view of histones isoforms explain how some of them can be modulated during late erythropoiesis with or without a visible impact on the total quantity of each histones family (like H1.2 and macro-H2A respectively).

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Keywords

SILAC, histones, erythropoïesis

Authors

Leduc Marjorie [1] [2], Guillonneau François [1], Mayeux Patrick [3]

1. 3P5 - Plate-forme protéomique (France)
2. Institut Cochin (France)
3. Institut Cochin, Dép. Immunobiologie et Hématologie (France)

Shotgun proteomics to decipher the advantages for Listeria monocytogenes of a biofilm vs planktonic mode of growth [P102]

Listeria monocytogenes is a foodborne pathogen and the causative agent of listeriosis, an infection with a mortality rate up to 20%. It is most of all a psychrotrophic biofilm forming bacterium capable of adapting, resisting and growing in harsh conditions, including those found in food industries as low temperatures and water availability. *Listeria* ability to survive refrigeration temperatures is directly associated with the majority of listeriosis cases being caused by food contaminated with this bacterium. Multiple genomic, transcriptomic and proteomic reports have duelled in understanding this adaptation at the physicochemical and biological changes. In all instances, temperature adaptation is in some way related to the overexpression or neosynthesis of a set of gene products aimed in particular at maintaining the fluidity and structural integrity of the cell membranes, some metabolic activities and the functioning of translational machinery. Here, three protein extractions methods [1], namely biotin-labelling, enzymatic shaving and cell fractionation where used in conjunction to explore the proteome of planktonic *L. monocytogenes* cells grown at three different temperatures (10°C, 25°C and 37°C). Plus, an in silico study was carried out using proteomic data previously obtained from biofilm cultures, in order to compare the two modes of growth at three different temperature settings. The combination of these data resulted in a *L. monocytogenes* proteome coverage of 32.6% for which we observed a significant remodelling of protein abundance as a function of temperature and mode of growth.

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Keywords

Listeria monocytogenes, Biofilm *vs* Planktonic, Shotgun proteomics.

Authors

Santos Tiago, Chambon Christophe, Viala Didier, Esbelin Julia, Hébraud Michel [1] [2]

1. Plateforme d'Exploration du Métabolisme, composante protéomique (France)
2. Université Clermont Auvergne, INRA, UMR MEDiS (France)

Subcellular protein fractionation for cysteine redox-proteomics analysis [P103]

Introduction

The systematic characterization of the cysteine redox state at the proteomic scale is a key step to understand the thiol-redox based molecular mechanisms. The major issue of this kind of analysis is the high percentage of reduced cysteines that could be 64% of the proteome [1]. Our group has already developed a protocol, called OcSILAC, aimed to improve the recovery of oxidized cysteines and quantify the reversible thiol oxidation considering also protein expression profiles. The aim of this study is to integrate the subcellular protein fractionation into the OcSILAC workflow.

Methods

The protocol uses the Thermo-Pierce" Subcellular Protein Fractionation Kit for Cultured Cells ". Reactions has been optimized with 2.5 x 106 wild-type Hela cells. Reduced thiols saturation has been monitored using the Thermo-Pierce Cy3-like maleimide by SDS-PAGE according our protocol [2]. Anaplastic Thyroid Cancer (ATC) 8505C cells were exposed to a small interference RNA to inhibit the expression of Bcl-2 Associated Athanogene 3 (BAG3) protein [3].

Results

In order to be compatible with cysteine-redoxomic analysis, the protocol should allow the saturation of reduced thiols after the cell lysis to circumvent air mediated oxidation, disulfide bonds shuffling and false positive assignations. We have found that subcellular protein fractionation, according to the manufacturer kit protocol, is not compatible with the complete alkylation of free thiols. Different reaction conditions have been tested for each subcellular fraction, obtaining a final protocol without altering the fractionation performances of the original workflow. The presence of artefactual oxidations induced during the subcellular fractionation has also been evaluated.

BAG3 protein is highly expressed in Anaplastic Thyroid Cancer [4]. Previous works showed that BAG3 could play a role in the redox cell homeostasis [4]. The subcellular fractionation protocol was successfully used to analyze the cysteines redox-proteome of BAG3 silenced ATC cells.

Conclusions

We optimized a subcellular fractionation protocol assuring the cysteine thiol saturation, the fractionation efficiency and the absence of artefactual oxidations. The protocol was useful to perform a redox-biology study.

Novel Aspect

At our knowledge this is the first validated redox compatible subcellular fractionation protocol.

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Keywords

Redox-proteomics, thiol-biochemistry, Anaplastic Thyroid Cancer

Authors

Chiappetta Giovanni [1], Shakir Shakir [1], Vinh Joelle [1]

1. SMBP (France)

Benchmarking of sample preparations and HR/AM instruments for the global profiling and the accurate quantification of trace-level host cell proteins impurities in therapeutic proteins. [P104]

Introduction

Host cell protein (HCP) impurities represent a major purification challenge during the biopharmaceutical process. Since they can reduce drug efficacy and/or induce adverse reactions in patients, guidelines require a maximum total HCP amount of 100 ppm in the final drug product [1]. ELISA assays are commonly used to monitor HCP. However, more accurate quantification and characterization of HCP are of highest importance. Introduction of high resolution/accurate masse (HR/AM) instruments, operating in DDA, PRM or DIA modes, appears as a promising alternative in this context.

Methods

The NIST mAb Reference Materiel 8671, a set of therapeutic mAb, and a series of HCP---containing samples with different degrees of purity [2] were analyzed on a Q---Orbitrap system using different quantification strategies. In the latter, a concentration---balanced mixture of SIS peptides of 10 HCP of interest was spiked. Isotope---dilution calibration curves were established in order to determine working dynamic ranges and lower limits of quantification of the targeted HCP.

Results

We proposed to assess the performances of HR/AM instruments for the individual identification and the precise quantification of trace---level protein impurities on samples collected over the biopharmaceutical process. DIA quantification first showed similar performances as XIC---MS1 quantification using a Top3 strategy [3] for the global profiling of HCP. Then, using the same DIA data, MS2---based quantification achieved comparable capabilities as PRM for the accurate quantification of the 10 targeted HCP. Detection of HCP present at sub---ppm levels could be achieved.

Besides the choice of the quantification method, the preparation of final drug substance samples is

particularly crucial and it should be finely optimized to cope with their inherent complexity --- up to 6 orders of magnitude between the mAb and the residual HCP. We compared several protocols, based on *in gel* digestion, and liquid non---denaturing and denaturing digestions, considering the number of retrieved HCP, the global HCP amount obtained and the preparation/analysis time.

Conclusions

This study demonstrates the high potential of HR/AM instruments operating in DIA mode

to perform both the global profiling of trace---level HCP and the accurate quantification of key HCP in a single analysis. We also proposed optimized protocols for tackling challenges

posed by final drug substance samples, and *in fine* improving HCP monitoring using proteomics analysis.

Novel Aspect

Innovative MS---based strategies for protein impurities monitoring during bioprocess development of

therapeutic proteins.

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Keywords

Host cell proteins, DIA, HR/AM instruments

Authors

Pythoud Nicolas [1], Bons Joanna [1], Cianférani Sarah [1], Carapito Christine [1]

1. LSMBO (France)

Analysis of changes in the cell wall of A. thaliana in response to salt stress through an integrative approach [P105]

Changes in the plant cell wall of Arabidopsis *sos* mutants (salt overly sensitive) under salt stress have been analyzed by a multidisciplinary approach using techniques from both cell biology, biochemistry and proteomic. Thus, phenotypic analyzes of roots with application of different NaCl concentrations were performed. A biochemical analysis of the cell wall components (celluloses, hemicelluloses, pectins) and the determination of pectin remodeling enzymes activities (PME, AE) made it possible to characterize the cell wall modifications. The degree of methylesterification and thus the modification of the pectins by immunolocalisation on cytological sections and confocal microscopy was analyzed. Finally, a differential proteomic approach with TMT labeling on *sos* mutants and wild ecotype GL has allowed to highlight the role of some cell wall remodeling enzymes in response to salt stress

Keywords

Arabidopsis, proteomics

Authors

Leschevin Maïté [1], Diakite Maya, Fournet Françoise [1], Lequart Michelle, Pageau Karine [1], Marcelo Paulo [2], Rayon Catherine [1]

1. Biologie des Plantes et Innovation (France)
2. Plateforme ICAP (France)

Relative quantification of highly complex samples using the timsTOF Pro [P106]

Nowadays, shotgun proteomic target protein mixtures with increasing complexity and interest in proteins with lower abundances, often available in limited amount. Thus, the benefits of the timsTOF Pro instrument developed by Bruker are essential to meet the needs of low biological samples amounts requiring deeper and faster proteome analyzes. The Parallel Accumulation Serial Fragmentation (PASEF) acquisition mode (*Meier F et al, 2018, Mol Cell Proteomics, 17:2534*) provides an increased speed and sensitivity in MSMS acquisition and pushes the limits of identification and quantification of proteins in low abundant and highly complex samples. Furthermore, the nanoElute-direct loading mode combined to the timsTOF Pro further enhances the performances shotgun proteomics. The new depths in proteome analysis reached with the timsTOF Pro need to revisit the sample preparation and the protein amount to be injected in the mass spectrometer. We will present the qualitative and quantitative results obtained with the timsTOF Pro using proteomic standard UPS1 spiked at different concentrations in a yeast background. First results obtained for highly complex biological samples, in collaboration with research teams from Paris-Saclay University, will be presented.

Authors

Cornu David [1], Sago Laila [2], Fellah Sophia [2], Redeker Virginie [3] [4]

1. Institut de Biologie Intégrative de la Cellule, Plateforme Proteomique SICaPS, CNRS, 91190 Gif-sur-Yvette, France (France)
2. Institut de Biologie Intégrative de la Cellule, Plateforme Proteomique SICaPS, CNRS, 91190 Gif-sur-Yvette, France (France)
3. Institut de Biologie François Jacob, Laboratoire des Maladies Neurodégénératives, CEA, CNRS, 92260 Fontenay-aux-Roses, France (France)
4. Paris-Saclay Institute of Neuroscience (France)

Development of a Parallel Reaction Monitoring Assay for Quantification of Autophagy [P107]

Autophagy is an intracellular catabolic process that promotes the recycling of organelles and cytoplasmic components, acting as a regulator of homeostasis and cellular metabolism. Several pathways can either positively or negatively regulate different steps of autophagosome formation and maturation, making autophagy a highly dynamic process. Due to its critical role in cellular quality control and metabolism, autophagy modulation has raised interest as possible therapeutic target for various human conditions ranging from age-related diseases, such as neurodegeneration and muscle frailty, to immunity and cancer.

The gold standard method to determine autophagy induction consists in quantifying microtubule-associated proteins 1A/1B light chain 3B (LC3) in its lipid-modified form (*i.e.,* LC3-II), and sequestosome-1 (p62) with western blotting. This methodology presents some limitations in terms of sample throughput for instance. Mass spectrometry (MS) and more specifically parallel reaction monitoring (PRM) could cope with such a technological constraint, by improving sample throughput but also providing precise quantification. PRM is suitable to quantify multiple proteins/peptides in complex matrices, and offers high sensitivity and selectivity.

We developed a PRM assay using heavy stable-isotope peptide standards and an Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer, to target autophagy in mouse muscles. We first investigated the quantification of both LC3-I and LC3-II. The specific peptides allowing the distinction of both proteoforms (*i.e.*, DEDGFLYMVYASQETFG and its phosphatidylethanolamine conjugate) revealed not well suited for MS analysis and could not be quantified. We concluded that a direct quantification of autophagy by measuring LC3-II concentration is not possible with MS.

However, we proposed autophagy assessment by measuring p62. A first cohort of mouse muscle samples was used to develop the assay and to optimize a PRM method. We then deployed the PRM assay to a second cohort of 15 mice: 5 untreated animals, 5 treated with colchicine (known to decrease autophagy) and 5 treated with spermidine (an autophagy activator). We showed that spermidine led to a decrease of p62 levels of more than 50% in mouse muscles. Following treatment with colchicine, the results indicated an increase of p62 protein in mouse muscles, meaning an autophagy decrease. This second animal cohort was also analyzed with the classical western blotting methodology. A coefficient of correlation $R2$ = 0.92 was obtained between western blotting and PRM measurements and a Bland-Altman plot further confirmed agreement between both assays.

Authors

Macron Charlotte [1]

1. Proteomics, Nestlé Research (Suisse)

Micro Pillar Array Columns: A novel robust chromatography platform for deep and reproducible proteome coverage [P108]

Current state of the art bottom-up proteomics relies on use of 50 to 75 cm long capillary LC columns coupled to high resolution mass spectrometers to analyze protein samples from tissues, body fluids or cell lysates. Typically, micrograms of digested protein material is injected and separated using nano flow rates and long solvent gradients. However, only marginal gains in identifications have been observed with gradient time beyond 240 minutes with traditional packed bed columns. Furthermore, reproducibility and robustness of packed capillaries may be a challenge to routine use.

To overcome these limitations, PharmaFluidics introduces the µPAC™ (micro Pillar Array Column) technology: a unique and novel approach to producing a chromatographic support structure that builds upon micromachining chromatographic separation beds into silicon wafers. This results in columns with exceptional properties in terms of chromatographic performance, repeatability and reproducibility, flexibility and robustness.

The chromatographic performance of 200 cm long µPAC™ columns is first demonstrated in both nano- (0.3 µl/min) and capillary flow regimes (1 µl/min), for both short and long gradients using retention standards. Next the results obtained from 1 µl direct injection runs from a dilution series of HeLa-digests (ranging from 0.01 to 1 µg/µl), coupled to various high-end mass spectrometers in the different operational regimes are discussed demonstrating the high efficiency in terms of identifications in limited sample amounts using nanoflow (>3,000 proteoforms starting from 10 ng digest with a 240 min gradient) and capillary flow modes (> 4,300 proteoforms in 1 µg digest with a 60 min gradient run).

Long term stability and repeatability are illustrated from a 6 months continuous column evaluation running sequences of Cytochrome C standard, HeLa-digest and blanks (1 hour runs, 3526 injections in total, 1000 HeLa-digest injections) showing less than 2 % retention time variation for the Cytochrome C peptides over the six target analytes.

Finally, the column robustness is demonstrated using three sets of samples that are either contaminated with detergents routinely used in sample preparation (NP-40; Triton X-114) or contain precipitating compounds. Whereas these samples consistently prove to be problematic for packed bed columns in terms of retention time deterioration or column clogging, the µPAC™ columns show stable pressure profiles and a retention time variation below 1% (CV, n=40).

Keywords

nanoLC, chromatography, microchip column

Authors

Op De Beeck Jeff [1], Van Mol Kurt [1], De Malsche Wim [2], Desmet Gert [2], Jaouen Gilles [1]

1. PharmaFluidics (Belgique)
2. Vrije Universiteit [Brussel] (Belgique)

SLIM-labeling: principles, data processing workflows and applications in quantitative proteomics [P109]

We recently developed a new method to quantify proteome variations based on an original in vivo labeling strategy, the Simple Light Isotope Metabolic labeling (SLIM-Labeling) (Léger, Garcia et al. 2017). Cells are forced to incorporate all-12C-amino acids synthesized from a U-[12C] sole source of carbon. This results in a large increase of the intensity of the monoisotope ion of peptides and proteins, and therefore allows higher identification scores and protein sequence coverage in mass spectrometry experiments. The method initially developed for signal processing and quantifying the rate of incorporation of 12C into peptides was based on a multistep process that was found difficult to implement by many laboratories. It involved 1) extracting the intensity of isotopologues using the commercial software Progenesis QI for metabolomics, 2) aligning the “Features” files with the peptide identification files (Mascot files), 3) calculating the intensity ratio of the monoisotope to the intensity of all isotopologues (called Riso), 4) establishing for each peptide sequence the equation of the theoretical values of Riso calculated using the MIDAs application (Alves, Ogurtsov et al. 2014) as a function of 12C enrichment, after calculating the elemental composition of each peptide and therefore the exact number of carbon it contains, and 5) calculating the abundance of 12C in each experimental peptide by adjusting its experimental Riso with the theoretical Riso. The data were then filtered to eliminate outliers based on monoisotopic intensity, peptide size, and 12C composition derived from the adjustment. Protein abundance was calculated on the basis of the abundance of their three most intense peptides (Top-3). The lack of automated processing tools restricted the diffusion of this procedure.

To overcome these limitations, we developed a set of procedures based on open source resources, using in particular dedicated OpenMS modules (Röst, Sachsenberg et al. 2016) for peptide identification associated with a modified version of FeatureFinderIdentification (Weisser and Choudhary, 2017) to extract the features from the Gaussian fit of the intensities of every isotopologue chromatographic trace. Basically, the only experimental data required for high quality quantification are the abundance of M_0 and M_1, the monoisotope ion and the +1 isotoplogue, of the peptides with a high confidence identification. The computation of the 12C abundance of peptides and the molar fraction of NC and 12C peptides in multiplexing experiments is performed by implementing appropriate calculus modules in a KNIME working environment (Warr 2012, Fillbrunn, Dietz et al. 2017). We also present the theoretical basis for establishing appropriate filters to obtain high-quality processed data from experimental datasets. These new integrated tools provide a convenient framework for a larger use of the SLIM-labeling strategy.

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Authors

Nicolas Senecaut [1], Gelio Alves [2], Hendrik Weisser [3], Thibaut Léger [4], Samuel Terrier [4], Laurent Lignières [4], Lilian Yang-Crosson [1], Pierre Poulain [1], Gaëlle Lelandais [5], Yi-Kuo Yu[2] and Jean-Michel Camadro [1] [4]

1. Mitochondria, Metals and Oxidative Stress group, Institut Jacques Monod, CNRS – Université de Paris
2. "Quantitative Molecular Biological Physics" group, NCBI, NLM, NIH, Bethesda, USA
3. STORM Therapeutics Limited, Cambridge, UK
4. Proteomics Core Facility, Institut Jacques Monod, CNRS – Université de Paris
5. Institut de Biologie Intégrative de la Cellule, Orsay, France

Bioinformatics / Biostatistics / Chemometrics

New functionalities of the free ms.cheminfo.org tool for on-line processing of HRMS and extension to DNA biomolecules [P110]

Introduction

Cloud based services are becoming of main importance for many applications like collaborative working, large databases analysis and IoT (Internet of Things). Nevertheless, few scientific implementations are currently available online. Since the last 10 years, we are developing core functionalities to allow the processing of High Resolution Mass Spectra (HRMS) directly in the browser [1]. This is particularly useful for MS platforms since most of the users do not have access to commercial software. Recently, we compiled various in-house tools to solve usual tasks but also to treat very special requests involving complex projects that can be encountered in research laboratories. In particular, we recently extended the tool to the analysis of oligonucleotides, a domain for which, to our knowledge, there are no free and open-source programs.

Those tools, available on the website http://ms.cheminfo.org, are open-source (MIT licence), don't require any installation and are always up-to-date (just need to reload the web page).

Methods

The libraries are implemented in JavaScript and available on GitHub. The main library can be downloaded on https://github.com/cheminfo-js/molecular-formula. The code is thoughtfully tested using jest and each commit is automatically processed in order to check for any broken code. Currently, there are over 150 tests that cover more than 80% of the code. Code syntax is enforced by over 200 eslint rules, that yields to a code easy to read, maintain and release under a MIT license.

Results

Several tools available on http://ms.cheminfo.org were unified and recently updated. These applications are daily used in our mass spectrometry facility of EPFL, for a wide range of applications and projects. Among others:

· Core function to generate isotopic pattern distributions. It can be calculated from a molecular formula or a chemical structure (Drawn or imported), with the possibility to select isotope abundances and charges. Based on the customizable FWHM, the system generates the theoretical isotopic distribution that can be compared with experimental data and exported as high quality figures (SVG).

· Application to determine possible molecular formulas for a specific monoisotopic mass acquired by high resolution mass spectrometry (HRMS). It is possible to specify the ion types (polarity, number of charges, adducts, etc.), the molecular formula range for a given monoisotopic value but also to specify groups of atoms that, when combined, generate multiple combinations that will be matched and compared with experimental data. In addition now, the system returns the possible compounds as present in the PubChem database.

· ***Easycont*** to track classical contaminants inherent to the sample preparation such as detergents (Tween, SDS, Triton...), polymers (PEG, PPG...), contaminants from containers

(phtalates, plasticisers) and solvent interferences. The experimental mass spectrum can be rapidly screened against the updated ms.cheminfo.org database of contaminants. Easycont browses the results ordered by similarity (%) and gives information about each identified contaminant.

· ***Apm2s*** calculates theoretical fragment ions (*a, b, c, x, y, z* and internal fragments) from a given protein/peptide sequence with any user defined modification such as post translational modifications, chemical modifications, non-natural amino acids, ligands or metal ions bound to the protein, and matches each individual theoretical isotopic pattern to the experimental mass spectra to provide a list of matches with similarity score. Very recently, this exhaustive Apm2s matching tool provided an abundance of data which gave critical insights into cisplatin-ubiquitin binding [2] or Histidine targeting Ru-Au complexes [3] .

· The new oligonucleotide tool (***Aom2s***) for characterization of DNA/RNA fragment ions by CID. This tool calculates theoretical fragment ions (*a, a-Base, b, c, d, d-H20, w, x, y, z, z-CH2*), internal fragments (*a-Base/w* and *a-Base/y*) and Base loss from a given nucleic sequence. Similarly to Apm2s, this tool allows the user to define any modification and adduct.

Conclusions

Contrary to beliefs, JavaScript proves to be suitable for creating fast and efficient scientific tools from any Web browser and indeed are competitive with most vendor's applications. Many different analytical tools were gathered on the ms.cheminfo.org website such as isotopic pattern distributions generations, molecular formula finder from monoisotopic mass and several advanced tools to compare the experimental data to the automatically predicted isotopic distributions of thousands of species. The specific tool for oligonucleotide analysis, recently released, is still under validation. We are opened to any improvement suggested by any user who will be interested in using the tool, especially for large oligonucleotides. The applications associated with this automated workflow are endless and can certainly be of extremely valuable help for any user with internet access having complex high-resolution mass spectra data to analyze.

Novel Aspect

Complex problems involving MS measurements can not necessarily be solved using commercial solutions. In top of that, commercial softwares are often expensive, inflexible, and their installation and upgrade procedures are generally time-consuming. In order to use the versatile ms.cheminfo.org toolbox, the only mandatory application is a web browser already installed on a computer. The free and open source applications of ms.cheminfo.org are in constant development, quickly evolving with the researchers requirements.

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Keywords

HRMS, On-line tool, biomolecule fragmentation

Authors

Ortiz Daniel [1], Gasilova Natalia [1], Sepulveda Francisco [1], Patiny Luc [1], Menin Laure [1]

1. SSMI,EPFL (Suisse)

Prostar: Biostatistical processing of label-free quantitative proteomics data at peptide and protein levels [P111]

Introduction

Prostar is a software tool devoted to the processing of label-free relative quantitative proteomics data produced by bottom-up LC-MS/MS (liquid chromatography and tandem mass spectrometry) experiments. Built on R biostatistics routines and web-based graphical user interfaces (Shiny technology), it is free, open-source and distributed via the BioConductor initiative.

Methods

Prostar is fully maintained (it has tightly followed a 6 months release rhythm for several years). It is suited to a large type of users, ranging from occasional (with the stand-alone version) to systematic use on a large proteomics platform (thanks to its server deployment options). Finally, it was designed to fit to a large number of quantitative data formats, including MaxQuant and Proline.

Results

The three cornerstones of Prostar development are the following: First, it incorporates cutting-edge statistical algorithms tailored to best suit the specificities of proteomics data. These algorithms result from specific researches we have conducted over the past years on imputation methods, on statistical tests and on false discovery rate control. Second, all these algorithms are associated with adapted interactive visualization tools to help the user keep a critic eye on his data. Third, the user is guided through the various off-the-shelf algorithms that are proposed in Prostar menus. It is as important to have good algorithms as to use them in the appropriate order. This is why, the user is oriented through the numerous processing steps that form a coherent and optimized pipeline.

Conclusions

Prostar makes it possible to extract the most biologically relevant knowledge from quantitative data, either at peptide-level or at protein-level. Any new user can directly test a Prostar demo on a dedicated server before making the choice of installing it: To do so, please connect to http://www.prostar-proteomics.org/.

Novel Aspect

The newest developments of Prostar are presented, as well as a roadmap for future developments.

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Keywords

Quantitative proteomics; differential analysis; biostatistics.

Authors

Wieczorek Samuel, Borges Hélène, Giai Gianetto Quentin [1], Combes Florence, Brun Virginie [2], Jacob Laurent [3], Burger Thomas [4]

1. proteomics platform (France)
2. CEA/DRF/BIG/INSERM/UGA 1038 (France)
3. Laboratoire de Biométrie et Biologie Evolutive (France)
4. CEA Grenoble (France)

Identification of internal fragments in deconvoluted top-down MS data [P112]

Introduction

High energy fragmentation methods used with mass spectrometry of proteins or peptides produces not only terminal fragments but also internal fragments, among which only the small group of terminal ones is traditionally exploited. In the context of top-down analysis of entire proteins, the correct assignment of internal fragments is challenging but also important for increasing coverage and shedding light on localization of PTMs or ions associated to structural elements. The complexity of the data increases exponentially with size of proteins studied.

Methods

A new set of tools has been developed in R. Assuming co-presence of variable modifications next to non-modified peptides helps reducing the complexity linked to PTMs considerably. Using a novel approach a global identification FDR for terminal and internal fragments is estimated and an identification score based on multiple parameters (eg preferential fragmentation patterns) has been developed to further reduce false positives or ambiguous assignments.

Results

Using our tools is possible to identify and rate internal and terminal peptides within a given precision range(ppm), including various protein modifications (eg PTMs or ions associated to structural elements). To complement identifications, graphs showing the localization of identified fragments can be produced to interpret local enrichment when comparing to external information like known structural elements. All functions are very flexible and can be customized to a very high degree to adopt to different scenarios and biological questions. As example we show the analysis of FT-ICR recorded fragments from horse ADH dimers. The location of with confidence identified fragments complements well information about bound Zn^{2+} ions and protein structure from PDB.

Conclusions

The identification of internal fragments from entire proteins is very challenging due to the huge number of possible internal fragments, in particular with additional variable modifications. The tools presented allow treating multiple proteins simultaneously while offering customization (eg flexibility for PTMs or ions associated) and efficient scoring to reduce identification ambiguities due to the extremely crowded search space.

Novel Aspect

Our tools allow identification and rating of internal fragments from multiple full length proteins while offering high flexibility for variable and fixed modifications.

Keywords

Top-down MS, internal fragments, identification score

Authors

Raffelsberger Wolfgang [1], Barroso Kim [1], Morlet Bastien [1], Negroni Luc [1], Ruffenach Frank [1], Li Huilin [2]

1. Institut de Génétique et de Biologie Moléculaire et Cellulaire (France)
2. Sun Yat-Sen University (Chine)

An optimized method for the clustering of quantitative proteomics data [P113]

Introduction

Large-scale profiling of biological systems with multiple experimental perturbations became possible through label-free quantitative proteomics. This technique produces big datasets that can be clustered to highlight proteins behaving similarly, which can lead to the discovery of important biological information. Here, we sought to build the most “optimal” clustering strategy for quantitative proteomics data. For this, we have conducted in-depth comparisons of several clustering methods on “gold standard” datasets, which has led us to an optimized clustering method.

Methods

State-of-the-art clustering methods have been compared using different validation criteria and 4 PRIDE datasets where the “true” clusters have been known since proteins from one organism have been spiked into another at different concentrations. Beyond the choice of the clustering algorithm, this framework allows us to know if we must cluster the intensities of peptides or the aggregated ones of proteins, and the best way to deal with missing values.

Results

Our results indicated that traditionally used clustering algorithms in proteomics, consisting to impute missing values with small values and using a hierarchical clustering on protein intensities (see [1], [2] among others), give poor results when compared to other strategies. Moreover, starting from aggregated protein intensities instead of peptide intensities seems to be a less effective strategy. The results of comparisons between clustering algorithms from literature led us to an optimized strategy consisting in transforming the peptide intensities into vectors summarizing the dynamic between each studied biological conditions, and in computing a distance between them before to apply a clustering algorithm. From our results, an unsupervised penalized k-means clustering method from these distances give good performances both on the 4 standard datasets and on other datasets where the highlighted proteins were then validated by other biological experiments.

Conclusions

In this study, we performed extensive comparisons of clustering algorithms for quantitative label-free proteomics data. Our comparisons have led us to some conclusions about the wisest strategy to apply, from which a "smart" clustering algorithm has been built.

Novel Aspect

A new clustering algorithm is proposed for label-free quantitative proteomics datasets. It is compared with state-of-the-art clustering methods from the literature.

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Keywords

Biostatistics, Clustering, Label-free

Authors

Bellami Maxime [1], Matondo Mariette [1], Giai Gianetto Quentin [2] [1]

1. Mass Spectrometry for Biology Unit, Proteomics Platform, Institut Pasteur, USR CNRS 2000, Paris, France (France)
2. Hub Bioinformatics and Biostatistics, Computational Biology Department, Institut Pasteur, USR 3756 CNRS, Paris, France (France)

Proline features & capabilities for quantitative proteomics: an update [P114]

Introduction

The Proline software suite is routinely used to validate, extract and publish protein identification and quantification data obtained from label-free shotgun LC-MS analysis. Recent developments are now focusing on specific features required in commonly used proteomic strategies, notably to allow handling more complex experimental designs involving sample prefractionation or enrichment, analysis of post-translational modifications (PTMs) in bottom-up mass spectrometry analysis or relative quantification based on labeling methods.

Methods

New features have been implemented in Proline to deal with complex label-free quantitation experimental designs including peptide fractionation and/or specific PTM enrichment. Algorithms dedicated to TMT and SILAC quantification have also been developed and integrated into the software. Finally, a tool is available to characterize all post-translational modifications sites in identification and quantification datasets.

Results

Individual quantification datasets can be aggregated hierarchically into a higher level dataset. Abundances of each precursor ion quantified in the different fractions are summarized before being used to infer protein abundances. Since signal extraction from raw MS data is not repeated, the computational cost of this method is low.

Interestingly, this can be used for example to combine in a single dataset, the abundance of phospho-peptides obtained from an enrichment protocol together with the abundance of their corresponding protein calculated from unmodified peptides. Since the aggregation can be applied recursively, protein abundances can themselves originate from individual fractions, providing an in-depth quantification of a proteome. Abundances profiles can thus be compared and visualized to confidently identify up or down regulated PTM sites.

In addition Proline provides algorithms to perform TMT and SILAC quantification, allowing navigation and curation of the results similarly to what is done for label-free quantification.

Conclusions

The newly implemented features enlarge the scope of proteomics workflows handled by Proline. Results reliability must be carefully assessed, not only by statistically controlled procedures, but also through examination of the underlying data by experts [1]. That’s why a particular effort has been put on data visualization and curation to provide an efficient MS data analysis tool.

Novel Aspect

The implemented feature provides an algorithm to hierarchically and recursively combine quantitation datasets.

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Keywords

fractionation, PTMs, label-based quantification

Authors

Bouyssié David [1], Hesse Anne-Marie [2], Mouton-Barbosa Emmanuelle [1], Rompais Magali [3], Carapito Christine [3], Gonzalez De Peredo Anne [4], Couté Yohann [2], Dupierris Véronique [2], Romdhani Aymen [3], Burel Alexandre [3], Bruley Christophe [2]

1. Institut de Pharmacologie et Biologie Structurale (IPBS), UMR5089 CNRS - Université de Toulouse (France)
2. Univ. Grenoble Alpes, CEA, INSERM, IRIG, BGE, F-38000 Grenoble (France)
3. Laboratoire de Spectrométrie de Masse Bio-Organique (France)
4. Institut de pharmacologie et de biologie structurale (France)

Phasing two-dimensional (2D) Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) in both dimensions [P115]

Introduction

Phasing the transient in Fourier transform ion cyclotron resonance mass spectrometry leads to compute the Fourier transform in absorption mode allowing a gain in of resolution of two compared to the magnitude mode. In two-dimensional (2D) the number of peaks which can be detected is proportional to the resolution in both dimensions [1]. So phasing a 2D spectrum leads to an increase by up to four of the number of individual peaks which can be detected. We describe here a strategy for 2D FT-ICR MS spectrum phasing including spectrum acquired using Non Uniform Sampling (NUS) we recently described. For the fragment dimension we used a method inspired the ones previously published [2] and an original algorithm for the precursor dimension [3].

Methods

Experiments were performed on an SolariX XR 9.4 Tesla FT-ICR MS fitted with an Harmonized cell using gas-free fragmentation methods ECD or IRMPD. The different 2D FT-ICR spectra were by nanoESI at an analytical concentration of 1 to 10 pm.mL-1. All 2D FT-ICR spectra were performed with our optimized pulse sequence and recorded. Spectra with physical size up to 256k×1024 points (F1×F2) were recorded either as complete spectra or using Non Uniform Sampling (NUS) at different ratios from 1/32 to 1/2 respectively. NUS acquisitions were performed with a uniform random sampling distribution and deconvoluted with a recursive call to a denoising algorithm or a newly developed genetic algorithm.

Results

Two 2D processing is done in two phases. In the first one the series of classical 1D spectrum corresponding to the fragment dimension at increasing encoding delay is processes by Fourier transform, then a second series Fourier transform in the precursor dimension is performed. Finally the hypercomplex modulus is taken. Phasing the series of classical 1D spectrum was performed by a method inspired by the ones previously published the Alan Marshall’s and David Kilgour’s laboratories. The phasing needs to be slightly corrected when the encoding delay increases. At this stage another advantage beyond the expected increase in resolution is to decrease the memory footprint by a factor two as real and not complex number are stored. The phasing in the precursor dimension may be performed may be performed using the same method directly or after a recursive call to a denoising algorithm for 2D acquired using Non Uniform Sampling (NUS). Here also a real spectrum is obtained so the global memory footprint or intermediate disk storage is decreased by a factor four.

Conclusions

For 2D the resolution was increased by a factor two leading to the decrease of surface of the spots by a factor up to 4. For this second step we developed a new genetic algorithm which performs both Non Uniform Sampling (NUS) reconstruction and phasing at the same time using the Distributed Evolutionary Algorithms platform developed by the laboratory ICube (Strasbourg, France). All these algorithm were implemented on Graphical Processing Unit

(GPU) cards. Example and comparison of processing time with the two approaches will be presented.

Novel Aspect

Phasing 2D FT-ICR MS spectra is described here for first time using a genetic algorithm

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Keywords

Genetic programming, phasing, 2D

Authors

Abdulkarimova Ulviya [1], Haegelin Marc [2], Bray Fabrice [2], Jeannin-Girardon Anne [1], Rolando Christian [2], Collet Pierre [1]

1. Laboratoire des sciences de l'Ingénieur, de l'Informatique et de l'Imagerie (France)
2. Miniaturisation pour la Synthèse, l'Analyse et la Protéomique - USR 3290 (France)

Dealing with imputation-caused variance in label-free quantitative proteomics data [P116]

Introduction

Current statistical methods used in label-free quantitative proteomics rely on peptides' intensities. They do not take satisfyingly enough into account peptides, which intensities are missing in some conditions, although they might be particularly interesting in differential analyses. While imputation methods have been described and are currently applied in state-of-the-art quantification software tools, the imputation itself is not optimally considered downstream of the imputation step. Indeed, in current solutions, imputed datasets are considered as if they had always been complete and the uncertainty due to the imputation is not properly taken into account.

Methods

To impute missing values, multiple imputation's algorithms have been used. Estimation of interest parameters was done after Rubin's rules. Differential analysis was provided using moderated-t-test statistics and based on a peptide-intensity-based linear model.

The developed model was applied to a series of well-calibrated datasets generated in-house and nanoLC-MS/MS analyses were performed on a nanoAcquity UPLC coupled to a Q-Orbitrap mass spectrometer.

Results

A peptide-intensity-based linear model was written and parameters were estimated thanks to generalized least squares method. Multiple imputation algorithms to impute missing values and Rubin's rules to estimate imputation-caused variability were evaluated. Then, hierarchical Bayesian models were used in order to moderate the peptide's intensities' variance estimator. Finally, this estimator was included in moderated t-test statistics to provide differential analyses.

The developed model was successfully employed to a series of well-calibrated datasets generated in-house. They include a range of yeast background samples spiked with known amounts of UPS1 standard proteins, total HeLa, yeast and Arabidopsis lysates mixed in different ratios in order to mimic real differential analyses of complex proteomes.

Conclusions

While sophisticated imputation models have been published and are implemented in recent software solutions, their downstream consideration has not yet been properly addressed. The present work demonstrates the benefits of using our model including imputation-caused variability on well-calibrated datasets.

Novel Aspect

We suggest using multiple imputation's properties and include them in moderated t-tests techniques to deal with imputation-caused variability.

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Keywords

Label-free quantification, Missing values, Imputation-caused variability

Authors

Chion Marie [1] [2], Muller Leslie [1], Pythoud Nicolas [1], Carapito Christine [1], Bertrand Frédéric [2]

1. Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO), Institut Pluridisciplinaire Hubert Curien (IPHC), UMR7178, Strasbourg. (France)
2. Institut de Recherche Mathématique Avancée, CNRS UMR 7501, Université de Strasbourg, Strasbourg. (France)

PTMselect: optimization of protein modifications discovery by mass spectrometry [P117]

Discovery of protein modification sites relies on protein digestion by proteases and mass spectrometry (MS) identification of the modified peptides. Depending on proteases used and target protein sequence, this method yields highly variable coverage of modification sites. We introduce PTMselect, a digestion-simulating software which tailors the optimal set of proteases for discovery of global or targeted modification from any single or multiple proteins.

PTMselect was optimized to eliminate the tedious work of manually sorting and selecting peptides to choose digestion settings before performing any MS-driven PTM analysis. This software is designed for (1) optimization of global coverage of protein PTM sites, (2) optimization of protein PTM sites coverage with the highest probability to be modified and (3) optimization of target PTM positions coverage.

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2. Software and demo videos : https://sites.google.com/site/fredsoftwares/products/ptm-select

Keywords

software – protein modifications – proteases parallel digestion

Authors

Pont Frédéric [1]

1. Groupe de Protéomique du CRCT (France)

Recover-Fx, a useful tool to prefilter and visualize MS/MS datasets [P118]

Introduction

In high-throughput bottom-up proteomics datasets, a large number of MS/MS spectra remain unassigned after classical database searches [1]. Are these unassigned MS/MS spectra of bad quality or simply non-interpretable with classical database searches? Recover-Fx has been designed to extract/filter subsets of relevant spectra based on user preferences, in order to run alternative interpretations such as de novo searching, post-translational modification searches …

Methods

Recover-Fx is an MS/MS spectra viewer and extractor designed to extract subsets of spectra from peaklist files. It is used to filter out spectra based on user-adjustable variables. The main variable is the Useful Peak Number (UPN), which is the number of fragment peaks above a noise level defined by the user thanks to a variable called Emergence (E).

Recover-Fx has been developed in Java 8 and Maven, its source code is available on Github https://github.com/LSMBO/recover-fx.

Results

Recover-Fx gives the user the ability to easily extract assigned or non-assigned subsets of spectra or subsets of spectra containing specific features from peaklist files. New peaklists containing the subsets of "recovered" spectra can be quickly exported for further alternative interpretation strategies. It provides an Excel-like interface allowing to set/unset many different filters. Spectra can be visualized in full details and sequence tags can be computed from handpicked fragments.

Recover-Fx also allows the user to flag identified spectra via import of .csv database search results files. This functionality is particularly useful in a *de novo* sequencing pipeline as it allows to run a first search using a classical database strategy followed by a *de novo* interpretation focusing only on "high quality" and non-identified spectra.

All settings and filters can be saved in .json parameters files and can be applied in batch mode on numerous peaklists.

Conclusions

Recover-Fx is aimed to help users for the visualization, quality control and pre-processing of their MS/MS data. After parameters fine tuning on individual files, the user can apply optimized settings in batch mode on complete datasets.

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Keywords

MS/MS spectra filtering, unassigned spectra, noise level.

Authors

Romdhani Aymen [1], Carapito Christine [1], Burel Alexandre [1], Rompais Magali [1], Van Dorsselaer Alain [1]

1. Laboratoire de Spectrométrie de Masse BioOrganique, Université de Strasbourg, CNRS, IPHC UMR 7178, Strasbourg, France (France)

Glycobiology

Lipidyl cyclodextrins synthesis assisted by ball milling : reaction monitoring, mechanism investigation and structural characterization using mass spectrometry [P119]

Introduction

Modified cyclodextrins (CDs) have many applications in various fields such as nano-reactors for catalysis and nano-carriers for drug delivery. To valorize such derivatives often obtained as complex mixtures, the challenge is to perform their synthesis environmental friendly with cheap processes at industrial scale and also to control their characterization for process optimization, chemical reaction mechanisms understanding and quality control purposes.

Methods

In recent years, the so-called "enabling technologies" such as microwaves, ultrasound and ball mills have emerged in synthesis of CDs derivatives [1]. Although ball milling is commonly used for the preparation of complexes or microparticles, its application for CDs derivatization remains confidential [2-4]. In this work, we focus on mass spectrometry tools for monitoring the epoxy ring opening reaction in fatty acid methyl esters by CDs using ball milling.

Results

First, in order to highlight the methyl oleate epoxide ring opening by the β-CD, ESI-MS/MS experiments were performed to confirm the condensation of one or more lipidyl chain on the cyclodextrin glucose units. Then, the optimization of the ball milling conditions has been conducted by ESI-MS to determine the degree of substitution (DS), the presence of reagents or byproducts and the reaction yield**.** Subsequently, the regio and steroselectivity of the epoxy ring opening was investigated by UPLC-ESI-MS allowing the separation of 12 isomers for the DS=1 derivative. Finally, travelling ion mobility separations (TWIMS) [5] were investigated with the aims of DS measurements and isomers distinction. The optimized protocol was efficiently applied to other cyclodextrins (α-CD, γ-CD, HPα-CD, HPβ-CD, HPγ-CD) and we also carried out the saponification of methyl ester's L-CDs through lipase-catalysed reaction.

Conclusions

New bicatennary lipidyl CDs (L-CDs) were obtained in a single step without the use of polar aprotic solvents, which generate byproducts and are difficult to recycle. These compound are obtained as complex isomers mixture and we demonstrated the benefit of mass spectrometry techniques for their rapid and efficient characterization.

Novel Aspect

Structural analysis of new "green" cyclodextrin derivatives design for catalysis and drug delivery.

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Keywords

Ball milling, cyclodextrins, MS

Authors

Oliva Estefania [1], Mathiron David [2], Rigaud Sébastien [2], Bonnet Véronique [3], Djedaïni-Pilard Florence [3], Pilard Serge [2]

1. Plateforme d'Analyse Chimique de Strasbourg Illkirch (France)
2. Plateforme Analytique (France)
3. LG2A UMR CNRS 7378 (France)

New method for highly sensitive analysis of complex oligosaccharides based on filter aided sample preparation and mass spectrometry [P120]

Introduction

Plant gums are a highly used biomaterial in food but also in Cultural Heritage artworks for their binding properties. They are naturally occurring polysaccharides coming from various plant or seeds species. The polysaccharide structure is different according the gum, it can be linear or highly branched with various oligosaccharides such as hexose, pentose, deoxyhexose, hexuronic acid, *N*-acetylneuraminic acid. Their identification is usually based on the monosaccharide composition analyzed by GC-MS after hydrolysis leading to complex spectra and difficult identification. We proposed here a very sensitive a method to identify gums used in artworks based on our based saccharidic fingerprint by enzymatic digestion [1] using filter aided sample preparation [2] and nanoLC nanoESI MS.

Methods

We propose a new method based on filter aided sample preparation and mass spectrometry for their highly sensitive detection. For the digestion and the desalting of the polysaccharidic compounds we used Nanosep filters (Pall Corporation) with ultrafiltration membrane made of modified polyethersulfone as cellulose acetate membranes are degraded by sugar hydrolysis enzyme. In order to identify the structure of gums, a separative nano-Liquid Chromatography method based on a reverse phase nanocolumn (25 cm, id 75 micron) eluted with a acetonitrile/water gradient was developed. The digested sugars were labeled with ABBE (butyl-4-aminobenzoate) to enhance the ionization allowing acquiring spectra with good signal to noise ratio a nano-ESI-Orbitrap XL (ThermoFisher Scientific) in MS and MS/MS.

Results

Starting from as low as 100 microgramme of sample, the developed FASP method allowed the successful identification of different types of gums such as arabic gum composed of arabinogalactans, guar gum, locust bean gum composed of galactomannans and tragacanth gum composed of mixture of arabinogalactans and galacturomannans. Each type of gum is digested by specific enzymes. Firstly we develop an enzymatic digestion cocktail universal for all gums using common temperature, duration and buffer for digestion. The digestion was performed by the Filter Aided Sample Preparation using filters made of modified polyethersulfone as cellulose acetate membranes are degraded by sugar hydrolysis enzymes. The use of reverse phase nano liquid chromatographic separation associated with mass spectrometry for complex sugars is poorly described in the literature. Several amino reagents were tested for sugar derivatization and ABBE (butyl-4-aminobenzoate) was found the best as it optimized both ESI ionization and peak shape and width in chromatography. The complex mixture obtained after digestion composed of simple sugars such as hexose, pentose to more complex such as hexosamine, glucuronic acid is cleanly separated using ABBE derivatized sugars. The compounds with more ose units are eluted first. Using DDA (Data dependant analysis) CID fragmentation gave the exact sugar composition of each fragment, affording information about the complex gum structure.

Conclusions

Extension to Fluorophore-assisted carbohydrate electrophoresis (FACE) detection is under development for improving sensitivity. This method can have several applications: in archeology, such as the identification of oligosaccharides presents inside the inks, water colors, stuccos but also in biology for deciphering the change in sugar composition in different parts of plants. The method has been recently extended to the simultaneous detection on the same sample of proteins and saccharides in gums. Examples from different fields will be presented.

Novel Aspect

We developed a new, sensitive FASP method to analyze complex sugars and entrapped proteins in vegetal gums

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Keywords

Carbohydrates, Filter aided sample preparation, nanoLC-NanoESI MS/MS

Authors

Pruvost Amandine [1], Penverne Christophe [1], Szydlowski Nicolas [2] [1], Rolando Christian [3] [1]

1. Miniaturisation pour la Synthèse, l'Analyse et la Protéomique - USR 3290 (France)
2. Unité de Glycobiologie structurale et fonctionnelle (France)
3. USR 3290 MSAP, Université Lille 1, Sciences et Technologies (France)

Modifications of Biopolymers

Phospho-secretome characterization of Acinetobacter baumannii in biofilm [P121]

Introduction

Acinetobacter baumannii is one of the most problematic opportunist pathogen responsible for many infections worldwide [1]. It presents high adhesion abilities on any types of abiotic and biotic surfaces leading to biofilm development, a mode of growth conferring an additional protection against various treatments and allowing the infection relapse [2].

Characterization of post-translational modifications, such as phosphorylation, may be an interesting way to identify new therapeutic targets, like in a cancer [3], to eradicate this bacterium. Indeed, different examples have demonstrated the implication of phosphorylation in antibiotic resistance, pathogenesis, virulence and persistence in bacteria [4,5].

Methods

We studied S/T/Y phosphorylation in *A. baumannii* biofilm by using high-throughput proteomic approach coupling TiO_2 enrichment and high resolution/high-accuracy mass spectrometry. After peptide identifications, all identifications were manually checked to ensure the location of the modification.

Results/Conclusions

Here, Ser/Thr/Tyr phospho-secretomes of *A. baumannii* reference strain ATCC17978 in planktonic and biofilm modes of growth were characterized by using a proteomic approach. In biofilm, we identified a higher number of phosphoproteins (98 proteins, 137 phosphosites) than in planktonic (35 proteins, 52 phosphosites). Phosphorylated proteins identified in biofilm are involved in different biological processes like ion transport, adaptation, bacterial secretion and iron acquisition. These phospho-secretomes will be compared to those of the clinical *A. baumannii* strain AB0057 to potentially highlight specific proteins or regulation pathways for this virulent isolate.

Novel Aspect

This is the first phosphosecretome of *A. baummannii* biofilm lifestyle. It will be essential to better understand mechanisms involved during biofilm formation.

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Keywords

Phosphorylation, bacteria, biofilm

Authors

Massier Sébastien, Cosette Pascal, Jouenne Thierry [1], Dé Emmanuelle, Hardouin Julie [2]

1. CNRS UMR 6270 (France)
2. Polymers, Biopolymers, Surface Laboratory, UMR 6270 CNRS (France)

Extraction of Fibroin H from Bombyx mori cocoon and its use a composite reinforcement [P122]

Introduction

Silk fibroin from cocoon of *Bombyx mori* has remarkable mechanical properties, rivaling with high-performance synthetic polymers like Kevlar thanks to Fibroin Heavy chain which has a crystalline structure due to it highly repetitive sequence. In this project, we develop Fibroin H biopolymer as a composite reinforcement. For this, we propose a method to extract Fibroin H without degradation in order to be solubilized in photo or thermic polymerizable ionic liquid before its integration into resin to design one-step composites.

Methods

The cocoons were washed in a denaturant urea solution to remove the sericin, the resulting fibers were washed with water and solubilized in a 9.3 M LiBr solution. The solution was treated with TCEP before a dialysis against water. Enzymatic digestion was performed for analysis by LC-MS/MS (Q-Exactive). Fibroin H fibers were obtained after freeze-dry and were incorporated into ionic liquid solution. This result was mixed with styrene resin to make composite by thermic polymerization.

Results

SDS-PAGE profile confirms that the extracted fibroin solution contains a unique protein with a high molecular weight which corresponds, according to MS bottom up analysis, to 391 kDa Fibroin H. This results confirm the integrity and the purity of Fibroin H. The usual extraction method gives intact Fibroin H aqueous solution which is very unstable and rapidly show gelling or fibers reformation [1]. Here the addition of denaturing conditions gives a stable aqueous Fibroin H solution. Fibers obtained after freeze-dry the solution were integrated to specific ionic liquids at 80 °C without overheating at 100 °C [2] which prevents degradation. These ionic liquids can be deactivated by photo or thermic polymerization so the fibroin solubilization is reversible and the mixture Fibroin H/ionic liquid demonstrates an interest to be integrate into a resin for *in situ* fiber reformation during composite manufacturing. These composites were submitted to mechanical three-points flexion test and show a better reinforcement than conventional fibers thanks to biomimetic strategy.

Conclusions

Results demonstrated that Fibroin H was extracted, purified and modified without degradation. The intact Fibroin H fibers obtained can now be reversible solubilized in polymerizable ionic liquid in order to be used as a reinforcement for composite.

Novel Aspect

This work is considered to be the first used intact Fibroin H incorporated into ionic liquid for the reinforcement of composite thanks to *in situ* fibers Fibroin H reformation.

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Keywords

Bombyx mori silk, Fibroin H, composite reinforcement

Authors

Piotrowski Violaine [1], Bray Fabrice [1], Schuller Anne-Sophie, Rolando Christian [1]

1. Miniaturisation pour la Synthèse, l'Analyse et la Protéomique - USR 3290 (France)

Polymers

Combining tandem mass spectrometry with ion mobility spectrometry to reveal information encrypted in sequence-defined poly(alkoxyamine phosphodiester)s [P123]

Introduction

Sequence-defined synthetic polymers are efficient media to store and deliver information. In such polymers, information is encoded by using a set of two different co-monomers arbitrarily designated as the 0- and 1-bit of the ASCII alphabet [1]. To ensure reliable message reading by MS/MS [2], full sequence coverage is required and this can be best achieved by structural optimization aimed at simplifying their fragmentation pattern. However, such a high readability is not compatible with secret communications, so molecular design should also include some security level.

Methods

Poly(alkoxyamine phosphodiester)s were synthesized using an orthogonal strategy that employs successively phosphoramidite and radical-radical coupling steps. High resolution MS, MS^2 and IMS experiments were performed on a Synapt G2 Q-TOF mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization source operated in the negative ion mode and using nitrogen as the collision gas.

Results

To simplify the fragmentation pattern of poly(phosphodiester)s, MS/MS-assisted design was performed to produce poly(alkoxyamine phosphodiester)s (PAPs), which contained weak alkoxyamine bonds to make all coding phosphate groups MS/MS silent [3], and a second coding segment to increase storage density [4]. Two versions of the second segment were conceived to exhibit the same mass but not the same architecture, and could be distinguished by slight conformational variation of a internal fragments produced in MS/MS. Using one or the other versions, sequence-defined PAPs could hence carry secret messages that could no longer be deciphered by MS/MS alone but also required ion mobility to identify the encrypting key revealed by specific drift times measured for internal fragments [5].

Conclusions

Coupling ion mobility with tandem mass spectrometry allowed steganography to be combined with cryptography to improve the security level of information based on digital messages encoded in the backbone of synthetic polymers.

Novel Aspect

MS/MS-IMS of sequence-defined poly(alkoxyamine phosphodiester)s to sequence and decrypt encoded information.

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Keywords

Sequence-defined polymers, MS/MS sequencing, Ion Mobility Spectrometry

Authors

Amalian Jean-Arthur [1], Cavallo Gianni [2], Al Ouahabi Abdelaziz [2], Lutz Jean-François [2], Charles Laurence [1]

1. Aix Marseille Univ - Institut de Chimie Radicalaire (France)
2. Institut Charles Sadron (France)

High Performance Thin Layer Chromatography with Atmospheric Solid Analysis Probe Mass Spectrometry : off line coupling for Analysis of Polymers [P124]

Introduction

High performance thin layer chromatography (HPTLC) is a very efficient method to characterize and quantify compounds in mixtures, widely used in various fields [1]. In order to confirm the analyte identification and to provide structural information, the direct coupling of HPTLC to mass spectrometry can be performed. In this study, atmospheric solid analysis probe mass spectrometry (ASAP-MS) [2] is coupled to HPTLC for analyses of polymeric species and more particularly additives present in gasoline.

Methods

Solutions of PPG and surfactants prepared in toluene were applied to the HPTLC plate using ATS4 automated applicator (CAMAG). Elution was performed with a mixture of MeOH:toluene (40:60). Then, plate is dried under vacuum and each spot was scratched and put into a microtube. ASAP glass capillary was moistened with deionized water and then was dipped in the microtube. Mass spectrometry experiments were performed on a QTOF mass spectrometer (Synapt G2 HDMS, Waters).

Results

Direct ASAP and HPTLC-ASAP analyses were compared using polypropylene glycol (PPG) and polyisobutylene succinimide polyamine (PIB) as analyte molecules. Two ionization parameters, sampling cone voltage and desolvation gas temperature, were evaluated. The optimal ionization conditions are significantly different for ASAP or HPTLC-ASAP. In particular, higher temperature was required to produce ions from the silica gel HPTLC plate, i.e. for dissociating non-covalent bonds between analytes and stationary phase. In HPTLC-ASAP, fragmentation is less abundant even for high values of sampling cone. Therefore, the presence of stationary phase reduces the internal energy of the ions and limits the fragmentation. The effect of the nature of stationary phase was also appraised and the cellulose appeared to be more suitable than RP18 and silica gel phases. Indeed, the high temperature in the ionization chamber could lead to the cellulose pyrolysis allowing a more efficient desorption and an increase of the ionization yield.

Conclusion

This work demonstrated the interest of HPTLC-ASAP as an user-friendly technique for mass spectrometry analysis of polymeric species. The sample preparation was fast and few ionization parameters needed to be optimized. Nevertheless, strong interaction between analyte molecule and stationary phase could result in pyrolysis products or a poor desorption ionization process and could lead to complex mass spectra.

Novel aspect

Off line coupling between HPTLC and ASAP mass spectrometry is a method newly described for polymer analysis.

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Keywords

HPTLC. ASAP. Polymers

Authors

Hubert-Roux Marie [1], Beaumesnil Mathieu [2] [1], Mendes Siqueira Anna Luiza [2], Loutelier-Bourhis Corinne [1], Afonso Carlos [1], Racaud Amandine [2], Bai Yang [2]

1. Normandie Univ, FR 3038; IRCOF, (COBRA) - Université de Rouen, CNRS : UMR6014, INSA Rouen (France)
2. TOTAL, Centre de recherche de Solaize (France)

Environment, Geochemistry and Natural Resources

Use of passive sampling and high resolution mass spectrometry using a suspect screening approach to characterize emerging pollutants in contaminated groundwater and runoff [P125]

Introduction

Groundwaters are facing a decrease in their quality partly due to organic contaminants. These compounds occur at very low concentrations, hence there is a need to accumulate them in order to be able to detect them. The use of passive samplers such as the Empore™ disk (ED)-based sampler is able to provide a representative assessment of groundwater contamination. High resolution mass spectrometry (HRMS) coupled with a suspect screening approach allows the detection of a large number of compounds and enables comprehensive screening of pollutants to be facilitated (Schymanski et al., 2014b).

Methods

Deployment rigs designed to fit down the well bore holes, and holding 4 SDB-RPS and 4 SDB-XC Empore™ disks were deployed for 10 days at 5 sites located in the east of Lyon. After extraction with acetone and methanol, extracts were analysed by liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer. The target Analysis for Screening and Quantitation (TASQ)® 1.4 (Bruker Daltonics®) database (~ 2 500 pesticides and pharmaceuticals) was used for suspect screening data processing.

Results

Filtering of information was undertaken by comparing experimental and theoretical data for each compound. All precursor and product ions were evaluated using three criteria (i) retention time (ΔtR), (ii) m/z (Δm/z) and (iii) isotope pattern. Additionally, the detection of at least one product ion for each precursor ion was mandatory. Finally, 101 suspected compounds were selected. To get the level 1 confidence (Schymanski et al., 2014a), injection of a reference standard in the same analytical conditions for the measurement of retention time, MS and MS/MS spectra was carried out.

Over the 43 compounds to be confirmed, 40 were effectively confirmed, 19 pesticides and 21 pharmaceuticals. From the 40 confirmed compounds, 30 were detected in groundwater and 31 in runoff. Several of them were ubiquitous including caffeine, carbamazepine, diuron, DEET, lamotrigine, metolachlor and theobromine. Overall, pesticides were mainly found in groundwater unlike pharmaceuticals which were more detected in runoff waters.

Conclusions

The application of the suspect screening workflow is helpful in the search for contaminants.

Our results highlight the relevance of our strategy to characterize the occurrence of pesticides and pharmaceuticals in groundwater in a simple way: a wide list of suspects was obtained as the suspect screening approach has the ability to prioritize also the less well-known compounds.

Novel Aspect

This is the first time that an ED passive sampler is combined with a liquid chromatography/high resolution mass spectrometry analysis with a suspect screening approach for assessment of groundwater contamination.

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Keywords

Emerging polar pollutants, high resolution mass spectrometry, suspect screening.

Authors

Pinasseau Lucie, Fildier Aurélie, Wiest Laure, Volatier Laurence, Mermillod-Blondin Florian, Vulliet Emmanuelle [1]

1. Institut des Sciences Analytiques, UMR 5280 CNRS - Université de Lyon 1, 5 rue de la Doua, 69100 Villeurbanne, France (France)

Fast MS/MS proteotyping for screening new microorganisms from environmental samples. [P126]

Introduction

While MALDI-TOF proteotyping allows quick identification of pathogens from clinical samples, no method is till now available for quick screening of environmental isolates. We propose a new proteotyping method based on tandem mass spectrometry and in-house developed bioinformatics software that allows a quick identification of any microorganism, even hitherto taxonomically characterized.

Methods

Here, we optimized i) the protein extraction for prokaryotic and eukaryotic microorganisms based on bead-beating cell disruption and ii) the protein digestion for an automatized procedure. Paramagnetic-bead based sample preparation in 96-well plate format gives the method amenable to full-automatization for high-throughput analyses. In parallel, the MS/MS proteotyping informatic pipeline was improved for automated high-throughput analysis.

Results

We exemplified the power of the method by characterizing an impressive set of 1,100 new environmental isolates. These samples represent the highly diverse origins of the sampled microbial communities with the identification of prokaryotes, including *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and archaea, but also eukaryotes with *Ascomycota* and *Basidiomycota* phyla. Interestingly, the MS/MS proteotyping approach quickly highlights uncommon proteomic profile for several isolates which can be interpreted for taxonomical characterization of new branches of the Tree of Life. For two of such isolates, we confirmed the phylogenetic taxonomy after sequencing their genomes using a combination of Illumina and PacBio next-generation sequencing. We obtained two complete circular genomes and could annotated 3,664 and 4,019 genes, respectively. These two genomes confirm that they are representatives of a new genus belonging to the *Cellulomonadaceae* family and a new species belonging to the *Nevskia* genus, respectively.

Conclusions

The efficiency and timeliness of optimized sample preparation protocol allows treatment of a hundred of samples in just a few hours. Besides, we validate the atypical nature of the two isolates and show that our proteotyping methodology is really fast and powerful for taxonomy compared to genomics-based approaches.

Novel Aspect

The MS/MS fast screening allows selecting isolates of interest that can be further characterized to search novel catalytic activities of biotechnological interest.

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Keywords

Proteotyping, sample preparation, environmental microorganisms.

Authors

Hayoun Karim [1], Pible Olivier [1], Culotta Karen [1], Alpha-Bazin Béatrice [1], Armengaud Jean [1]

1. Laboratory Innovative technologies for Detection and Diagnostics (LI2D) (France)

Comparison of two asphaltene samples of similar origin by the analysis of extrography fractionation and subsequent analysis by FT ICR MS, GPC UV/ICP MS and PVT Quartz Crystal Resonator Cell [P127]

One of the most analytically challenging problems is to understand asphaltene macro and nanoaggregation issues encountered in the industrial-scale processing of very similar oils (same origin, but different reservoir and processing behaviors). Bulk analyses commonly fail to identify differences in these types of samples, as they have similar macroscopic properties, and the differences are often in trace-level components / aggregated components that are not captured in the analysis.

In hopes to reveal the molecular-level differences between the samples and further understand potential structural differences (island vs. archipelago mass distributions) and its subsequent effect on aggregation states, extrography [1] fractionation was performed on both asphaltene samples and the fractions analyzed in the QCR cell, and by FT-ICR MS and GPC ICP MS analysis.

The extrography fractionation has been shown to isolate less polar species in the earlier eluting fractions (lower aggregation tendency) and more polar (higher heteroatom-containing species) in the later eluting fractions (higher aggregation tendency). The difference in aggregation tendency has also been shown to be directly related to not only heteroatom content, but also structure, as early eluting species are frequently dominated by island type (single aromatic core) structure and the later eluting species by archipelago (multi-aromatic core) structures.1 Molecular-level differences can only be see on the extrography fractions of asphaltenes by direct infusion analysis by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR MS), or Gel Permeation Chromatography Inductively Coupled Plasma Mass Spectrometry (GPC ICP MS). Differences between the 2 samples were also detectable by Quartz Crystal Resonator analysis. Thus, there are detectable differences in asphaltene flocculation points and deposition trends.

Extrography allows to understand the macro and nanoaggregation issues encountered in the industrial-scale processing and focus on the role of the “archipelago” (later eluting) fraction plays in this complex phenomenon. In fact, the “archipelago” fraction shows to be the more important part to understand the difference between asphaltenes.

The novel aspect of this works is to create a new method of separation in order to understand the differences between asphaltenes samples

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Keywords

Asphaltene, mass spectrometry, extrography

Authors

Mejia Aurora [1], Carrier Hervé [2] [1], Radji Sadia [3], Daridon Jean-Luc [1], Barrère-Mangote Caroline [4] [2], Bouyssière Brice [3] [2], Moulian Rémi [4] [3] [2], Chacon Martha [5] [2], Rodgers Ryan [5] [2], Giusti Pierre [4] [2]

1. Laboratoire des Fluides Complexes et leurs Réservoirs (France)
2. TOTAL RC – CNRS Joint Laboratory C2MC, Complex Matrices Molecular Characterization (France)
3. Laboratoire de Chimie Analytique Bio-inorganique et Environnement (France)
4. TOTAL RC (France)
5. National High Magnetic Field Laboratory (États-Unis)

Study of the Phospholipid Composition of Brewery spent grain Extracts by Mass Spectrometry [P128]

The brewing sector (≈ 4,000 breweries in the European Union) generates significant volumes of co-products that are currently undervalued. Brewery spent grains (BSG), residues of the cereal fermentation, are the main co-product of these breweries (90% of the volume). Today, around 60% of the BSGs are used for animal feed but the other 40% are not valued (i.e. spreading on agricultural land). The use of dried grain residues as raw materials for the production of new molecules of interest for the food, chemical, pharmaceutical, and cosmetic sectors could offer new outlets for these co-products which, in spite of several studies [1], remain poorly characterized.

In the framework of the Cross-border Program for European Territorial Cooperation INTERREG VA "Greater Region" [2], the BIOVAL project (2014-2020) aims to develop the valorization sector of brewery grains in the context of a circular economy. This requires previously to characterize as finely as possible the molecular content of the BSG.

Here we present the analysis by Mass Spectrometry (MS) of the lipid composition of dried grains studied during the project. The first analyses were carried out on lipid extracts by GC-MS in which fatty acids were detected, mainly palmitic and linoleic acids. More complex lipid families were highlighted and characterized by direct infusion Electrospray Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (ESI FTICR-MS) and Ion Trap Tandem Mass Spectrometry (ESI-ITMSn). The complex lipid profiles of the different BSG extracts were then conducted by liquid chromatography (RPLC and HILIC mode) coupled with IonTrap Mass spectrometry (LC IT-MSn). These extracts showed significant amounts of phospholipids such as acylphosphatidylglycerols and acylphosphatidylethanolamine principally substituted by the palmitic and linoleic acids. Moreover, for some BSG extracts, very interesting lipids have been highlighted such as cyclic phosphatidic acids known to be, for example, specific inhibitors of DNA alpha polymerase [3]. The presence of these molecules indicates that the grains could actually contain valuable compounds of interest for the inhibition of cancer cell invasion and metastasis.

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Keywords

Brewery spent grains; Mass Spectrometry; phospholipids.

Authors

Zaiter Ali [1], Carre Vincent [1], Dupire François [2], Aguedo Mario [3], Bockstal Lauris [3], Jacquet Nicolas [3], Thiel Werner [4], Richiling Elke [5], Weingart Pascal, Saynisch Ranya [4], Tchoumtchoua Job [6], Chaimbault Patrick [1]

1. Laboratoire de Chimie et Physique - Approche Multi-échelle des Milieux Complexes (France)
2. Laboratoire Lorrain de Chimie Moléculaire (France)
3. University of Liège - Gembloux Agro-Bio Tech Biomass and Green Technologies (Belgique)
4. Technische Universität Kaiserslautern Lehrgebiet Anorganische Chemie (Allemagne)
5. University of Kaiserslautern Department of Chemistry/Division of Food Chemistry and Toxicology (Allemagne)
6. CELABOR (Belgique)

Apport des techniques d'analyses couplées à l'étude des constituants de l'huile essentielle de Conocephalum conicum [P129]

L'analyse de mélanges complexes s'effectue classiquement par le couplage « en ligne » d'une technique chromatographique avec une technique d'identification spectrale. Cette procédure est privilégiée lors d'analyse «de routine », cependant, dès lors que l'étape d'identification se complexifie, la procédure nécessite un fractionnement de l'échantillon qui peut se poursuivre jusqu'à la purification d'un constituant afin d'aboutir à son élucidation structurale [1].

La bibliothèque de spectres de masse et d'indices de rétention développée au laboratoire pour l'analyse des compositions chimiques des mélanges complexes volatils comprend à ce jour, plus de 1000 références. Malgré cela, elle s'est avérée inopérante pour l'étude de la composition chimique de l'huile essentielle de *Conocephalum conicum* puisque seulement 60% de la composition chimique, soit 36 constituants ont été identifiés.

Cette communication présente les développements analytiques mis en oeuvre pour identifier les constituants moléculaires de l'huile essentielle absents des bibliothèques de références. Après plusieurs fractionnements 7 composés ont été concentrés et leurs structures ont été élucidées par RMN mono et bidimensionnelle. Quatre d'entre eux, le 3,4-diméthyoxystyrène, l'épi-presilphiperfolan-1-ol, le dactylol et le spathulénol ont été identifiés par comparaison de leurs données spectrales expérimentales de RMN du carbone-13 avec celles décrites dans la littérature. Les identifications du bicyclogermacrèn-14-al et du 1,6-humuladièn-10-ol ont nécessité une étude RMN bidimensionnelle. L'identification du dernier composé a nécessité une attention toute particulière, du fait de son instabilité chimique.

Avec un ion moléculaire à *m/z* 222 et un ion à *m/z* 204 caractéristique de la perte d'une molécule d'H2O, ce dernier présentait un spectre de masse IE caractéristique d'un alcool sesquiterpénique, en accord avec la proposition « Conocéphalénol » avancé par la bibliothèque littérature. Cependant le spectre de RMN du carbone-13 enregistré dans le CDCl3, ne présentait aucun carbone caractéristique d'une fonction alcool, suggérant plutôt la présence d'un composé hydrocarboné. Notre étude combinée MS - RMN a permis de confirmer l'instabilité du conocéphalénol [2] réaliser l'attribution des valeurs de déplacements chimiques du carbone-13 de trois brasiladiènes connus comme produits de dégradation du conocéphalénol et observer la dégradation vers un produit non encore observé, le brasila-1(9),5-diène. En complément, notre travail a permis de décrire les données spectrales de RMN du carbone-13 du conocéphalénol dans le CDCl3 puisque en mélange avec le bicyclogermacrèn-14-al, l'instabilité de l'alcool est contrôlée.

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Authors

Pannequin Anais [1], Tintaru Aura [2], Muselli Alain [1], Desjobert Jean-Marie, Costa Jean

1. Laboratoire de Chimie des Produits Naturels, (France)

2. Aix-Marseille Université (France)

Chemical investigation of Senecio transiens (Rouy) Gamisans essential oil, an endemic species from Corsica [P130]

The genus *Senecio* (Asteraceae family) is one of the largest genus worldwide that include more than 1500 species [1]. In Corsica, 10 species have been reported and among them, 3 are endemic status: *S. rosinae*, *S. serpentinicola* and *S. transiens* [2].

Senecio transiens is a crasulescent plant (4-40 cm high) commonly localized on cliffs and coastal dunes of the Corsican west littoral. It was rare on the eastern coast where it was only found around the Cap Corse (North of Corsica) and the localities of Porto Vecchio and Bonifacio (South of Corsica). The plant geographical distribution was linked to environmental conditions: it grows only on the area exposed to the sea sprays and on the stones or beach dunes near to sea [3].

The chemical composition of *S. transiens* essential oils from 30 Corsican locations was investigated using gas chromatography (GC-FID), gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR). Seventy components, which accounted for 91.2% to 98.3% of the total amount were reported and two new bisabolene derivatives named 8-acetoxybisabolen-1,9-diol and acetoxybisabolene oxide have been discovered for the first time.

A statistical study of the chemical results highlighted the chemical variability of *S. transiens* essential oil compositions. The three clusters were discriminated according to their chemical compositions and the chemical clustering was correlated to the morphological polymorphism of the plant:

- Cluster 1 included 12 sample oils dominated by 3-isopent-2-enyl-methoxyacetophenone (32.2-63.3%), and all the samples were arborescent specimens with middle size (10-20 cm),
- Cluster 2 included 9 samples characterized by the occurrence of both bisabolane derivatives. These sample plants were more slender and higher (20-40 cm),
- Cluster 3 included 9 samples exhibited highest amounts of euparin (18.2-33.2 %), α-pinene (5.3-8.3%), myrcene (11.3-16.3 %) and nonene (12.3-29.5%). The plant materials were small size samples (4-8 cm).

Our study highlighted the gain of analytical techniques as well as Mass Spectrometry and Magnetic Nuclear Resonance to the study and the comprehension of the vegetal biodiversity.

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Keywords

Senecio transiens; bisabolene derivatives, Mass spectrometry.

Authors

Andreani Stéphane [1]

1. Sciences pour lénvironnement (France)

Chemical investigation of Corsican propolis and Populus nigra var. italica buds essential oil [P131]

Propolis is a resinous product collected by honeybees from buds and/or barks of some plants. It is used to protect the colony from diseases and parasites, particularly to inhibit bacterial growth [1]. It is reported that propolis has antibacterial, antioxidative and antifungal activities [2]. *Populus nigra* is known as an important ressource of propolis. Some studies showed the presence of terpenoid and phenolic compounds in *P. nigra* extracts [3]. Only few studies focused on the volatile compounds of *P. nigra* buds [4, 5], and the essential oil of Corsican propolis was never been studied.

In this work, essential oil composition analysis of five propolis and five *P. nigra* var. *italica* buds samples was carried out using GC-FID and GC-MS. The objective of this study was to characterize the essential oil compositions of Corsican propolis and its possible raw material; the resineous secretion of buds from *P. nigra* var. *italica*. The chemical relationships between the hive propolis and plant material were also established on the basis of volatile composition.

The propolis essential oil was characterized by 107 volatile compounds. This oil was dominated by oxygenated sesquiterpenic compounds (27.9 – 36.0 %) with guaiol (5.8 %), *β*-eudesmol (3.6 %) and bulnesol (3.5 %) as main components. The three same main constituents were reported in oil of *P. nigra* var. *italica* buds amounting to 19.4 %, 3.8 % and 20.9 % of the total oil, respectively. Otherwise, a moleluar, named *(E)*-5-phenyl-3-penten-1-ol, had been identified in popolar oil by mass spectrometry and NMR. To the best of our knowledge, the occurrence of this molecular as a natural product is reported for the first time. 43 other common volatile compounds such as *α*-bisabolol, hinesol, *γ*-eudesmol were also reported on propolis and *P. nigra* var. *italica* oils.

These results suggested that the buds of *P. nigra* var. *italica* are one of the principal raw material for the production of Corsican propolis by honeybee. The study of chemical variability of propolis and corresponding plant material could be used to establish the complex botanical origins of propolis.

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Keywords

essential oil, propolis, *Populus nigra*

Authors

Yang Yin [1]

1. Sciences pour l'environnement (France)

Proteomic identification of ovarian fluid components able to extend fish egg viability [P132]

Introduction

The control of egg quality is a major issue for the development of a sustainable aquaculture in the context of an increasing demand for aquaculture food products. In most fish species, the egg ability to be fertilized exhibits a rapid and dramatic decrease after ovulation. In contrast, salmonid fishes can hold their eggs after ovulation for several days without any significant decreased in egg viability.

The EggPreserve project aims at identifying proteins in ovarian fluid that are responsible for preserving egg viability and ability to be fertilized after ovulation in salmonids.

Methods

Ovarian fluids were desalted and concentrated by diafiltration and then fractionated by UHPLC using sequential chromatography (Gel Filtration and Anion Exchange). The different fractions were systematically assessed for egg quality with a biological test and proteins in fractions of interest were identified by mass spectrometry (Tims TOF Pro; Bruker).

Several ovarian fluids proteome of salmonid and non-salmonid species were also compared by mass spectrometry.

Results

The 30 Gel Filtration fractions of ovarian fluids were assessed by incubation with ovulated eggs before ovulation. We identified 3 groups of fractions allowing the conservation of the eggs, and a group of fractions having the opposite effect. A second fractionation using Anion Exchange was performed for 2 previous groups of Gel Filtration fractions preserving eggs viability. These fractions were also tested on ovulated eggs.

In parallel, mass spectrometry analysis on all active fractions resulting from both chromatographic runs allowed the identification of proteins of interest, probably involved in egg quality. Moreover we qualitatively compared the ovarian fluid proteome of different salmonid and non-salmonid species, based on orthologous analysis and identified proteins specifically present in salmonid species which could be involved in preserving egg viability.

Conclusions

We have initiated a mass spectrometry analysis of ovarian fluid proteomes from salmonid and non-salmonid species in order to increase our understanding of ovarian fluid protein composition. We have identified proteins that could probably be involved in the enhancement of egg viability in salmonid species compared to non-salmonid species. Our work will help deciphering of the biological mechanisms of egg quality control in fish.

Novel Aspect

This project can have a direct, and possibly rapid, impact for the aquaculture sector with the marketing of a commercial synthetic fluid that mimics ovarian fluid.

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Keywords

Ovarian Fluid, Fractionation, Egg quality

Authors

Guével Blandine [1], Guého Aurélie [2], Rime Hélène [2], Com Emmanuelle [1], Lavigne Régis [1], Montfort Jérôme [2], Labbé Catherine [2], Bobe Julien [2], Pineau Charles [1]

1. Protim (France)
2. LPGP (France)

Characterization of new emergent per-fluoroalkyl environmental contaminants by mass spectrometry [P133]

Introduction

Per-fluorinated compounds as per-fluoroalkyls (PFAs) are very stable chemicals and possess lipophobic and hydrophobic properties useful for combating fuel fires as additives in fire-fighting foams. Their physicochemical properties lead the PFAs to be placed at the interface between the foam and the fuel and block the arrival of oxygen. The PFAs concentration of groundwater and surface water near the areas where they were used makes them emerging contaminants. Some studies also showed that PFAs can cause liver and kidney damages. Their analysis is consequently required.

Methods

Even if PFAs are industrially produced by well-known processes, side reactions lead to the formation of a huge number of by-products, which are only partly known. Consequently, it was chosen to adopt a not targeted approach. After adequate dilution in a water/methanol solution (50/50 ; v/v), acetic acid (1%) or ammonium (1%) was added before to conduct analysis by (+) or (-) ESI. FT ICR-MS was used to ensure high mass resolution and high mass measurement accuracy.

Results

More than two hundred different peaks were evidenced in the study of three concentrates of commercial foams produced by different vendors. High measurement accuracy achieved by FT ICR-MS in 2XR mode (in the underdo ppb range) and the fine isotopic structure ensured to unambiguously assigned a chemical formulae to each feature observed in the 450 – 1500 m/z range. Kendrick mass defect clearly highlighted repetitive units in the structure of the detected PFAs, which ensured to have some insights on the used industrial process. The main part of the studied PFAs were from co-polymerization of ethylene, tetrafluoroethylene and acrylamide. Among the evidenced PFAs, a significant part of them was still reported and tandem mass spectrometry ensured to confirm their structure. Nevertheless, one concentrate displayed PFAs, which were not still described. The combination of MS/MS and MS/MS/MS experiments gave powerful insights to obtain structural information. Such PFAs are the association of a per-fluorinated domain connected to an oligomeric acrylamide backbone by a thiol link.

Conclusions

ESI FT-ICR mass spectrometry demonstrates that commercial foams combined a significant number of different PFAs, which only part of them are known. This study ensures to highlight still unknown ones. Moreover, the structural analysis of unknown PFAs ensures to highlight specific fragmentation pattern which can be used for their quantitative analysis by LC MS/MS.

Novel Aspect

The fine characterization of per-fluorinated alkyl complex mixture was achieved by positive and negative ESI combine to FT-ICR ultra-high resolution mass spectrometry.

Keywords

Per-fluorinated contaminants, Non-targeted analysis, ESI FT-ICR MS

Authors

Trzpit Quentin [1], Carre Vincent [1], Boiteux Virginie [2], Dauchy Xavier [2], Aubriet Frédéric [1]

1. Laboratoire de Chimie et Physique - Approche Multi-échelle des Milieux Complexes (France)
2. ANSES - Laboratoire d'Hydrologie de Nancy (France)

Clinical Mass Spectrometry

Innovative nanoflow liquid chromatography HRMS approach opens new perspectives for high throughput doping control analyses [P134]

Doping control analytical workflows often involve an extraction procedure followed by narrow bore liquid chromatography coupled to tandem mass spectrometry. Over the past decade, this approach underwent several breakthrough improvements in terms of throughput and precision thank to ultra-high-performance liquid chromatography (UHPLC) and high-resolution mass spectrometry (HRMS). First generation nanoflow liquid chromatography (nanoLC) significantly improved sensitivity at the expanse of robustness and analysis throughput. Indeed, the use of such technology was often associated with the risk of retention time fluctuation and time-consuming sample loading. These constraints limited the use of nanoLC for horseracing drug testing purposes to confirmatory analysis of a few polypeptides such as recombinant human erythropoietins (rHuEPOs).

The recent advances in nano-separation through innovative sample trapping combined to controlled elution and on-column refocusing is now available by means of the Evosep One system which fills the gap between conventional high-throughput UHPLC and high-sensitivity nanoLC. Our results demonstrate that the adaptation of our rHuEPOs and Growth Hormone validated methods to the Evosep One improved robustness and throughput with three to five-fold time saving, without compromising the analytical performances. Further developments are focused on the confirmatory analysis and non-targeted screening analysis of small molecules and protein/peptide-based drugs in different horse matrices.

Novel Aspect

Method development in high throughput nanoLC-MS/MS for doping control analyses

Keywords

nanoLC-MS/MS, Horse doping control, High throughput analysis

Authors

Delcourt Vivian [1], Garcia Patrice [1], Chabot Benjamin [1], Popot Marie-Agnès [1], Bailly-Chouriberry Ludovic [1]

1. Laboratoire des Courses Hippiques (GIE-LCH) (France)

New approach for the detection of bisphosphonates in horse plasma by IC-MS/MS [P135]

Bisphosphonates are prohibited drugs according to article 6 of the International Agreement on Breeding, Racing and Wagering of the International Federation of Horseracing Authorities (IFHA) and the International Equestrian Federation (FEI). These compounds are used for the treatment of navicular and related diseases in horses and are divided in two groups: non-nitrogen-containing bisphosphonate drug (*e.g.* clodronic acid) and nitrogen-containing bisphosphonate drug (*e.g.* zoledronic acid). Their hydrophilic properties and high affinity for the bone matrix make control of their use quite difficult. Today analysis of such compounds is based on a solid phase extraction (SPE) using 96-well plates coated with Oasis weak anion exchange (WAX) sorbent on a Biotage® Extrahera™ system followed by detection using UHPLC-MS/MS on a TSQ Quantiva™ mass spectrometer (Thermo Scientific™) after methylation with trimethyl orthoacetate (TMOA) in acetic acid.

To get rid of the derivatization step a new approach using ion chromatography linked to mass spectrometry is considered. Analyses are conducted on an ICS-6000 HPIC system coupled to a TSQ Altis™ (Thermo Scientific™). Horse plasma preliminary results show good performances of the method for the detection of non-nitrogen-containing bisphosphonates and nitrogen-containing bisphosphonates with limit of detection (LOD) below 1 ng/mL.

Use of Ion Chromatography for the detection of bisphosphonates in horse plasma.

Keywords

IC, Bisphosphonates, Horse doping control

Authors

Garcia Patrice [1], Pinetre Justine [1], Jaubert Murielle [1], Perot Isabelle [1], Popot Marie-Agnès [1], Bailly-Chouriberry Ludovic [1]

1. Laboratoire des Courses Hippiques (GIE-LCH) (France)

Nanobioanalytical studies and proteomic analysis of human platelet-derived extracellular vesicles [P136]

Introduction

We developed a NanoBioAnalytical (NBA) platform [1,2] combining three techniques: Surface Plasmon Resonance (SPR) technique, Atomic Force Microscopy (AFM) and Mass Spectrometry (MS) for on-chip, label-free, characterization of platelet extracellular vesicles (PEVs) in their crude sample. Extracellular vesicles (EVs) are attracting considerable interest in the scientific community due to their role in intercellular communication. The NBA platform combines different techniques in order to better understand the features that can influence their potential haemostatic functions.

Methods

PEVs were collected by centrifugation from human platelets, through freeze-thaw cycles or by the activation with the calcium ionophore A23187 (Calo). Their capture on a biochip presenting multiple arrays of ligands was monitored in real time with surface plasmon resonance imaging (SPRi) and the size and morphology of the captured objects were evaluated by atomic force microscopy. In addition, a proteomic analysis of the captured EVs on the biochip was carried out.

Results

The NBA platform enabled the capture of PEVs subsets on a chip, through interactions on anti-integrin alpha-IIb (CD41), anti-platelet glycoprotein Ib alpha chain (CD42b), anti-integrin beta-3 (CD61), anti-P-selectin (CD62P) and anti-CD9 protein antibodies, as well as on annexin V through the interaction with phosphatidylserine (PS). The best capture rate was observed on α-CD41 antibodies, followed by α-CD9 and annexin V. The AFM analysis demonstrated that the diameter of the majority of the particles laid in the range of 30-50 nm. PEVs prepared with CaIo were approximately 20% larger comparing to PEVs obtained from frozen-thawed platelets. The nano-liquid chromatography-tandem mass spectrometry analysis revealed more than 100 proteins (from 300 ng of EVs captured). Proteins detected in PEVs are involved in hemostasis (platelet cell adhesion molecule), cell adhesion (glycoprotein Ib and integrins), coagulation (coagulation factor XIII) and platelet activation (filamin A) pathways.

Conclusions

The NBA platform is a versatile tool for determination of EVs number and the identification of specific EVs subsets in complex biological samples. These advances will allow establishing a relationship between EVs structure and composition and their physiological functions. The perpectives include the development of a bioreactor for coculture of platelets and cancer cells followed by the study with the NBA platform of the cell-to-cell communication mediated by EVs.

Novel Aspect

NBA platform offers a sensitive and label free method to characterize EVs in a large dynamic concentration range that matches with EVs concentration ranges in body fluids.

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Keywords

Proteomics, nanocharacterization, biochip

Authors

Maes Pauline [1], Maximova Ksenia [2], Obeid Sameh [2], Godon Alexandre [3], Lecompte Thomas [3], Boireau Wilfrid [2], Rageot David [1], Fontana Pierre [3], Elie-Caille Céline [2], Bonhomme Fanny [3]

1. CLIPP Platform (France)
2. FEMTO-ST Institute (France)
3. Geneva Platelet Group, Faculty of Medicine, University of Geneva, Geneva, Switzerland (Suisse)

Development of an HPLC-MS / MS method for the quantification of 3-O-methyl glucose in plasma, a marker of intestinal absorption of glucose [P137]

Introduction

Intestinal absorption of glucose is a well-documented phenomenon. It is characterized by the use of analogous glucose markers. "D-Xylose test" is used clinically to explore the absorption capabilities of the proximal small intestine. Nevertheless new questions emerge concerning its use because of its physico-chemical properties different from that of glucose (pentose *vs* hexose). Recently, 3-Me-Glc has been selected to more closely mimic intestinal glucose absorption [1]. The dosage of 3-Me-Glc remains poorly documented [2]. We propose in this process a rapid quantitative analysis.

Methods

Proteins are precipitated with a cooled methanolic solution. After centrifugation, the supernatant is evaporated under a stream of nitrogen. This is used by the optimized mobile phase for the chromatographic separation of Glc, 3-Me-Glc, 3-Me-13C6 Glc and Xyl. The selective elution of the compounds is carried out using the HILIC column, bridge X BEH amide 2.5 µm; 3 x 100 mm, using an elution gradient with a mobile phase composed of a 10 mM ammonium formate buffer and acetonitrile and a flow rate of 0.4 ml/min [3].

Results

The procedure for optimizing the analysis method required selected parameters of the detection conditions (direct injection, FIA mode) and elution conditions (stationary phase, mobile phase and HPLC-MS/MS coupling). The specific transitions of each compound made it possible to release fragment ions whose intensities are sufficiently intense for facilitated quantification of 3-O-Me-Glc. Coupling to the chromatographic system allows separation in less than 3 minutes for an analysis time of 5 minutes of the three components of the mixture (3-O-Me-Glc, Glc and Xyl).

Pre-validation of the method was implemented with selectivity, limit of detection and limit of quantification as well as response function and linearity as criteria.

The method was applied to the preliminary assay of pig plasma that received a mixture of glucose and 3-O-methyl glucose by intra-duodenal administration. A kinetic study carried out on short times validates the model.

Conclusions

A method for plasma assay of 3-O-methyl glucose was developed by HPLC-MS/MS. The analytical procedure proves to be sensitive and rapid in order to determine the 3-O-methyl glucose, a marker of the intestinal absorption of glucose. It was applied to a kinetic study performed on pig plasma after intra-duodenal administration of glucose and its absorption marker.

Novel Aspect

This detailed analytical study demonstrates the value of measuring 3-O-methyl glucose as a marker of glucose uptake

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Keywords

3-O-methyl glucose, plasma, Mass Spectrometry

Authors

Kouach Mostafa [1], Zubiaga Lorea [2], Descat Amandine [1], Daoudi Mehdi [2], Leprovost Savignien [1], Hecquet Paul-Emile [1], Pattou François [2], Goossens Jean-François [1]

1. PSM "Plateau de Spectrométrie de Masse" de l'EA7365 GRITA "Groupe de Recherche sur les formes injectables et les technologies associées" (France)
2. UMR1190 "Recherche translationnelle sur le diabète" (France)

Combination of Nematic Protein Organization Technique (NPOT®) and proteomics for drug-target identification: example of INOV-001, a Hydroxychloroquine (HCQ) analogue for the treatment of triple-negative breast cancer [P138]

Introduction

The pharmaceutical industry is constantly under economic pressure because of high attrition rate of drug candidates. The drug development process is mainly jeopardized by problems of toxicity and/or inefficiency of drug candidates during clinical trials, mainly due to the poor understanding of the drug clinical mode of action.

To tackle those major challenges, Inoviem Scientific has developed the concept of **drug-upgrade** based on its proprietary technology, NPOT® [1,2,3] coupled with proteomics, that aims at the identification of the therapeutic (ON-) or toxic (OFF-) drug targets.

Methods

NPOT® was performed on hydroxychloroquine (HCQ) and HCQ analogue (INOV-001) to decipher ON- and OFF-targets. The NPOT® heteroassemblies were analysed using nanoLC-MS/MS: nanoACQUITY UPLC-TripleTOF 5600 (Waters-Sciex).

Efficacy of increased doses of INOV-001 was evaluated in combination or not with Docetaxel *in vivo* on a MDA6MB-231 xenograft tumor model in nude mice. Tumor growth was measured over a 32-day period.

Results

A drug-upgrade program has been applied on hydroxychloroquine (HCQ, Plaquenil®), an already marketed drug used as anti-malaria and in systemic lupus erythematous (SLE) but inducing irreversible retinopathies.

Thanks to our proprietary technology NPOT®, we managed to identify HCQ therapeutic (ON-) targets and toxic (OFF-) targets, responsible for retinal toxicity (RT). We have developed INOV-001, a novel analogue with higher efficacy in SLE context and reduced RT. We further discovered that INOV-001, when combined with first-line chemotherapy treatment, is able to induce a significant reduction of tumor volume in an *in vivo* model of triple negative breast cancer (TNBC), the most aggressive form of breast cancer. Hence, INOV-001 potentiates the effect of chemotherapy in the treatment of TNBC.

NPOT® experiments on INOV-001 in the context of TNBC are ongoing to understand the drug mode of action in this new therapeutic area.

Conclusions

These results confirm that using highly sensitive LC-MS/MS strategy to decipher NPOT®-derived interactomes is a powerful approach to improve our understanding of drug mode of

action and clinical ON/OFF targets that would result in novel chemical entities (NCE) from marketed drugs.

Novel Aspect

Inoviem Scientific has developed a labelfree technology allowing to isolate and in combination with proteomics to identify the mode of action of a clinical compound.

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Keywords

NPOT®, interactomics, drug-upgrade

Authors

Bazel Isabel [1], Mopin Laura [1], Schneider Justine [1], Spenlé Caroline [2], Eschbach Judith [1], Peter Jean-Christophe [3], Amouroux Rachel [1], Bagnard Dominique [2], Eftekhari Pierre [1]

1. Inoviem Scientific, Illkirch-Graffenstaden (France)
2. INSERM U 1109, MN3T Laboratory, Labex Medalis (France)
3. Laboratory of Excellence Medalis (ANR-10-LABX0034_MEDALIS), Institut de Biologie Moléculaire et Cellulaire (France)

Proteomic Characterization of Juvenile Neuroblastoma [P139]

Introduction

Proteomic characterization of tumor cells and tissues is gaining increasing interest. Tumor-focused proteome investigations serve to yield novel insights into tumor biology. We performed quantitative proteome profiling of n = 58 juvenile neuroblastomas (NBLs). Our cohort included all INSS Stages (I – IVs), reflecting both high as well as low SIOPEN risk stratification. The direct and system-wide protein-level analysis by tandem mass-spectrometry is the preferred approach for the discovery of protein signatures.

Methods

FFPE tumor tissue specimens were used for mass spectrometry based proteomics, further highlighting their suitability for proteome-wide profiling studies. We performed several batches of TMT-11plex labelling combined with high pH reversed phase HPLC fractionation. For statistical analysis, the measured intensities were normalized using a combination of the Voom and the ComBat function in R.

Results

In first pilot experiments our optimized FFPE tissue protocol for the NBL samples yielded over 2000 identified and quantified proteins in 3 different patients. We than applied the protocol to the 53 patient's available samples and were able to cumulatively identify and quantify over 2000 proteins. Preliminary data analysis showed drastic batch effects, which we were able to overcome by optimized data analysis and using Combat batch normalization. The normalized values were used for a variety of statistical tests in collaboration with the Institute for Medical Biometry and Statistics in Freiburg. Preliminary results of a multivariate analysis show a set of five proteins allowing for a good risk-of-death stratification.

Conclusions

This data set provides novel insight into NBL biology and stratification. Improved relative survival / risk stratification could lead to optimized and individualized treatment. Large scale proteomic studies can be correlated with previous genomic and transcriptomic studies to gain more comprehensive understanding of the disease.

Novel Aspect

Including 53 patients, this cohort reflects a valuable and rare opportunity to investigate this controversially behaving tumor.

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Keywords

Clinical proteomics, FFPE tissue proteomics, Neuroblastoma proteomics

Authors

Fahrner Matthias [1] [2] [3] [4] [5], Erdélyi Zoltán [6] [5], Bronsert Peter [4] [7], Froehlich Klemens [1] [4], Hess Moritz [8], Micsik Tamás [9], Garami Miklós [6], Schilling Oliver [4] [7]

1. Spemann Graduate School of Biology and Medicine (SGBM), Albert-Ludwigs-University Freiburg (Allemagne)
2. Faculty of Biology, University of Freiburg, Freiburg (Allemagne)
3. Institute of Molecular Medicine and Cell Research, Faculty of Medicine, University of Freiburg, Freiburg (Allemagne)
4. Institute of Surgical Pathology, Medical Center – University of Freiburg (Allemagne)
5. contributed equally (Allemagne)
6. 2nd Department of Pediatrics, Faculty of Medicine, Semmelweis University, Budapest (Hongrie)
7. German Cancer Consortium (DKTK) and Cancer Research Center (DKFZ), Heidelberg (Allemagne)
8. Institute of Medical Biometry and Statistics, Faculty of Medicine and Medical Center of the University of Freiburg, Freiburg (Allemagne)
9. First Department of Pathology and Experimental Cancer Research, Faculty of Medicine, Semmelweis University, Budapest (Hongrie)

Towards the development of a bitterness predictive tool for agrofood protein enzymatic proteolysis: a sensomics approach [P141]

Introduction

Enzymatic hydrolysis of proteins is widely used in the food industry to improve functional properties such as solubility, emulsification and taste, or to prepare extensively hydrolyzed proteins for nutritional properties [1]. The protein hydrolysates also have a health beneficial purpose. For example, hydrolysed caseins are frequently used in infantile milk formulation for children with a milk allergy [2]. Although milk is characterized by a sweet taste, when obtaining hydrolysates of milk proteins, a bitter taste with some off flavors are often detected. The aim of my PhD thesis project focuses on the control of enzymatic hydrolysis of milk proteins to obtain low to non-bitter protein hydrolysates because it limits their use.

Methods

Three enzymatic hydrolysis were made in triplicates from milk proteins by following a biotechnological protocol developed within the company INGREDIA. Samples are collected at the end of the hydrolysis and were subjected (i) to size-exclusion chromatography to establish the peptide size profiles, to UPLC/MS-MS analyses for peptide identification using PEAKS Studio software, to principal component analysis with Progenesis QI for proteomics and were (ii) characterized by sensory analysis.

Results

Sensory test confirmed the bitterness difference between the three hydrolysates and the PCA performed from UPLC/MS-MS data revealed differences in terms of peptide heterogeneity. Some hypotheses found in the literature concerning the characterization of the bitter peptides are validated.

Conclusion

By combining the different approaches mentioned above, these findings constitute the first step of the conception of a peptide-based bitterness predictive tool to guide the development of "non"-bitter agrofood protein hydrolysates.

Novel aspect

Using sensory analysis and concomitantly, the peptide characterization by UPLC-MS/MS and bioinformatics, it should be possible to predict whether a hydrolysate will be bitter or not by studying the amino acid composition of all its peptides.

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Keywords

Sensomic, hydrolysate, bitterness

Authors

Daher Dahlia [1] [2], Froidevaux Gianni [1], Deracinois Barbara [2], Wattez Elodie [1], Dantin Justine [1], Mandoux Antoine [1], Baniel Alain [1], Froidevaux Renato [2], Chollet Sylvie [2], Flahaut Christophe [2]

1. Ingredia S.A. 51 Av. Lobbedez - CS 60946, 62033 Arras Cedex - France (France)
2. Université de Lille. INRA. Université d'Artois. Université Littoral Côte d'Opale. ISA, EA 7394 - Institut Charles Viollette, F-59000 Lille, France (France)

Urinary steroid profiling by gas chromatography-mass spectrometry [P142]

Introduction

Steroid profiling by mass spectrometry approaches consists in the simultaneous measurement of several steroid molecules in a biological sample, allowing an optimal characterization of steroidogenesis alterations, particularly in the context of adrenal pathologies. Urinary steroid profiling has thus been shown to be particularly useful in the diagnosis of adrenal pathologies [1,2,3]. We describe here the optimization and validation of a gas chromatography-mass spectrometry (GC-MS) approach, allowing the determination of a profile of 19 steroid urinary metabolites.

Methods

Urinary samples preparation required several steps including enzymatic hydrolysis, liquid-liquid extraction and derivatization of steroid metabolites. Retention time and mass spectrum of each steroid metabolite were determined by injection of the corresponding external standard in fullscan mode. Linearity, repeatability and reproducibility of the method were further evaluated.

Results

Optimized conditions of urine samples preparation were as follows: enzymatic hydrolysis with arylsulfatase/glucuronidase, first derivation in methoxyamine/pyridine and second derivation in trimethylsilyimidazole. R2 of the 19 calibration curves ranged from 0.983 to 0.998. Coefficient of variation (CV) of repeatability and reproducibility ranged respectively from 2.4% to 14.4% and from 3.2% to 13.6%. As previously described we confirmed with this approach, the increase in steroid precursor metabolites in urinary samples from patients with adrenocortical carcinoma.

This method was applied for the first time to urinary samples from patients with Cushing disease, highlighting a global activation of steroidogenesis in Cushing disease, characterized by an increase of glucocorticoids (THE, THE), androgen (Etiocholanolone), glucocorticoid and androgen precursors (PT, THS) urinary metabolites.

Conclusion and novel aspect

We propose here a complete methodology of urinary steroid profiling by GC-MS, analytically validated in terms of linearity, repeatability and intermediary fidelity. This method is biologically validated by using samples from patients with adrenal carcinoma. This approach gives new insights into the characterization of steroidogenesis alterations, including in Cushing disease.

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Keywords

Urinary steroids, GC-MS, adrenal pathologies

Authors

Bonnet Fidéline, Pages Alice, Zientek Corinne, Gaillard Catherine, Bertherat Jérôme, Guibourdenche Jean, Menet Marie-Claude [1]

1. Laboratoire d'hormonologie (France)

Macrophages reactivation inside glioma microenvironments through PC1/3 inhibition associated with TLR3 activation [P143]

Glioma are common brain tumor and some of the most aggressive. They are currently treated with surgical resection, chemotherapy and radiotherapy. But, despite of these treatments, their immunosuppressive and invasive nature makes it impossible to eradicate them completely and the prognosis stay poor. In this way, therapeutic strategies need to be developed against this immunosuppression. Inside immunosuppressive tumor environment, infiltrated macrophages present an anti-inflammatory phenotype promoting tumor growth and suppressing cytotoxic function of T cells. One of the new therapeutic strategies is the reprogramming of macrophages into pro-inflammatory phenotype to trigger the immune response against the tumor. For this purpose, we are targeting the proprotein convertases (PCs) which are enzymes implicated in tumors development. We have previously shown that proprotein convertase 1/3 (PC1/3) inhibition led to an abundant secretion of immune factors and orient macrophages towards pro-inflammatory [1,2] and anti-tumoral phenotype [3]. Macrophages activation is enhanced when PC1/3 inhibition is combined with Toll Like Receptors (TLRs) stimulation [3, 4]. Previous studies have also revealed that after TLR3 stimulation, macrophages secrete factors that exerted a toxic effect on glioma cells [5]. Thus, we decided to study the activation state of macrophages and their ability to secrete anti-tumor factors after a co-treatment with TLR3 receptor agonist, Poly (I:C) and PCs inhibitor.

First, the effect of PCs inhibition on rat C6 glioma cell line was investigated. A viability test was performed by MTS test and reveals that PCs inhibitor decreased the viability of C6 glioma cell line. Moreover, glioma display a significant invasive nature. This is one of the causes of resistance to treatment. Therefore, we also performed invasive tests on spheroids and mixed glioma cells/macrophages spheroids. This test showed that PCs inhibitor decreased the invasion of C6 glioma cell line. To go further, mixed glioma cells/macrophages spheroids were treated with PCs inhibitor and PCs inhibitor penetration into the spheroids was studied by Mass Spectrometry Imaging. This experiment showed that PCs inhibitor penetrates inside the mixed spheroids and decreased their invasion. Thereby, PCs inhibitor has an effect on both glioma cells, by decreasing their viability and invasion, and on macrophages by decreasing their secretion of tumor growth related protein.

The association between TLR3 activation and PCs inhibition shows good anti tumoral activity on rat glioma cell line. The results of this study could lead to a new therapeutic strategy for glioma treatment.

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Keywords

Glioma, macrophages, cell reprogramming

Authors

Rose Melanie [1], Duhamel Marie [1], Rodet Franck [1], Salzet Michel [1]

1. Protéomique, Réponse Inflammatoire, Spectrométrie de Masse (PRISM) - U1192 (France)

Benchmarking of Biomarker Discovery Workflows in large patient cohorts using LC-MS/MS based proteomics [P144]

Introduction

The discovery of biomarkers using proteomics is challenging due to a number of factors, including undersampling, technical and biological variance and the choice of statistical analysis tools [1]. While spike-in datasets are powerful tools to benchmark biomarker discovery, current datasets lack realistic patient to patient heterogeneity. To overcome these limitations, we will measure a large dataset which reflects realistic protein expression variance, while also providing ground truth of differentially abundant proteins.

Methods

Pilot Studies: In the first experiment, different amounts of E.coli proteins were spiked into a static human background (HEK lysat) over 10 replicates. In the second experiment, different amounts of 400 synthetic peptides were spiked into a static E.coli background over 45 replicates. Both datasets were measured label-free and TMT-labeled. Analysis was carried out using MaxQuant followed by Ttest statistics, LIMMA and linear mixed models.

Results

The identification of fragment spectra allows for the reliable differentiation between E.coli and HEK proteins and therefore can be used to control the quantitation accuracy of all proteins. Following quantitation, we performed linear regression and controlled the separation of slopes between E.coli proteins and HEK proteins. As expected [2], TMT datasets showed ratio compression, yet were still able to separate slopes at deeper proteome coverage as compared to label-free quantitation. In the next analysis step, the samples were grouped and compared using tests for statistically differentially expressed proteins. Receiver operator curves were created using the information of spiked-in concentrations to evaluate the performance of different analysis workflows and labelling techniques.

Conclusions

A major pitfall in optimizing statistical tools using these type of data is the potential overfitting of parameters to a given arbitrary spike-in experiment. While conventional analysis pipelines have been shown to perform well in cell culture experiments, biomarker discovery pipelines have not yet been benchmarked with realistic ground truth datasets. In order to overcome this limitation, we will create a dataset with realistic patient to patient variability while also providing information on ground truth.

Novel Aspect

We use pilot studies to create a dataset with realistic patient heterogeneity to benchmark real biomarker discovery workflows and choice of statistical tools.

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Keywords

Biomarker Discovery, Benchmark

Authors

Froehlich Klemens [1] [2], Kook Lucas [3] [4], Bronsert Peter [2], Timme-Bronsert Sylvia [2], Schilling Oliver [2]

1. Spemann Graduate School of Biology and Medicine (SGBM), Albert-Ludwigs-University Freiburg (Allemagne)
2. Institute of Surgical Pathology, Medical Center – University of Freiburg (Allemagne)
3. Epidemiology, Biostatistics & Prevention Institute, University of Zürich (Suisse)
4. Functional Genomics Center, University of Zürich, ETH Zürich (Suisse)

High throughput urinary free cortisol determination by solid phase extraction on line with liquid chromatography and tandem mass spectrometry [P145]

Introduction

The concentration of urinary free cortisol, which is representative of that of plasma free cortisol, is the best criterion for the diagnosis of hypercortisolism. We present here the development and validation of a method for the determination of urinary free cortisol, by HPLC coupled downstream to tandem mass spectrometry and upstream to an on-line solid liquid extraction. This method makes up for the lack of specificity of the immunoassays usually used and answers to the high rate of requests for determining this parameter at the level of a hormonology laboratory.

Methods

The previously centrifuged urine is mixed with the internal standard solution (heavy isotope labeled cortisol) before dilution 10 times in water. The mixture is then injected directly onto a Turboflow column (C2) and then analyzed in reversed phase chromatography using a silica based C8 stationary phase and a gradient of methanol in water. The specific detection of cortisol and its internal standard is achieved by tandem mass spectrometry.

Results

The elimination of interfering molecules, tested with patient urines, is ensured by the nature of turboflow phase, liquids of loading, washing and elution (extraction recovery of 90%), by the choice of the stationary phase in the analytical column, the composition of the mobile phase (H_2O/methanol) and the slope of the elution gradient (10.6 %/min) as well as the choice of transitions during the detection by the mass spectrometer. The linearity of the method is studied from 28 to 790 nmol/L ($y=0.62x+1.15.10^{-2}$, $r^2=0.9993$). The repeatability, on three pools (n = 35) of urine of patients at 48.0, 302.9 and 629.8 nmol/L (average values) has CVs between 3.3 % and 4.1%, and intermediate fidelity, on three quality controls (n = 5) at 70.6, 167.0 and 487.0 nmol/L, CVs from 1.1 to 8.9% and bias from 0.6% to 15%. This method is correlated with that used previously in the laboratory (liquid/liquid extraction followed by immunoanalysis (XL Liaison DiasorinR) (y=0.8076x-59.04, r=0.92, n=40). There is also no interference with steroids commonly administered to patients.

Conclusions

The presented method is fast and selective. Sample preparation is easy since it consists of a single dilution, which can be automated. The analysis time, from solid liquid extraction to MS tandem detection, is 6 minutes. The method is validated analytically in terms of linearity, fidelity, precision and correlation with the method previously existing at the laboratory.

Novel Aspect

Our method uses a rapid on-line solid liquid extraction, which is not the case with methods described in literature, with similar chromatographic separation time [1,2,3].

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Keywords

High throughput, urinary free cortisol, tandem MS

Authors

Menet Marie-Claude [1], Bonnet Fideline, Gaillard Catherine, Zientek Corinne, Mosnier Pudar Helen, Guibourdenche Jean

1. Laboratoire d'hormonologie (France)

A sensitive detection of synthetic IGF-I analogs using Mass Spectrometric Immunoassay and nano-ultra-high-performance liquid chromatography coupled to High Resolution Mass spectrometry [P146]

Since 1999, Insulin-like Growth factor-I (IGF-I) and its analogs have been listed as banned substances by the International Olympic Committee. The present study describes a rapid and sensitive analysis of IGF-I analogs, Long-R3-IGF-I, R3-IGF-I and Des(1-3)-IGF-I, readily available black market products on several websites and used by athletes for their growth-promoting action.

In this work, a top-down analytical method has been developed for IGF-I analogs detection for anti-doping purposes. Briefly, the sample preparation consisted of IGF-I analogs immunoaffinity purification using a specific polyclonal rabbit anti-human IGF-I antibody. After isolation, the target substances were directly analyzed by a nano-ultra-high-performance liquid chromatograph device coupled to a High-Resolution Mass spectrometer. The combination of immunopurification, nano-ultra-high-performance liquid separation and high resolution/high accuracy ensured sensitivity, specificity and selectivity. Analytical performances were studied using spiked samples in human serum: linearity (0-200 ng/mL, R2>0.99), specificity, detection limits (below 0.5 ng/mL), recovery (48-62%), accuracy (<25% at 0.5 ng/mL) and robustness.

In order to prove and validate the applicability of the developed analysis, a single intramuscular administration of each analog was carried out in rats and kinetic elimination was studied from 4h post injection to 36h post-injection. R3-IGF-I and Des(1-3)-IGF-I were detected until 16h post administration (0.2-2 ng/mL) and Long-R3-IGF-I was detected until 4h post administration (0.7-1.5 ng/mL). In addition to the intact molecule Long-R3-IGF-I, three N-terminally truncated degradation products were observed: Des(1)-Long-R3-IGF-I, Des(1-10)-Long-R3-IGF-I and Des(1-11)-Long-R3-IGF-I. Des(1-11)-Long-R3-IGF-I was detected until 16h after administration while the other two were detected only until 4h after administration. No metabolites for Des(1-3)-IGF-I and R3-IGF-I were identified. This administration study clearly demonstrated the applicability of this developed method for anti-doping purposes.

Keywords

IGF-I analogs, nano-UHPLC, HRMS

Authors

Mongongu Cynthia [1], Buisson Corinne [1], Coudoré François [2], Audran Michel [1], Marchand Alexandre [1]

1. AFLD, Département des analyses, 92290 Châtenay-Malabry (France)
2. Laboratoire de Neuropharmacologie, INSERM UMRS 1178, 92290 Châtenay-Malabry (France)

Implantable cardiac defibrillator shock-induced pathophysiological changes : A proteomic approach on a sheep model [P147]

Introduction

Implantable cardiac defibrillators (ICD) are meant to fight life-threatening ventricular arrhythmia and are associated with a reduction of mortality in humans[1]. However, shocks are independently associated with an increased mortality [2] but very few pathophysiological pathways are described [3–5]. For a better understanding of this toxicity, after implantation of ICD, we performed global quantitative proteomics experiments based on two approaches: a direct tissue profiling by MALDI-TOF MS combined to Top-down (TD) MS and bottom-up analyses.

Methods

ICD were implanted in sheep. Animals were distributed in two groups of five each: « electrical shock group » where five maximal energy shocks were delivered and «control group» where the right ventricular lead was placed at the apex without any delivered therapy or induced arrhythmia. Myocardial tissue was collected at the right-ventricle (RV) apex near to the tip of the lead (subgroup « near », N=5 in each group) and at the RV basal region (subgroup « far », N=5 in each group).

Results

Molecular profiles in the range 1,000–30,000 m/z were obtained using myocardial sections in both « near » and « far » regions of the two groups by MALDI-TOF MS. 100 mean spectra were collected for each region. 191, 203, 198 and 215 m/z were detected for control «near» and «far», electrical shock «near» and «far» groups respectively. A Principal Component Analysis was able to clearly discriminate the two groups in both regions. Differential analysis characterized 20 and 27 peaks between control and electrical shock, in the «near» or in the «far» region, respectively. Identification by TD MS is under progress on a representative sample from the electrical shock «near» group.

Preliminary bottom-up MS analyses by GeLC-MS/MS (without SDS-PAGE fractionation) showed evidence of cell lysis, down-regulation of proteins involved in energy metabolism, detoxification of reactive oxygen species, calcium regulation and sarcomere constituant. A more exhaustive study of each group after SDS-PAGE fractionation is under progression.

Conclusions

Nowadays, all proteomic approaches (tissue profiling and bottom-up) revealed modifications of myocardial proteome after ICD electrical shocks. This myocardial injury seems to be multifactorial and to involve common pathways described in heart failure. Further analyses are in progress to support these preliminary results using TD and bottom-up MS for identification and quantification.

Novel Aspect

This is the first global approach to understand toxicity of ICD-electrical shocks.

It provides a toxicity profile which will permit to compare novel defibrillation methods.

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Keywords

Implantable cardiac defibrillator; electrical shock ; proteomics

Authors

Labas Valérie [1] [2], Bodin Alexandre [3], Teixeira-Gomes Ana-Paula [1] [4], Tomas Daniel [1] [2], Combes-Soia Lucie [1] [2], Gomot Gilles [1] [2], Elleboudt Frederic [1] [2], Moussu Christian [1] [2], Lecompte François [1] [2], Bisson Arnaud [3], Babuty Dominique [3], Fauchier Laurent [3], Clementy Nicolas [3]

1. Plate-forme CIRE (Chirurgie-Imagerie-Recherche-Enseignement)-Pôle d'Analyse et d'Imagerie des Biomolecules (PAIB) (France)
2. Physiologie de la Reproduction et des Comportements [Nouzilly] (France)
3. Service de Cardiologie (France)
4. Infectiologie Santé Publique (France)

Towards a better understanding of the mechanisms of action of platinum-based drugs for the improvement of peritoneal carcinomatosis treatment protocols: study of the in vivo interactions of oxaliplatin with proteins [P148]

Introduction

Peritoneal carcinomatosis are common evolution of tumours from the gastrointestinal or gynaecological organs spread throughout the abdominal cavity. Treatments are systemic administration of a Pt-drug followed by maximal surgical resection sometimes completed by HIPEC with oxaliplatin. Thus the Pt-drug can reach tumors *via* blood circulation and/or by direct contact with tissues, leading us to suppose different mechanisms of action of Pt. Even so the clinical efficiency of this treatment is observed, biochemical interactions of Pt-drug with proteins are poorly understood.

Methods

Among mass spectrometry techniques, ICP MS can be used notably for total metal content determination and metal speciation analysis in complex matrices. First total analysis of Pt over time after treatment is carried out by direct infusion of samples (plasma, blood, …) into ICP MS. Then, non-denaturating gel electrophoresis followed by Laser Ablation coupled to ICP MS allows the study of *in vivo* Pt interaction with plasmatic proteins of treated patients.

Results

The total analysis by ICP MS of Pt in biological fluids (blood, plasma, …) shows that the maximal concentration was found at day 1 and tend to slowly decrease until day 5. The same trend was observed for 11 patients. « Free » Pt or bound to small proteins (< 30 k Da) can be detect 30 min after the treatment and then Pt is bound to proteins. LA-ICP MS on Gel Electrophoresis allows the detection of possible Pt transporters in human plasma. For example, Pt binds proteins at ca 130-230 kDa, 95 kDa and between 60 and 40 kDa. The kinetic of the binding of Pt with proteins is studied until day 5 after treatment. Although some Pt-protein peaks are common to several patients, different Pt-protein patterns are observed between patients.

Conclusions

LA-ICP MS on Gel Electrophoresis will allow a better identification of possible platinum transporters. Work on LC-ICP MS and LC-ESI MS/MS is planned for proteins identification.

Novel Aspect

Our work presents the study of *in vivo* interaction of oxaliplatin with proteins, and notably proteins of much lower abundance.

Keywords

Oxaliplatin, protein, ICP MS

Authors

Larroque Marion [1], Arnaudguilhem Carine [2], Sgarbura Olivia [3], Quenet François [3], Carrère Sebastien [3], Bouyssiere Brice [2], Larroque Christian [4], Mounicou Sandra [2]

1. ICM/ Unité de Recherche Translationnelle, Campus Val d'Aurelle (France)
2. CNRS/ UNIV PAU & PAYS ADOUR, INSTITUT DES SCIENCES ANALYTIQUES ET DE PHYSICO-CHIMIE POUR L'ENVIRONNEMENT ET LES MATERIAUX, UMR 5254, 64000, PAU, France (France)
3. ICM/ Service Chirurgie A2, Campus Val d'Aurelle (France)
4. IRCM/INSERM U896, ICM Val d'Aurelle, 208 rue des Apothicaires, 34296 Montpellier, France (France)

Proteomics evidence of specific IGKV1-8 association with cystic lung light chain deposition disease [P149]

Introduction

We previously reported a new form of LCDD presenting as diffuse cystic lung disorder that differs from the usual systemic form, with respect to the age, the male/female ratio, the involved organs, and the hematologic characteristics. We also demonstrated that the light chains were produced by an intrapulmonary B-cell clone and, that this clone shared a stereotyped antigen receptor IGHV4-34/IGKV1. However, we analyzed only 3 patients. Herein, we conducted a retrospective study including lung tissue samples from 24 patients with pulmonary LCDD (pLCDD) matched with samples from 13 patients with pulmonary AL kappa amyloidosis used as controls. Mass spectrometry-based proteomics identified immunoglobulin kappa peptides as the main protein component of the tissue deposits in all patients. Interestingly, in pLCDD, IGKV1 was the most common kappa family detected (86.4%) and, IGKV1-8 was overrepresented compared with pulmonary AL kappa amyloidosis (75% vs 11.1%, p=0.0033). Furthermore, IGKV1-8 was predominantly associated with a diffuse cystic pattern (94%) in pLCDD. In conclusion, high frequency of IGKV1-8 usage in cystic pLCDD constitutes an additional feature arguing for a specific entity distinct from the systemic form that uses preferentially IGKV4-1.

Methods

Patient population and controls : we conducted a retrospective study including lung samples from 24 patients with localized pLCDD identified from 2004 to 2018, retrieved from our collection. The samples from 13 patients with pulmonary AL kappa amyloidosis (pAL amyloidosis) identified during the same period served as controls (12 localized forms and 1 systemic form). The diagnosis of LCDD and AL amyloidosis was established on biopsy specimens or explanted lungs using Congo red, immunofluorescence, electron microscopy and/or MS-based proteomics. Based on computed tomography, the patients were categorized into diffuse cystic disorder, solitary nodule, bronchial and bronchiolar involvement, and diffuse interstitial disease without cysts. Patients' consents were obtained according to our Institutional Review Board.

Mass spectrometry-based proteomic analysis: a 10 µm-thick section of formalin-fixed paraffin-embedded tissue was mounted on slides and stained with hematoxylin-eosin. Hundred thousand µm2 of deposits were selected by laser microdissection. Proteins were extracted from the collected material in ammonium bicarbonate buffer, reduced with dithiothreitol, and alkylated with iodoacetamide. Then, proteins were digested into peptides with trypsin and analyzed by nanoscale liquid chromatography (nanoLC) coupled to tandem MS (MS/MS) using an Ultimate 3000 RSLCnano system coupled to an LTQ-Orbitrap Velos mass spectrometer. Data were processed with Mascot against human entries of the SwissProt protein database augmented with known protein sequences from human IGVL genes obtained from ImMunoGeneTics database. Validation of results was performed through a false-discovery rate set to 1% at protein and peptide-sequence match levels determined by target-decoy search using the in-house-developed Proline software (http://proline.profiproteomics.fr/). The spectral count metrics was used to rank the proteins and peptides according to their relative abundance in the sample.

Statistical analysis: differences between groups were assessed using Chi-square or Fisher's exact test for qualitative variables and Mann-Whitney U test for continuous variables. Two-sided p-values of less than 0.05 were considered statistically significant.

Results

Patients with pLCDD were younger (51y vs 67y, p=0.0001) and more likely to be female (62.5% vs 15.4%, p=0.006) than pAL amyloidosis. All pLCDD patients had deposits restricted to the lung. Cystic lung disease was the dominant presentation for pLCDD compared to pAL amyloidosis (75.0% vs 15.4%, p=0.0005). Mass spectrometry-based proteomic analysis identified immunoglobulin kappa peptides as the main protein component of the tissue deposits in each patient. The kappa peptides belonging to the constant region were found in 23 (95.8%) and 13 (100%) patients with pLCDD and pAL amyloidosis, respectively. The combination of APOE, SAP and APOA4 known as universal amyloid tissue markers was not found in pLCDD. We were able to identify peptides from an IGVL gene in 20 (83.3%) patients with pLCDD and in 8 (61.5%) patients with pAL amyloidosis. In pLCDD, IGKV1-8 was the most common IGKVL gene detected (75%), and IGKV1 was the most common kappa family

detected (86.4%). Peptides from IGKV1-8 were overrepresented in pLCDD compared with pAL amyloidosis (75% vs 11.1%, p=0.0033).

Furthermore, it was predominantly associated with a diffuse cystic lung pattern (94%) while it was not found in the 4 pLCDD patients with isolated nodules. In pAL amyloidosis, IGKV1-8 was observed in one patient with diffuse cystic lung disease. The present study confirms that mass spectrometry based-proteomics is an accurate method for the molecular characterization of LC deposits in pLCDD. It further highlights that IGKV1-8, a gene rarely used in the normal B-cell repertoire, is significantly associated with pLCDD in its cystic form.

Conclusions

There are few data in the literature about IGVL gene and light chain family used in LCDD. In the systemic form of LCDD, sequencing of pathogenic light chain in two series indicated an overrepresentation of the IGKV4 family and IGKV4-1 subgroup. IGVK1-8 was not identified. The potential role of the variable region in tissue deposition was documented by showing that amino acid changes in this region is sufficient to promote deposition in the kidney, liver, spleen and heart of mice expressing a human LCDD IGKV4 chain. All these findings added to our results highly suggest the contribution of the light chain variable domain in organ tropism in LCDD. In the same way, several studies based on PCR sequencing of the bone marrow plasma cells support that organ involvement in AL amyloidosis may be partially related to the immunoglobulin light chain repertoire of the clone. Recently, these results were strongly consolidated with the investigation of IGVL gene usage by LC-MS/MS among 821 AL amyloidosis. Interestingly, in this large cohort, IGVL was reported in 14 localized pAL kappa amyloidosis. IGKV3-20 was the most common IGVL identified (35%) and none of them matched with IGKV1-811. Beyond the role of immunoglobulin gene usage in organ tropism, the preferential usage of IGKV1-8 in cystic pLCDD strongly upholds the belief of an antigen-driven process already suggested by the previous identification of a stereotyped IGHV4-34/IGKV1 receptor expressed by the pulmonary B-cell clone. Finally, our study sheds light on the link between IGKV1-8 and diffuse pulmonary cystic disorder, especially LCDD raising the question of the IGVL gene

implication in the development of this particular pattern. Limitations of our study include the sensitivity of mass spectrometry to detect IGVL gene products. There is still room to do better by improvement of sequence template libraries and peptide identification algorithms. In conclusion, we provide evidence that IGKV1-8 is highly associated with cystic pLCDD. This represents an additional feature arguing for a specific entity distinct from the usual systemic form.

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Keywords

LCDD (lung light chain deposition), proteomics

Authors

Camus Mylène [1], Hirschi Sandrine, Mal Hervé, Prevot Gregoire, Chenard Marie-Pierre, Stern Marc, Reynaud-Gaubert Martine, Gilhodes Julia, Burlet-Schiltz Odile, Brousset Pierre, Colombat Magali

1. Institut de pharmacologie et de biologie structurale (France)

A combined laser microdissection and proteomic analysis method to explore tumoral heterogeneity. [P150]

Introduction

Hepatocellular adenomas (HCA) are rare benign tumors, usually developing in women using oral contraception. We focused on beta-catenin-mutated HCA (b-HCA) in which different types of b-catenin mutations have been identified associated with variable risk of malignant transformation. Glutamine Synthetase (GS) is as a biomarker of the different levels of b-catenin pathway activation. By immunohistochemistry, a very frequent GS positive rim in all type of b-HCA indicate a protein expression heterogeneity into tumors that we investigated by proteomic analysis.

Methods

The Oncoprot platform (http://www.tbmcore.u-bordeaux.fr/oncoprot/) developed a method combining laser microdissection and mass spectrometry analysis to compare the proteomic profiles of tumors [1,2]. This procedure has been optimized for the study of FFPE tissue sections even on small material (1mm² over 5µm thick).

Results

In inflammatory b-HCA, the GS and C-reactive protein (CRP) delimit the same area, which shows that this GS rim belongs to the tumor. Thanks to laser microdissection, we isolated and compared in parallel beta catenin mutation and proteomic profiles of the center of the tumor (T) and the GS positive rim of 5 cases of each type b-HCA (exon 3, exon S45, exon 7/8) also with the corresponding non-tumoral (NT) liver. By Sanger sequencing we found the same mutation profiles in the rim and center of the tumor. As expected, proteomic profiles showed a high T/NT expression ratio of GS in b-AHC ex3 non-S45 and low in b-AHC ex3S45 and b-HCA ex7/8 with an increase in the GS rim/center ratio in the latter two. Among the proteins whose expression is different between the rim and the center, the expression of CYP1A2 protein has been systematically positively regulated in the border, particularly in b-HCA ex3S45 and ex7/8 b-HCA, as confirmed by immunohistochemistry analysis with a label exactly superposed on that of GS.

Conclusions

We showed an intratumoral heterogeneity of HCA with specific pathways deregulated in this GS positive rim while the mutation of beta catenin was the same. We thus confirm the powerful capacities of our method combining laser microdissection and mass spectrometry to explore the tumoral proteomes.

Novel Aspect

The selection of the tissue area of interest by laser microdissection makes it the ideal tool for in-depth exploration of tumor proteomic heterogeneity.

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Keywords

Tumoral heterogeneity proteomics

Authors

Di-Tommaso Sylvaine [1] [2], Cappellen David [1], Dupuy Jean-William [3], Dugot-Senant Nathalie [4], Bioulac-Sage Paulette [1], Balabaud Charles [1], Saltel Frédéric [1] [2], Raymond Anne-Aurélie [1] [2]

1. BaRITOn INSERM1053, Bordeaux (France)
2. Plateforme Oncoprot, TBM-Core US005 (France)

3. Plateforme Protéome - Centre Génomique Fonctionnelle Bordeaux, Université de Bordeaux (France)
4. Plateforme d'histopathologie, TBM-Core US 005, Bordeaux (France)

Development and validation of an HPLC-MS/MS method in depleted plasma for the quantification of bile acids [P151]

Introduction

Bile acids are the main constituents of bile. They play a key role in the digestion of lipids, but also affect various physiological and pathological functions [1]. They have been identified as ligands of many signaling pathways modulating the expression of key metabolic genes and many physiological mechanisms via the activation of specific receptors such as the Farnesoid X Receptor (FXR), a nuclear receptor, and a membrane receptor coupled to a G protein, TGR5 [2]. Due to their diversity and their varied roles, it is interesting to be able to determine bile acids profiles in plasma. Thus, it is possible to appreciate the variation of these profiles in different metabolic and physiological contexts (diabetes, obesity, pregnancy ...) by individually quantifying bile acids. A HPLC-MS/MS method was developed to quantify 27 bile acids. This method was validated using depleted plasma.

Methods

Plasma was depleted of bile acids with NORIT activated charcoal and was used as a biological matrix for the preparation of validation and calibration standards [3]. Plasma proteins from the samples were precipitated with cold methanol/acetonitrile 50/50 (v/v) (4°C). After centrifugation, the supernatant was removed and evaporated under a stream of nitrogen until a dry residue is obtained. This is used by the optimized mobile phase for the chromatographic separation of 27 bile acids and their 5 internal standards (CA-d4, GCA-d4, TCA-d4, CDCA-d4, GCDCA-d4). The selective elution of the compounds is carried out using a C18 column, Luna 5 µm; 3 x 250 mm, using an elution gradient with a mobile phase composed of a 20 mM ammonium acetate buffer, pH = 8 and a mixture of acetonitrile/methanol 70/30 (v/v) and a flow rate of 0.5 mL/min [4].

Results

Plasma samples preparation was optimized and extraction recoveries were higher than 94%. The MRM transitions of each bile acid were optimized and coupling to the chromatographic system allowed separation of the 27 bile acids and their internal standards in 41 minutes. Thanks to the bile acid plasma depletion procedure, the analytical method was validated in terms of selectivity, accuracy, response functions and linearity. Detection limits were below 1 nM and quantification limits were lower than 15 nM. An accuracy profile was drawn for each bile acid.

Conclusion

A method for plasma assay of bile acids was developed and validated by HPLC-MS/MS. The analytical procedure proves to be robust and sensitive in order to determine the bile acids profiles in plasma. Accuracy profiles were drawn for each analyte.

Novel Aspect

This study described a validated method for the quantification of 27 bile acids in depleted plasma.

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Keywords

Bile acids, plasma, activated charcoal

Authors

Descat Amandine [1], Kouach Mostafa [1], Sauty Laura [1], Hecquet Paul-Emile [2], Vallez Emmanuelle [2], Muhr-Tailleux Anne [2], Goossens Jean-François [1]

1. Université de Lille (France)
2. Université de Lille-INSERM (France)

Investigation of crucial sample preparation steps in the proteomics workflow [P152]

Introduction

Sample preparation is a crucial step for quantitative proteomics analysis to ensure repeatability and robustness of the results. There is no universal sample preparation method and every single sample would certainly benefit from a dedicated protocol optimisation. However, a few unavoidable steps and questions remain, especially when dealing with non serum-free cell cultures, regarding efficient protein extraction and contamination. In this context, we have evaluated the extent of contamination of bovine serum proteins in human cell cultures and have tested different protein extraction buffers.

Methods

Increased concentrations of bovine serum were applied on B lymphocytes cell cultures (from 1 to 20 %), using at least three culture replicates per condition. Cells were washed 5 times with cold PBS before pelleting. RIPA and Laemmli buffer were used to evaluate yield, efficiency, repeatability and coverage of protein extraction. All samples were prepared using stacking gels before nanoLC-MS/MS analysis. The latter were performed on a NanoAcquity TripleTOF 5600 coupling (Waters, Sciex) and on a nanoAcquity Q Exactive + coupling (Waters, Thermo Fisher Scientific) using 105 minutes gradients. Data were interpreted using Proline, Maxquant and Prostar softwares.

Results

Overall, identification and quantification results demonstrated that with five PBS washes, serum proteins can be efficiently removed and are very low abundant in the samples. With 1% serum, bovine proteins contamination was estimated at 2.5 %, while it was estimated at 3.3 % for cultures with 20% serum.

Total amounts of bovine proteins were approximated using extracted ion chromatogram intensities of bovine proteins and fractions of total signals were calculated. With 1% of serum, bovine proteins contamination was estimated at 8 %, while it was estimated at 11 % for cultures with 20% serum.

The total numbers of validated proteins follow the same trend: 1730 proteins were validated with 1% serum and 1600 were validated with 20% serum with a good repeatability for each condition. In conclusion, no major difference is observed when working with high or low amount of bovine serum and our results confirm the proteomics workflow compatibility of serum-fed cell cultures when efficient washing steps are undergone.

RIPA and Laemmli buffers showed similar results regarding the number of validated peptides and proteins as well as the localization of identified proteins in lymphocytes B cells. However, the extraction yield is much higher with Laemmli type buffer when compared to RIPA buffer. The protein overlapping between both buffers is higher than 80%. The quantitative differential analysis with Prostar reveals that over the total of 2443 quantified proteins only 91 were significantly differentially expressed, representing a minor percentage of less than 4%.

Conclusions

In conclusion, our results demonstrated the compatibility of using bovine serum in cell cultures even in high amount (up to 20%) provided sufficient and efficient washes are performed. We have also demonstrated that Laemmli extraction remains a very good way to extract highly complex protein mixtures for further proteomics analysis.

Novel Aspect

This work investigates two key aspects in the proteomics sample preparation workflow, namely the compatibility of non serum-free cell cultures and protein extraction efficiency using various extraction buffers.

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Keywords

Sample preparation – protein extraction – cell culture media

Authors

Hirschler Aurélie [1], Pythoud Nicolas [1], Tranchant Thibaud [2], Delalande François [1], Carapito Raphaël [3], Carapito Christine [1]

1. Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO), Institut Pluridisciplinaire Hubert Curien (IPHC), UMR7178, Strasbourg. (France)
2. Laboratoire d'ImmunoRhumatologie Moléculaire, INSERM UMR_S1109, Centre de Recherche d'Immunologie et d'Hématologie, Faculté de Médecine, Fédération de Médecine Translationnelle de Strasbourg (FMTS), Strasbourg. (France)
3. Laboratoire d'ImmunoRhumatologie Moléculaire, INSERM UMR_S1109, Centre de Recherche d'Immunologie et d'Hématologie, Faculté de Médecine, Fédération de Médecine Translationnelle de Strasbourg (FMTS), Strasbourg. (France)

Application of capillary electrophoresis hyphenated to mass spectrometry for the determination of acetaminophen and its main metabolites in urine of patients included in a clinical trial [P153]

Introduction

After major abdominal liver surgery, acetaminophen is used to treat post-operative pain. Unfortunately, as hepatic function can be altered by reduction of hepatocyte volume and oxydative stress [1], it could induce an overdose.

Hence, a clinical trial has been initiated in order to assess the metabolism of acetaminophen administered after hepatic resection.

The aim of this work was to develop and validate a rapid and sensitive CE-ESI/MS-MS method for quantitation of acetaminophen and its metabolites in urine samples of patients included in the clinical trial.

Methods

Firstly, optimization of the method, *i.e.* MS detection conditions (sheath liquid composition; MS parameters) and CE separation conditions (BGE pH and concentration; sample preparation method) has been performed. Secondly, the method has been validated according to the validation guidelines of SFSTP [2-4]. Accuracy profiles were assessed using NeoLiCy® software.

Results

Optimized method lies on a detection of analytes in positive ESI mode using a methanol-ammonium hydroxyde (0.1%) (50:50, v/v) mixture as sheath liquid and a 9 min separation after anodic injection, using an ammonium acetate solution (40 mM, pH 10) as BGE. It was shown that the dilution solvent and the dilution factor to use for sample preparation are critical parameters to avoid peak splitting, to gain in sensitivity and then to obtain an effective analysis method. Hence, two successive analyses using two different dilution factors have to be carried out in order to quantify all metabolites. The method has been validated using the accuracy profile approach with a total error (accuracy) included in the ± 20 % range. Thereby, the method allows the quantitation of acetaminophen and acetaminophen mercapturate in the range (0.1 – 1 mg/L), and of acetaminophen sulfate, methoxy-acetaminophen, acetaminophen glutathione and 3-methylthio-acetaminophen in the ranges (0.5 – 5 mg/L), (0.025 – 0.4 mg/L), (9.22 – 30 mg/L) and (0.073 – 0.4 mg/L), respectively.

Conclusions

A CE-MS/MS method was developed and validated for the quantitation of acetaminophen and its metabolites in urine samples. It was successfully applied to the analysis of urines of the first patients included in the clinical trial. The differences in concentrations observed according to the membership of the different inclusion groups, show that the method will be sufficiently discriminating to obtain relevant information on metabolism after liver resection.

Novel Aspect

First CE-ESI/MS-MS method validated to quantify acetaminophen and five metabolites in urine. Use of CE *versus* HPLC permits to quantify two additional metabolites.

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Keywords

Capillary electrophoresis-mass spectrometry, Acetaminophen metabolites, Urinary samples

Authors

Lecoeur Marie [1], Descat Amandine [1], Lebuffe Gilles [2] [1], Foulon Catherine [1]

1. Univ. Lille (France)
2. CHU Lille (France)

Stable Isotope-Labelled Morphine to Study in vivo Central and Peripheral Morphine Glucuronidation and Brain Transport in Tolerant Mice [P154]

Chronic treatments can have an important impact on metabolic enzymes leading to physiological adaptations. Morphine metabolism in the liver has been extensively studied but morphine metabolic processes in the central nervous system are poorly characterized. Long-term morphine treatment is limited by the development of tolerance, resulting in a decrease of its analgesic effect. Whether or not morphine analgesic tolerance affects *in vivo* brain morphine metabolism and blood-brain barrier (BBB) permeability remains a pending question. Our aim was to characterize the *in vivo* metabolism and BBB permeability of morphine after long-term treatment at both central and peripheral levels. Mice were injected with morphine or saline solution for 8 consecutive days to induce morphine analgesic tolerance. On the ninth day, both groups received a final injection of d3-morphine (morphine bearing three ^{2}H). LC-MS/MS was used to quantify morphine, its metabolite morphine-3-glucuronide (M3G) and their respective d3-labelled counterparts in blood, urine, brain and liver samples. We found no significant differences in morphine CNS uptake and metabolism between control and tolerant mice. This suggests that morphine analgesic tolerance is not linked to an increase of morphine glucuronidation into M3G or an alteration of BBB permeability.

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Authors

Ivan Weinsanto [1], Alexis Laux-Biehlmann [1], Jinane Mouheiche [1], François Delalande [2], Virginie Chavant [1] [3], Florian Gabel [1], Alain Van Dorsselaer [2], Sarah Cianferani [2], Yannick Goumon [1] [3]

1. CNRS UPR3212, Institut des Neurosciences Cellulaires et Intégratives, Centre National de la Recherche Scientifique and University of Strasbourg, Strasbourg, France
2. CNRS UMR7178, Laboratoire de Spectrométrie de Masse BioOrganique, IPHC-DSA, Centre National de la Recherche Scientifique and University of Strasbourg, Strasbourg, France
3. Mass Spectrometry Platform, CNRS UPR3212, Institut des Neurosciences Cellulaires et Intégratives, Centre National de la Recherche Scientifique, Strasbourg, France

Structural Biology and Biophysics

Structural Mass Spectrometry: A Versatile and Orthogonal Tool for Drug Discovery [P155]

Introduction

In the past decade, Mass Spectrometry has become one of the most powerful and valuable tools both as a stand-alone and as a complementary technique to other biophysical technologies in drug discovery.

Methods and Results

From protein production QC to structural studies, MS and Native MS in particular are used routinely at NovAliX as an orthogonal and problem solving tool. In combination with other techniques or as a support, it provides key insight into target properties and accelerates our research programs.

Native MS is an information rich technique which only requires minimal sample quantity and allows screening and characterization of protein-ligand and protein-protein interactions measurements in gas phase.

Conclusions

Our recent examples on hot targets clearly demonstrate an orchestration of binding and competition studies led by Native MS in synergy with HDX-MS, SPR binding study and crystallography to validate orthosteric/allosteric site.

Keywords

Native MS, Structural MS, biopharma

Authors

Debaene Francois [1]

1. NovAliX (France)

Tau fibrils interact with Na+/K+-ATPase and AMPA receptors at the cell membrane of neurons: from interactomics and cross-linking to functional validation [P156]

Introduction

In neurodegenerative tauopathies including Alzheimer's disease (AD), the microtubule-associated protein Tau aggregates into fibrillary tau assemblies and accumulates into intracellular deposits. These Tau fibrils propagate from one neuron to another, seed and amplify the aggregation of endogenous Tau [1,2]. In this prion-like propagation process, molecular interactions of exogenous Tau fibrils with the plasma membrane of recipient neurons plays a key role and are not well known.

Methods

Using an optimized proteomic-based interactomic approach, we identified proteins interacting with extracellularly applied Tau fibrils, at the cell surface of neurons. Pull-downs were performed using neurons exposed to biotin-labeled recombinant Tau fibrils, without and with cross-linking, and streptavidin beads. Fibrillar Tau interactors were identified using LC-MS/MS and label-free relative quantification between non exposed neurons and neurons exposed to Tau fibrils.

Results

We have reported that fibrillar Tau forms clusters at the cell membrane following lateral diffusion. With our proteomic screen we have analyzed the synaptic and plasma membrane interactome of Tau fibrils. Among the proteins with extracellular domains we have identified, are the α3-NA+/K+-ATPase (NKA) and the key AMPA and NMDA neurotransmitter receptors. Interaction with these key interactors of Tau fibrils was validated by immunoprecipitation and cross-linking. We have further observed that the consequence of the clustering of Tau at the cell membrane is a reduction in the amount of α3-NKA and an increase in the amount of GluA2-AMPA receptor at synapses. Finally, we have shown evidences for cross-talk between Tau and αSynuclein fibrils, with an enhanced clustering and synaptic localization of Tau fibrils in presence of αSynuclein fibrils [3].

Conclusions

Our results suggest that Tau and αSynuclein fibrils, that often co-exist in AD patients brains, cross-talk at the plasma membrane, imbalance neuronal homeostasis and potentiate deleterious processes involved in AD onset.

Novel Aspect

Biotinylation of insoluble protein complexes combined to cross-linking at the cell membrane highlighted protein interactions, further validated by functional studies.

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Keywords

Interactomic, Alzheimer, Membrane Protein Receptors

Authors

Redeker Virginie [1], Shrivastava Amulya [1], Pieri Laura [1], Bousset Luc [1], Triller Antoine [2], Melki Ronald [1]

1. Institut de Biologie François Jacob, Laboratoire des Maladies Neurodégénératives, UMR9199 (France)
2. Institut de Biologie de l'ENS, École Normale Supérieure (France)

Oxidative peptide dimerization studied by liquid chromatography, mass spectrometry, ion mobility [P157]

Introduction

Within the cell, proteins can be subjected to a number of reactive oxygen species (ROS), which can lead to modifications of the proteins. Structural characterization of the oxidative modification of proteins can be carried at various levels (bottom-up, top-down proteomics). Covalent binding between proteins was described as a specific modification of some proteins, such as the human Centrin-2 (Cen-2) proteins, which bears a C-ter tyrosine residue. We show based on short features of the Cen-2 systems that, contrary to previous knowledge, multiple dimerization structures coexist.

Methods

Short elements of the Cen-2 protein (tyrosine, tyrosine-d2, P5 – 5 residue C-terminal peptide, P12 – 12 residue C-terminal peptide) were subjected to gamma ray radiolysis at various doses in a 60Co irradiator, in condition (N2O bubbling, NaN3) that lead to the controlled formation of the N3• radical. Reaction products were analyzed by LC/MS, LC/IMS/MS, LC/MS/IMS/MS (Waters Synapt G2Si instrument). Deuterium labelling experiments were performed in solution.

Results

From radiolysis experiments using the OH• radical on a short, 12 amino acid peptide of the C-terminal extremity of Cen-2, with UHPLC/IMS/MS separation, more than 200 separate reaction products were observed. Among these, multiple forms of covalent dimers were observed, separated in LC/IMS. To simplify the system, the N3• radical was used, as it is known to react mostly with tyrosine. These experiments confirmed the formation of multiple forms of covalent dimers, bridged by the tyrosine group, for P12, P5 as well as for tyrosine. This is in strong contrast with previous literature which had indicated only one major dimerization reaction product observed for tyrosine and tyrosine containing peptides. The dimer composition evolves with time. CCS values have been measured for each of the dimer species. To better understand the structure and mechanisms of formation, d2-tyrosine was used, as well as radiolysis in D2O, and transfer to D2O conservation buffer, to probe the labile hydrogen displacements along the reaction pathway.

Conclusions

A new class of covalent dimers formed under oxidative stress conditions have been identified using LC/IMS separation on tyrosine, and short C-terminal peptides of the Cen-2 protein. This questions the current literature on oxidative protein dimerization and suggests looking more precisely at the existence of such dimeric structures *in vivo* as they could play several roles in the oxidative damage on proteins.

Novel Aspect

LC/IMS/MS used to separate, characterize and probe the mechanism of oxidative dimerization of peptides and proteins.

Authors

Billault Isabelle [1], Gatin Anouchka [1], Sicard-Roselli Cécile [1], Van Der Rest Guillaume [1]

1. Laboratoire de Chimie Physique (France)

Transient multimers modulate conformer intensity ratios of prion protein monomer through conformational selection. [P158]

Introduction

Only a few analytical methods, such as NMR 1 or molecular dynamic simulation allow the study of the conformational landscape of proteins. An alternative of these techniques relies on « native » electrospray ionization mass spectrometry that has become a valuable tool for the study of protein assemblies in the past years. More recently, implementation of ion mobility device on commercially mass spectrometry instruments has led to a renewed interest for this technology in the field of structural biology with the description of prion protein conformational properties.

Methods

Native mass spectrometry experiments were performed on a QToF instrument equipped with a Traveling Wave Ion Mobility (TWIM) guide. All samples were infused through the ESI source at 5 µl/min flow rate and analyzed in the positive ion mode over the 500 to 5000 m/z mass range. Reliable and accurate detection of native proteins was obtained by optimizing instrumental and biochemical parameters.

Results

From the peculiar observation that prion protein neurotoxicity could relate to alternate PrPSc isoforms whose production is concentration dependent or to the existence of several PrPSc strains we hypothesized that protein concentration can promote changes in monomer Prion protein conformational landscape apart from mechanisms occurring during the formation of neurotoxic oligomers. We demonstrate that recombinant prion protein exists in a conformational equilibrium. The conformers' intensity ratios were also shown to be dependent on protein concentration through the formation of transient multimers via conformational selection. The study of prion protein mutants that follow dedicated oligomerization pathways demonstrated that the conformers' relative intensity ratios are modified, thus reinforcing the assertion that the nature of conformers' interactions orient the oligomerization pathways. Such a result can further be viewed as the « signature » of an aborted oligomerization process.

Conclusions

This discovery sheds a new light on the possible origin of prion protein diseases, namely that a change in prion protein structure could be transmitted through the formation of transient multimers. The implication of transient multimers having different compositions in conformers could thereby explain the selection of a transient multimeric type that could be viewed as the precursor of PrPSc responsible for strain apparition.

Novel Aspect

Quantitative ion mobility allowed revealing the prion protein intensity conformer modulation by protein concentration via transient multimers formation.

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Keywords

Native mass spectrometry, Ion mobility, Quantitation, Prion conformers

Authors

Van Der Rest Guillaume, Rezaei Human, Halgand Frédéric [1] [2]

1. Laboratoire de Chimie Physique (France)
2. Laboratoire de Chimie Physique DÓrsay (France)

Tuning ETD Top-Down MS parameters on a UHR-QTOF instrument to improve protein fragmentation coverage [P159]

Introduction

Our group is engaged in the characterization of covalent protein-ligand complexes and chemically labelled proteins in the context of metastasis. Building a comprehensive picture of specific reaction *vs* side reactions is essential to the development of drugs that covalently bind their target protein. To this end, we investigate methods for the exhaustive identification and quantification of modified residues [1]. We set out to explore whether electron transfer dissociation (ETD) top-down mass spectrometry in an UHR-QTOF could provide this full characterization.

Methods

ETD Top-Down was conducted on an ESI-UHR-QTOF Maxis ETD instrument. To monitor parameter optimization, three outputs were considered: fragmentation coverage, intensity of precursor remaining and percentage of annotated fragment ions. We focused on input parameters most likely to improve sequencing capabilities: size and sequence of the protein, charge state, transmission parameters, precursor intensity, precursor/reactant ratio, and duty cycle.

Results

On Orbitrap instruments, it has been shown that multiple parameters can affect the outcome of the ETD reaction and that parameter codependence is common [2]. In designing a strategy to limit the time and amount of sample consumed, we considered 2-way matrices to find the combined optimum for two parameters. Since this type of approach limits us to 2 parameters, we decided to proceed with a experiments (DOE) approach. DOE proved useful in identifying the most relevant parameters to optimize, and could speed up the process of optimum parameter value selection. DOE however also turned out to be sensitivity to prior knowledge about the system. The results of our strategy show that, upon optimization and depending on the protein size and sequence, FC varies from 70% to 100%, meaning that a comprehensive map of modified residues cannot be reached for certain proteins using this approach only.

Conclusions

DOE approaches can be useful tool for QTOF MS instruments optimization as they feature a reasonable number of parameters to adjust. Although ETD top-down MS may not provide of a comprehensive map of modified residues of proteoforms for all proteins, there is room for further improvements. For less favorable cases, gathering enough sequencing information for localization may require the use of complementary fragmentation methods (collision-induced dissociation, photodissociation), middle-down or bottom-up approaches.

Novel Aspect

We designed a DOE-based optimization strategy to accelerate the adjustment of parameters for efficient localization of protein modifications by ETD Top-Down MS on a UHR-QTOF.

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Keywords

top-down, ETD, modification

Authors

Gabant Guillaume [1], Nemcic Matej, Pinetre Justine, Cadene Martine [1]

1. Centre de Biophysique Moléculaire (France)

What aspects of nucleic acid structures are preserved upon electrospray ionization? [P160]

Introduction

Native mass spectrometry is a promising technique for structural biology and biophysics but, if we want to infer structural information from gas-phase methods by taking advantage of the mass separation, one must know which aspects of the structure is preserved or not upon ionization. Here we address this question for various nucleic acid structures (duplexes, quadruplexes, i-motifs,…), and infer general principles that should be transferrable to proteins and protein complexes.

Methods

We used drift tube ion mobility spectrometry, cyclic traveling wave ion mobility spectrometry, infrared ion spectroscopy, circular dichroism ion spectroscopy, gas-phase structural calculations (comparing force field MD, semi-empirical and DFT calculations), CCS trajectory model calculations in helium, and vibrational frequencies calculations.

Results

DNA and RNA double helices get significantly more compact in the gas phase than they are in solution, at least at the low charge states naturally obtained in native MS conditions (100-150 mM NH4OAc) [1]. Cyclic ion mobility spectrometry shows that activation promotes compaction and peak broadening. While force field molecular dynamics suggest a significant structural distortion to account for the experimental collision cross section, higher-level DFT calculations and semi-empirical calculations suggest that compaction can occur while preserving base pairing and stacking [2]. Infrared spectroscopy confirms the formation of new (hence, non-native) phosphate-phosphate hydrogen bonds. The charge state attained by electrospray ionization greatly influences the gas-phase structures obtained, and at some critical charge state, activation causes extension instead of compaction [3]. DNA/RNA G-quadruplexes are the most resistant to gas-phase denaturation, as the G-quartet core is well maintained [4].

Conclusions

Nucleic acid secondary structures, which depend on hydrogen bonding motifs between the bases, are well preserved in the gas phase. However, tertiary structures can be masked by loop and domains rearrangements. At typical “native” charge states, compaction predominates, and this can mask some aspects of the solution 3D structures as some loops and domains wrap around the core and mask some details.

Novel Aspect

Better understanding of the rules of ion rearrangements allows us to draw a more sensible interpretation of ion mobility spectrometry results.

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Authors

Rosu Frédéric [1], Daly Steven [2], Ghosh Anirban [2], Khrostenko Nina [3], Palmer Martin [4], Berden Giel [5], Oomens Jos [5], Gabelica Valérie [3]

1. Institut Européen de Chimie et Biologie (IECB) (France)
2. Acides Nucléiques: Régulations Naturelles et Artificielles (ARNA) (France)
3. Acides Nucléiques: Régulations Naturelles et Artificielles (ARNA) (France)
4. Waters corp. (Royaume-Uni)
5. Radboud university [Nijmegen] (Pays-Bas)

Deconvolution of isotopic patterns in 2D-FTICR Mass Spectrometry of peptides and proteins [P161]

Introduction

Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR MS) allows to accurately measure the mass over charge ratio of molecular ions in the gas phase. It affords a high resolution and mass accuracy, decreasing the ambiguity of assignments, very important for MS applications. A general procedure was recently proposed to perform a pattern recognition of the classical isotopic pattern, efficiently extracting the monoisotopic mass and charge state for 1D FTICR MS [1]. The goal is to explore the possibility to extend this approach to the recently expanding 2D FT ICR MS case [2].

Methods

2D FTICR MS knowing a recent renewal with the increase of computer capacities [3] produces 2D maps datasets, stored as matrices, obtained from a sample mixture, from which fragment ions are collected after three pulse sequences. Values are stored into a structured hierarchical HDF5 file, not images. The HDF5 format allows to map and to access the data without memory load. The deconvolution program, dictionary-based, is written in python and relies on the SPIKE library.

Results

To implement the deconvolution algorithm on the whole 2D dataset at the highest possible resolution, it is necessary to perform the calculations by chunks due to the huge size of data. From the dataset, a list of chunk coordinates of fixed sized in points is generated, with size chosen to get a fast processing and to be large enough to accommodate full isotopic patterns. For each zone, the deconvolution pattern is computed with a sliding approximation, using the averagine model, to estimate a typical molecular formula compatible with the local m/z, and a fast algorithm to estimate an isotopic pattern [4]. The result of averagine model on a given mass is a set of positions and their correspondent intensities presenting the abundances of present isotopes with their positions along the mass axis [5]. Pattern recognition and monoisotopic analysis is successfully performed in a reasonable time considering the size and complexity of the dataset. 20.4 hours were needed on a desktop computer to get a result over a whole 4k x 256k real points dataset.

Conclusions

It was shown that the method previously proposed for the analysis of 1D MS spectra and accurate determination of monoisotopic values by isotopic pattern matching [1], can be extended to 2D FTICR MS experiments. It was implemented and successfully applied on a real dataset, stored in HDF5 format, within a compatible time and on a desktop computer. The code used in this work is open-source, and available on a GitHub repository.

Novel Aspect

The Primal-Dual Splitting Algorithm is used for the fast analysis of composite diagonal isotopic patterns over hierarchical very large datasets produced with 2D FTICR MS.

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Keywords

Mass Spectrometry, Isotopic Pattern, 2D FT ICR MS

Authors

Duciel Laura [1], Delsuc Marc-André [1], Cherni Afef [2]

1. Institut de Génétique et de Biologie Moléculaire et Cellulaire (France)
2. Institut de Mathématiques de Marseille (France)

Unveiling the post-translational modifications of the human 6-O-endosulfatase HSulf-2 by mass spectrometry [P162]

Introduction

The human 6-O-endosulfatase HSulf-2 catalyses the regio-selective hydrolysis of the 6-*O*-sulfate group from the glucosamine residues within sulfated domains of heparan sulfate (HS). This unique and original post-biosynthetic modification of the cell surface HS proteoglycans modulates their interaction properties in the extracellular matrix. Numerous studies point out the role of HSulf-2 in crucial physiological processes as well as in pathological conditions, particularly in cancer. However, the structural organization of Hsulf2 remains poorly understood [1].

Methods

The investigation of Hsulf-2 PTMs was mainly done by a CID/ETD approach. The CID fragmentation of HSulf-2 glycopeptides generated diagnostic ions for glycan chains, allowing the identification of their structure. Ions fragmentation of glycopeptides by ETD allowed backbone sequencing and the assignment of the glycan chain position. Moreover, we have investigated the presence of disulfide bonds by cysteine labeling and ETD analysis.

Results

Our goal is to clarify the structural organization of Hsulf-2 along with its action mechanism, in order to develop selective inhibitor. The used CID/ETD combined strategy leads to the identification of *N*-glycosylation composed of high mannose glycan structures. In addition to *N*-glycans, an unprecedented *O*-glycosylation was also discovered in HSulf-2, consisting in an anionic sulfated polysaccharide chain. The composition of this unusual carbohydrate Post Translational Modification (PTM) has been investigated by enzyme depolymerization of the polysaccharide and LC (HILIC) - ESI Mass Spectrometry (MS) analysis. Furthermore, HSulf-2 results from a maturation process, which includes a cleavage by a furin protease to yield the mature enzyme as a two-chain protein likely joined by covalent bonds. Using an ETD approach, we have been able to identify several disulfides bonds within the long chain and between Hsulf-2 chains.

Conclusions

A combination of deglycosylation and proteomic analysis had previously indicated that at least 4 of 12 potential sites were glycosylated [2]. We report here the accurate location of *N*-glycan chains along the HSulf-2 sequence and their structure by using CID and ETD. Finally, the identification of inter-chain disulfide bonds is a key step toward understanding the protein folding and the specific recognition of the sulfated polysaccharide substrate.

Novel Aspect

We report the first MS analysis of HSulf-2 aiming to unravel PTMs, which either decorate (*O*- and *N*-glycosylation) or are involved in the protein folding of this endosulfatase.

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Keywords

Glycosylation, Protein, PTM

Authors

Bilong Mélanie, Seffouh Ilham, El Masri Rana [1], Vives Romain R. [1], Gonnet Florence [2], Daniel Régis [2]

1. Institut de Biologie Structurale (France)
2. Laboratoire Analyse et Modélisation pour la Biologie et l'Environnement (France)

MS2MODELS: probing protein interaction networks by MS-based proteomics and structural data integration [P163]

Introduction

Mass spectrometry (MS) has become essential for characterizing molecular species and their interactions. Most of the time, proteomic studies stop at listing the interacting proteins, without performing the analysis of the identified sequences. This is a wasted opportunity when considering the fact that structural and evolutionary aspects provide a powerful analysis framework for biologists: e.g. for interpreting patients mutations that interfere with assemblies, setting up directed mutagenesis and functional dissection experiments, or virtual screening.

Methods

The MS2MODELS proteomics pipeline integrates structural biology to MS data, in order to enhance the analysis of the protein-protein interaction networks. The homology-based detection of relevant structures from the Protein Data Bank (PDB) [1] is carried out with HHsearch [2]. Annotations of homomultimeric complexes, as well as interaction data from BioGRID [3] and the eukaryotic linear motifs (ELM) [4] resource are also integrated into the analysis.

Results

We have used MS2MODELS on several MS datasets containing up to hundreds of proteins. Thanks to the integration of structural information, the pipeline is able (i) to identify true positives in MS data by validating interactions within the input list of proteins, and (ii) to find additional partners that are either below the MS detection threshold (false negatives) or not detected at all. Moreover, MS2MODELS indicates the potential involvement of each input protein into a homomultimeric complex. The pipeline comes with an easy-to-use web interface. Thus, the protein-protein interaction networks can be conveniently visualized in a web browser. Although MS can detect protein complexes, it cannot identify the protein residues involved in the interactions. This is why MS2MODELS offers the possibility to visualize the 3D structure of each partner within the context of its complex. The structure of the latter is either experimental or predicted, depending on its availability in the PDB or the Swiss Model Repository [5], respectively.

Conclusions

The MS2MODELS project shows the interest of integrating protein structure data to the analysis of interactomes. In this way, MS2MODELS may benefit the community of biologists working on macromolecular interactions, with important applications such as the analysis of pathological dysfunctions related to altered molecular interactions or isoforms. Further development will focus on predicting the 3D structure of multiprotein complexes.

Novel Aspect

We propose an innovative pipeline that integrates MS-based proteomics data with experimental and computational structural biology for interactome analysis.

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Keywords

Interactomics; Bioinformatics; Protein structure

Authors

Postic Guillaume [1], Marcoux Julien [2], Andreani Jessica [3], Guerois Raphael [3], Rey Julien [4], Cianférani Sarah [5], Mouton-Barbosa Emmanuelle [6], Vandenbrouck Yves [7], Labesse Gilles [8], Schiltz Odile [9], Tufféry Pierre [10]

1. INSERM UMR-S 1133, Université de Paris, RPBS, Paris 75205, France (France)
2. Institut de pharmacologie et de biologie structurale (France)
3. Laboratoire de Biologie Structurale et Radiobiologie (France)
4. INSERM UMR-S 1133, Université de Paris, RPBS, Paris 75205, France (France)
5. Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO), Institut Pluridisciplinaire Hubert Curien (IPHC), UMR7178, Strasbourg. (France)
6. Institut de pharmacologie et de biologie structurale (France)
7. Exploring the Dynamics of Proteomes (EDyP), BGE/U1038, INSERM/CEA/Université Grenoble Alpes (France)
8. CBS, Univ Montpellier, CNRS, INSERM, 34090 Montpellier, France (France)
9. CNRS UMR5089 Institut de Pharmacologie et de Biologie Structurale, 118 route de Narbonne, 31077 Toulouse, France (France)
10. INSERM UMR-S 973, Université Paris Diderot, Sorbonne Paris Cité, RPBS, Paris 75205, France (France)

A novel thioether network revealed by MS in antimicrobial RumC peptides. [P164]

Introduction

A major public health challenge today is the resurgence of microbial infections caused by multidrug-resistant strains. In the search for novel antimicrobial molecules, the human gut microbiome is an under-explored potential trove of valuable natural molecules, such as the ribosomally-synthesized and post-translationally modified peptides (RiPPs) [1].

Here, we used mass spectrometry to characterize an antimicrobial sactipeptide family from the human symbiont *Ruminococcus gnavus* [2], revealing a previously undescribed double-hairpin folding essential for activity.

Methods

MS, HCD MS/MS and finally PRM have been used in the top-down mode to analyze three types of samples: (i) Ruminococcins C1 to C5 purified from the caecal contents of rats mono-associated with *Ruminococcus gnavus* E1, (ii) an heterologously produced form of Ruminococcin C1 peptide, and (iii) specific point mutants of heterologously produced Ruminococcin C1. Heterologously produced Ruminococcin C1 peptides maturated by a specific sactisynthase were also analyzed.

Results

We first characterized the natural and active form of these sactipeptides, demonstrating that their maturation consists in the cleavage of a N-terminal leader sequence and the formation of 4 intramolecular thioether bridges involving the 4 cysteines present in their sequence.

Tandem MS analyses allowed to identify the 4 other residues involved in these linkages. Our results notably highlighted the specificities of MS2 fragmentation, using HCD mode, induced by the presence of thioether bridges in the analyzed peptides and resulting in the production of unusual ions.

The characterization of point mutants allowed to map precisely the residues involved in each thioether bridge.

Altogether, these analyses revealed a novel thioether network in these natural antimicrobial peptides, inducing a specific folding into two distinct structured domains, both containing two thioether bridges. Interestingly, this structure is essential for the antimicrobial activity of the Ruminococcin C peptides.

Conclusions

Combination of efficient purification, heterologous production and maturation of Ruminococcin sactipeptides with advanced MS characterization allowed a fine characterization of the structure-function dependency of these bacteriocins.

These natural peptides with remarkable antimicrobial activity against pathogenic Clostridia, including multidrug-resistant strains, should be of considerable interest for healthcare and biotechnology applications.

Novel aspect

The analysis of the natural and heterologous peptides by MS followed by the analysis of the point mutants sample conducted to the characterization of a novel thioether network, which creates a previously undescribed double-hairpin folding.

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Keywords

Bacteriocins, post-translational modification, structure

Authors

Kieffer-Jaquinod Sylvie [1], Chiumento Steve [2], Roblin Clarisse [3], Lafond Mickael [3], Coute Yohann [1], Duarte Victor [2]

1. CEA Grenoble IRIG/BGE/EDYP (France)
2. Université Grenoble Alpes (France)
3. Aix-Marseille Univ (France)

Structural characterization of EPAC1 by HDX-MS [P165]

Introduction

EPAC1 (Exchange Protein Activated by cAMP 1) is a transmembrane protein that activates small GTPAses by stimulating exchange of GDP by GTP [1]. EPAC1 is involved in many cell functions is proposed as a therapeutic target for many human diseases, namely cardiovascular diseases. EPAC1 is activated by 3',5-adenosine monophosphate (cAMP). As no complete 3D structure of EPAC1 exists, we used Hydrogen/Deuterium Exchange coupled to Mass Spectrometry (HDX-MS) [2] to investigate EPAC1 conformational changes upon cAMP activation in presence or absence of liposomes as membrane mimics.

Methods

HDX of EPAC1 was carried out with or without liposomes (100-fold excess) and/or cAMP (300-fold excess).

Samples were incubated in 95% deuterated buffer.

Analyses were realized with a Q-TOF Synapt G2Si HDMS (Waters) coupled to a CTC PAL robot (Leap Technologies) and an Acquity UPLC (Waters).

DynamX 3.0 (Waters) and MEMHDX were used to validated peptides.

Results

The comparison of deuterium uptake between EPAC1 and EPAC1/cAMP shows conformational modifications into DEP (Disheveled / Egl-10 / Pleckstrin), CNB (Cyclic Nucleotide Binding) and GEF (Guanidine Nucleotide Exchange) domains. Indeed, solvent accessibility decreases with cAMP in CNB domain, especially at its fixation site. A part of GEF domain is also less accessible with the ligand.

With liposomes, all domains are impacted by cAMP. However, the difference of deuterium uptake is very significant for CNB domain (fixation site of ligand): cAMP decreases solvent accessibly.

All EPAC1 domains show a significant difference of deuterium uptake with liposomes.

Finally, the liposomes cause a difference of deuterium uptake for all domains of EPAC1 with cAMP, but the GEF domain is the most impacted.

Conclusions

HDX-MS was able to highlight regions of EPAC1 affected upon cAMP binding in presence or absence of liposomes to mimic the membrane.

Novel Aspect

HDX in liposome environment

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Keywords

HDX-MS, structural MS, membrane protein

Authors

Del Nero Elise [1], Sartre Candice [2], Bourguet Maxime [1], Ferrandez Yann [2], Cherfils Jacqueline [2], Cianférani Sarah [1]

1. Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO), Institut Pluridisciplinaire Hubert Curien (IPHC), UMR7178, Strasbourg. (France)
2. Laboratoire de Biologie et Pharmacologie Appliquée, CNRS and Ecole Normale Supérieure, Paris-Saclay, 94235 Cachan (France)

Probing peptide conformationnal dynamics coupling lasers ans mass spectrometry [P166]

Previous studies have shown that, in a certain family of peptides including a trypto-phan and a histidine, it was possible to photo-induce an intramolecular proton transfer. It was also shown that, in the peptide sequence HGGGW, this proton transfer between tryptophan and histidine lasts over a few hundreds of microseconds [1]. Eventually, it was shown by comparison with molecular dynamics, that this internal proton transfer was kinetically limited by the peptides conformational dynamics. Building on those previous results, we want, in the study reported here, to use the proton transfer time constant as a signature of the peptide conformational dynamics. A pump-probe setup was designed, coupling two lasers and an LTQ linear ion trap, to measure the proton transfer time constant.

In particular, we propose to analyze the e_ects of di_erent parameters _ peptide size, backbone rigidity and secondary structure _ on the conformational dynamics of those peptides. In order to assess e_ects of backbone rigidity we will compare di_erent peptide sizes and glycine chaines vs. proline and alanine.

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Authors

Bouakil Mathilde [1]

1. Institut Lumière Matière [Villeurbanne] (France)

Overcoming unusual challenges in the characterization of monoclonal antibodies by Mass Spectrometry in early and late phase [P167]

Different modalities of monoclonal antibodies are pushing the limits of characterization by mass spectrometry. With this new class of biomolecules, new critical quality attributes need to be carefully monitored to ensure product quality as efficacy and safety, in particular absence of immunogenicity.

From developability in early phase, during process development and routine monitoring in first clinical phases, Mass Spectrometry is more widely used to monitor post translational modifications or to identify and monitor unwanted HCPs.

Several case studies will illustrate the first stages of development of monoclonal antibodies and the characterization of unusual modifications (such as sulfation, oxidations, additional glycosylation) .

The enzymatic desulfation of the antibody allowed to generate different levels of sulfation to monitor the impact on biological activities. The oxidation need to be carefully monitored as all the components of a formulation buffer can impact its level. Finally, a presence of an additional glycosylation on one chain complexify the global pattern and need a monitoring of separate glycoforms on each chains.

In addition to traditional methods (intact mass in denaturing conditions and peptide mapping), the use of non denaturing native SEC-MS method for the investigation of structural heterogeneity complete the toolbox for the in-depth characterization of atypical behavior.

Authors

Lechat Nelly [1]

1. BioAnaltytics (BioA) department France (France)

Combining Hydrogen/Deuterium exchange (HDX) and chemical crosslinking (XL) with mass spectrometry to get insight into the specific association of the coactivator Med1 with the VDR-RXR heterodimer [P168]

Introduction

The Mediator complex is involved in transcriptional machinery recruitment through its association with transcription factors such as nuclear receptors [1]. While much has been discovered about the Mediator and its association with transcriptional machinery, how it bridges the RNA Pol II to other transcription factors and the role of Med1 coactivator, the largest subunit of the Mediator complex, are far less understood. Here we report the characterization of Med1 association with the VDR-RXR heterodimer using structural methods based on mass spectrometry: HDX-MS and XL-MS.

Methods

HDX-MS was automatically conducted on UPLC system with HDX technology coupled to a Synapt G2Si HDMS (Waters). A range of five H/D exchange times was used (0 min to 30 min). Deuterium uptakes for identified peptides were validated manually using DynamX 3.0 and statistically using MEMHDX [2]. XL-MS data were acquired on a Q-Exactive Plus Orbitrap (Thermo) using MS cleavable crosslinkers in 50, 100 and 200-fold molar excess. Data treatment was performed using MeroX [3].

Results

In this work, we performed HDX-MS and XL-MS experiments with Med1 (50-660) fragment to characterize its binding to VDR-RXR heterodimer. Firstly, we compared deuterium incorporation rates of VDR-RXR with and without the presence of Med1 (50-660). Numerous regions of VDR and RXR were observed to be protected from H/D exchange upon Med1 binding. All of these regions are located in the same area of the heterodimer. Then, XL-MS experiments allowed to identify a total of 42 crosslinks including 11 intra-RXR, 12 intra-VDR, 6 intra-Med1, 12 inter-VDR/RXR and 1 inter-Med1/RXR crosslinks. Intra-Med1 crosslinks will be used as distance constraints for the modelisation of Med1 structure. More interestingly, we obtained a inter-Med1/RXR crosslink located in the same region protected from HDX, highlighting that Med1 (50-660) strongly impacts the structural dynamics of this region when it binds to VDR.

Conclusions

The structural dynamics of the complex observed by HDX-MS and the interaction site obtained with XL-MS have confirmed the VDR-RXR heterodimer regions affected upon Med1 (50-660) binding. Combining HDX-MS and XL-MS allowed getting closer to the structure of the complex and to the understanding of the interaction network between Med1 coactivator and VDR-RXR heterodimer.

Novel Aspect

HDX-MS and XL-MS allowed to characterize VDR-RXR and Med1 binding interface, involving several identified regions of VDR, RXR and Med1.

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Keywords

Structural biology, HDX, Crosslinking

Authors

Bourguet Maxime [1], Hessmann Steve [1], Belorusova Anna [2], Rochel Natacha [2], Cianférani Sarah [1]

1. Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO) (France)
2. Institut de Génétique et de Biologie Moléculaire et Cellulaire (France)

Oxidative stress and human centrin 2-P17XPC complex [P169]

Introduction

Human centrin 2 (Hscen 2) is an extremely sensitive protein to ionizing radiation, which result in its oligomerization [1]. By its implication in the processes of centrosomes duplication [2] and DNA repair [3], it appears crucial to understand its radio-induced modifications. When DNA is damaged, Hscen 2 interacts with another protein, XPC (Xeroderma Pigmentum Group C Protein), to form a complex that initiates DNA repair.

Methods

Aqueous samples of proteins were irradiated with γ rays in order to produce oxidizing radicals through water radiolysis. Characterization of the complex formed between Hscen 2 and a 17 amino acids peptide from XPC (P17XPC) was achieved through microcalorimetry, fluorimetry and native mass spectrometry. Radio-induced modifications were studied through electrophoresis and LC-MS. The major damage, namely Hscen 2 dimerization, was investigated in more details.

Results

In the chosen conditions, Hscen 2 complexes P17XPC with a stoichiometry of 1:1. A blue-shift in the fluorescence emission spectrum of the P17XPC tryptophan residue is consistent with the complex formation. Mass spectrometry revealed a non-covalent preformed dimer of Hscen 2 which disappears in the presence of P17XPC (1 equivalent) in favor of the complex.

After γ irradiation, oxidation products of Hscen 2-P17XPC were compared to those of Hscen 2. Oligomerization still appears as the major modification: the dimer formation can be easily detected on the gel and mass spectrum. But its dimerization yield decreases in the presence of P17XPC. A new band in the gel suggests a covalent bond between both partners.

Conclusions

Complexation of Hscen 2 and P17XPC in the γ irradiation conditions was confirmed by mass spectrometry highlighting a preformed dimer of the isolated protein which fades away when the complex is formed. We evidenced a new radio-induced covalent bond between Hscen 2 and P17XPC, in agreement with the Hscen 2 dimerization yield decline.

Novel Aspect

Full characterization of oxidative degradation products of a Hscen 2-P17XPC complex was performed by mass spectrometry coupled to ion mobility.

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Keywords

Oxidative stress, Human centrin 2, Native MS

Authors

Gatin Anouchka [1], Billault Isabelle [1], Van Der Rest Guillaume [1], Duchambon Patricia [2], Sicard Cécile [1]

1. Laboratoire de Chimie-Physique d'Orsay (France)
2. Institut Curie (Orsay) (France)

Quantitative crosslinking-MS and native MS applied to the dynamic peroxisomal translocon [P170]

Introduction

Peroxisomes emerged in the recent years as versatile and dynamic organelles, which are involved in severe human diseases [1]. Lacking organellar DNA, peroxisomes import nuclear-encoded matrix proteins depending on the physiological demands of the cell. The import is performed by a complex and highly dynamic import machinery termed translocon [2]. Cytosolic matrix proteins are captured by a soluble receptor, which in turn interacts with the membrane-associated docking complex comprised of Pex14p and Pex13p and in the yeast *S.cerevisiae* Pex17p and Dyn2p [3].

Methods

We first used recombinantly expressed proteins and chemical crosslinking (XL) as well as native MS to study homo-oligomerization of Pex14p and Dyn2p. Then we applied a label-free and targeted quantitative crosslinking-MS (QXL-MS) workflow to analyze crosslinked products of specific oligomeric states of these proteins. Finally, we used XL-MS to study native protein complexes affinity-purified from detergent-solubilized membrane fractions of yeast cells.

Results

In a first stage of this work, we localized essential homo-oligomerization domains of Pex14p. Furthermore, we identified and mapped interaction sites that are unique for a distinct oligomeric state of the protein in a reproducible and statistically robust manner. In addition, the dynamic nature of Pex14p homo-oligomerization was characterized and we gained insight into the so far unknown functional role of the soluble Dyn2p, which occurs as a dimer in the wildtype form. A H58K mutation within the dimerization interface of Dyn2p abrogates its homo-oligomerization [4]. Additional XL-MS data of protein complexes affinity-purified from cellular membranes showed a detailed network of contact sites between Pex14p, Pex17p and Dyn2p. By comparing the *in vitro* experiments of single components with the *in vivo* data and using *in silico* prediction, the docking complex will be discussed in terms of possible structural arrangements of its functional units.

Conclusions

Studying homo-oligomerization of proteins by XL-MS can result in ambiguous or even misleading information about interaction sites. With the novel workflow presented here, which takes MS intensities of replicates into account, we gained reproducible and statistically robust data of contact sites. Combining this high-confidence data with conventional XL-MS data of protein complexes, native MS and *in silico* predictions enables new insights into complex structures.

Novel Aspect

Quantitative cross-linking MS paired with native MS and conventional XL-MS of protein complexes resulted in novel insights into structural aspects of the peroxisomal translocon.

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Keywords

native MS, Quantitative XL-MS, Peroxisomal Translocon

Authors

Wendscheck Daniel [1], Drepper Friedel [2] [1], Bender Julian [1], Buergi Jerome [3], Wilmanns Matthias [3], Warscheid Bettina [1] [2]

1. Institute of Biology, University of Freiburg (Allemagne)
2. Signalling Research Centres BIOSS and CIBSS, Freiburg (Allemagne)
3. EMBL Hamburg (Allemagne)

Characterization of the keratinolytic activity of the Bacillus species N3 strain on poultry feathers. [P171]

Introduction

Feathers constitute a major by-product of poultry industry with a high potential as source of proteins, peptides and amino acids [1]. Feather keratins are highly cross-linked fibrous proteins, insoluble in water and not easily hydrolysed by common agrofood proteolytic enzymes. The content in cysteine and the 3D-structure of feather keratins explain the high proportion of disulphide bridges and hydrogen bonds, leading to a packed quaternary structure hard to degrade. Nevertheless, feather keratins can be efficiently hydrolysed by some keratinases produced by micro-organisms [2].

Methods

Sterile poultry feathers were incubated during 6 days in the presence of two *Bacillus licheniformis* strains: PDW-1 (ATCC® 53757) and LBAE-N3. The keratinolytic activity of the supernatant was measured using keratine azure as a substrate. The culture media was centrifuged at different incubation days and the recovered solubilized peptides were subjected to a size-exclusion chromatography and analyzed by UPLC/MS-MS. Peptides were identified using Peaks Studio and a PCA have been performed with Progenesis QI to highlight the differences in peptide contents between feather hydrolysates.

Results

Mass balances, protein contents and size-exclusion chromatography profiles of culture supernatants demonstrate clearly the keratinolytic capability of the N3 *Bacillus* strain on *Gallus gallus* feathers. As expected, the identified peptides come only from the feather keratins but feather molecular structure and molecular mass of feather keratins limit the peptide identification. The Venn diagram resulting from the comparative analysis of identified peptides for each sample (N3 and PWD1 cultures respectively) reveals a low recovery in terms of peptide sequences between hydrolysates. This difference is also highlighted by the PCA performed using Progenesis QI. Finally, N3 strain Keratinase(s) purification is ongoing.

Conclusions

Feather keratins are packed fibrous proteins and are resistant to physical and (bio)chemical environmental factors. Here, we demonstrate that the N3 strain degrades the mixture of α- and β-keratins, that appears to preferentially express in different feather parts [3]. The generated peptides are qualitatively and quantitatively abundant but a large part of them are identified by *de novo* approach suggesting a peptide size inferior to 5-6 amino acids.

Novel Aspect

Study of keratinolytic properties of the N3 *Bacillus licheniformis* strain and characterization of generated peptides for the valorisation of poultry feather waste into added-value products.

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Keywords

Feather keratins, biotechnological proteolysis, peptide characterization

Authors

Deracinois Barbara [1], Allayaud Fanny [2], Zaidi-Ait Salem Meriem [2], Lefebvre Dominique [2], Marcato-Romain Claire Emmanuelle [2], Girbal-Neuhauser Elisabeth [2], Flahaut Christophe [1]

1. Institut Charles Viollette (ICV) - EA 7394 (France)
2. Laboratoire de Biotechnologies Agroalimentaire et Environnementale (France)

Combining native and H/D exchange mass spectrometry for the structural characterization of PPARg loss-of-function mutants [P172]

Introduction

The peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor involved in the regulation of transcriptional activity [1]. Two mutations of PPARγ (F310S and H494Y) located in the ligand-binding domain (LDB) have been shown to reduce its transcriptional activity in basal bladder cancer, inhibiting the release of corepressors (CoR) and the recruitment of coactivators (CoA). Here, We performed a MS-based characterization of PPARγ loss-of-function mutants using a combination of native mass spectrometry (MS) and hydrogen-deuterium exchange MS (HDX-MS).

Methods

Native MS and titration experiments involving PPARγ, coregulators and ligands at different ratio were performed on a Synapt G2 HDMS (Waters).

HDX-MS experiments were performed using a Q-TOF Synapt G2Si HDMS (Waters) coupled to a CTC PAL robot (Leap Technologies) and an Acquity UPLC (Waters). PPARγ mutants and wild type (wt) proteins were deuterated from 0 to 10 min with a 95% deuterated solution. DynamX 3.0 (Waters) and MEMHDX were used to validate peptides.

Results

We used native MS to understand how the mutations impair PPARγ activity. Native MS revealed that both PPARγ mutants strongly destabilize the interaction with the CoA (with a slightly higher effect of F310S), as lower amounts of 1:1 PPARγ:CoA complexes were detected compared to wtPPARγ. In presence of the GW1929 agonist ligand, the destabilizing effect is emphasized. Conversely, native MS highlighted a strong stabilization of PPARγ/CoR interactions for both mutants either alone (F310S again slightly more effective than H494Y) or in presence of the agonist (rosiglitazone) or antagonist (T0070907).

To better understand the conformational changes induced by PPARγ point mutations on the overall structure, we next conducted HDX-MS experiments. Both mutants present a significant difference in D uptake in several regions of PPARγ LBD (H3, H5 and H8 helixes) which correspond to the binding regions of the coregulators [2]. HDX-MS results provided explanations for differences observed in PPARγ/coregulator interactions by native MS.

Conclusions

Altogether, our results confirmed the complementarity of native MS and HDX-MS to better understand structure-function studies of two PPARγ mutant proteins, allowing the identification of structural elements that underpin their loss-of-function and should help develop potent molecules to transactivate PPARγ as therapeutic strategies.

Novel Aspect

Combination of native MS and HDX-MS for structure-function studies of two loss-of-function PPARj mutants involved in basal bladder cancer.

References

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Keywords

HDX-MS, Native MS, structural MS

Authors

Ley Marie [1], Beji Syrine [2], Rochel-Guiberteau Natacha [2], Cianferani Sarah [1]

1. Laboratoire de Spectrométrie de Masse BioOrganique (France)
2. Institut de Génétique et de Biologie Moléculaire et Cellulaire (France)

Structural analysis of the Type VI bacterial secretion system using native mass spectrometry [P173]

Introduction

To cause pathogenesis, bacterial pathogens have evolved a broad repertoire of macromolecular machineries that are able to deliver specific effectors and toxins. Among those, the type VI secretion system (T6SS) is a contractile nanomachine that mainly comprises a tail structure anchored to the cell envelope by a membrane complex. A very important element is the baseplate, which serves as the tail assembly platform. In this work, we used native mass spectrometry to precise the structure of this multiprotein complex and complement cryo-Electron Microscopy (cryo-EM) data.

Methods

In enteroaggregative *E. coli*, the baseplate complex is composed of the TssK, F, G and E subunits. The whole complex was purified and buffer exchanged with 500 mM ammonium acetate by size-exclusion chromatography or with biospin6 (3 times). Samples were nano-electrosprayed using a TriVersa NanoMate (Advion Biosciences) coupled to a Synapt G2-Si mass spectrometer (Waters Corporation).

Results

We first checked the integrity of all subunits by analyzing the complex in denaturing conditions. For all subunits, the experimental molecular masses obtained were very close to the theoretical ones, indicating that they have been expressed with the expected sequence and length. The complex was then measured in native conditions to preserve all noncovalent interactions during the transfer into gas phase. A mass of 499,490 Da (± 400 Da) was obtained indicating an unexpected stoichiometry: TssK6F2GE. The intact complex was then dissociated using different types of approaches (Collision Induced Dissociation & Surface Induced Dissocation). Various sub-complexes were obtained showing the presence of a dimer of trimers for TssK. Our results also indicated that TssE was more loosely bound to the complex and thus probably localized at the periphery of the complex. These topological data were used to complement the cryo-EM previously obtained for which some assignments remained elusive. All subunits could finally be assigned to the structure.

Conclusions

Native mass spectrometry allowed both the stoichiometry of the baseplate complex of the Type VI secretion system of enteroaggregative *E. coli* and elements of topology to be obtained. All data were used to complement cryo-EM data, showing the added value of combining structural methods to precisely decipher the structure of complex bacterial nanomachineries that are key-pathogenic factors.

Novel Aspect

Native MS complements cryo-EM data to determine the structure of a key-pathogenic bacterial factor.

References

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Keywords

Native mass spectrometry, Structural biology, Type VI secretion system

Authors

Malosse Christian [1], Rey Martial [1], Cherrak Yassine [2], Rapisarda Chiara [3], Pellarin Ricardo [4], Bouvier Guillaume [4], Bardiaux Benjamin [4], Allain Fabrice [4], Cascales Eric [2], Fronzes Rémi [3], Durand Eric [2], Chamot-Rooke Julia [1]

1. Unité Spectrométrie de Masse pour la Biologie, Institut Pasteur, CNRS USR2000, Paris, France (France)
2. Laboratoire d'Ingénierie des Systèmes Macromoléculaires, Institut de Microbiologie de la Méditerranée, UMR7255, Aix-Marseille Université – CNRS, Marseille, France (France)
3. Institut Européen de Chimie et Biologie, University of Bordeaux, Pessac, France (France)
4. Unité Bioinformatique Structurale, Institut Pasteur, CNRS UMR3528, C3BI USR3756, Paris, France (France)

Probing conformational changes of an integral membrane therapeutic target in human cancer upon binding with a cellular protein by HDX-MS [P174]

Introduction

The integral membrane solute carrier under study has emerged as an important target in cancer therapy but its molecular and pharmacological mechanisms remain poorly understood. The lack of cell-surface markers targeting this transporter and their low selectivity drive research towards the development of new selective molecules with improved therapeutic potential. Here, we use HDX-MS to gain insights into the conformational and dynamic changes of the transporter upon binding with a cellular protein. Both wild-type and mutated transporters (to increase stability) were studied.

Methods

We mixed 20 pmol of proteins with D2O buffer and quenched at defined time points with a cold acidic solution. Quenched samples were injected into a cooled UPLC HDX system, online digested using pepsin, desalted before being separated onto a C18 column in 10 min. Mass spectra were acquired on a Synapt G2-Si HDMS mass spectrometer with electrospray ionization. Identification, deuterium uptake calculation and statistical analysis were performed with Mass Spec Studio [2].

Results

After 3 min of online pepsin digestion and desalting, a sequence coverage of more than 60% was obtained for both the mutant and wild-type constructs. Numerous peptides were identified from regions of the transporter close to the cytoplasm or extracellular loops. Very interestingly, highly hydrophobic domains buried in the membrane were also covered. Deuterium uptake variations were clearly detected upon binding of the cellular protein and highlighted conformational changes of the membrane transporter. A strong decrease of the deuterium uptake (more than 12%) was observed in the extracellular loop suggesting a direct binding of the cellular protein to the transporter, as expected. In the meantime allosteric effects were also detected in membrane domains far from this extracellular loop and in regions presumed to play a key role in the transport of neutral solutes. Moreover HDX-MS indicates a similar behavior for the wild-type and the mutant constructs upon interaction with the cellular protein, which is very encouraging.

Conclusions

HDX-MS identified the binding site of the cellular protein onto the transporter and probed effects on its conformation. This protein seems to be a valuable candidate for future development as a cell marker targeting this transporter. Combined with other approaches HDX-MS data pave the way for the design of new selective molecules with alternative anti-cancer mechanisms. HDX-MS also testified the validity of the more stable mutated construct as a reasonable model.

Novel Aspect

Identification by HDX-MS of a promising candidate as cell marker and drug delivery system towards an integral membrane solute carrier involved in cancer cell proliferation.

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Keywords

HDX-MS, Membrane proteins, Cancer

Authors

Dhenin Jonathan [1], Rey Martial [1], Kumar Anand [2], Reyes Nicolas [2], Chamot-Rooke Julia [1]

1. Unité Spectrométrie de Masse pour la Biologie, Institut Pasteur, CNRS USR2000, Paris, France (France)
2. Mécanismes des protéines membranaires, Institut Pasteur, CNRS UMR3528, Paris, France (France)

Investigating the conformation and dynamics of the CyaA toxin by an integrative structural biology approach [P175]

Introduction

The adenylate cyclase toxin (CyaA) is one of the primary virulence factors of *B. pertussis*, the causative agent of whooping cough [1]. CyaA is a 1706-long multi-domain protein required in the early stages of respiratory tract colonization. The molecular mechanism involved in the toxin translocation through the plasma membrane of eukaryotic cells remains largely unknown. Herein, we used Hydrogen/Deuterium Exchange Mass Spectrometry (HDX-MS) in combination with other biophysical approaches to understand, at the structural level, the mechanism of cell intoxication by CyaA.

Methods

SEC-SAXS and CD data were collected on the SWING and DISCO beamlines at the SOLEIL synchrotron. HDX-MS experiments were performed using 80% excess deuterium. Proteins were labelled at 20°C and quenched samples were injected into a cooled nanoACQUITY HDX UPLC™ connected to a Synapt™ G2-Si HDMS™ mass spectrometer (Waters). HDX data were extracted using DynamX 3.0. Statistical analysis was performed with MEMHDX [2] using a 1% FDR. All analyses were performed in triplicate.

Results

CyaA is produced as an inactive pro-toxin and converted into its cytotoxic form upon acylation. Our results reveal that CyaA acylation contributes to the refolding of CyaA into a functional cytotoxic state [3]. A disorder-to-order transition within the receptor-binding domain (RD) favors the secretion of CyaA. In the absence of calcium, RD adopts an intrinsically disordered state with a diameter appropriately sized for transport through the narrow channel of the T1SS. Once secreted, the C-terminal domain of RD binds calcium and initiates the folding into a compact structure larger than the secretion channel, thus ensuring the irreversible exit of the toxin [4]. After translocation into the cells, the catalytic domain (AC) of CyaA is activated by binding calmodulin (CaM). A 75-residue-long stretch within AC undergoes a disorder-to-order transition upon CaM binding. This local folding is associated with long-range allosteric effects that stabilize the catalytic site, whilst preserving the flexibility of the catalytic loop required for activity [5].

Conclusions

Disorder-to-order transitions regulate the biological activity of many proteins containing intrinsically disordered regions but remain challenging to analyze at the structural level. Using an integrative structural biology approach, we showed that such transitions were critical for the secretion and the activation of the CyaA toxin. The acylations also contribute to the folding of the CyaA toxin into a functional cytotoxic state.

Novel Aspect

Use of an integrative structural biology approach to decipher the mechanism of cell intoxication of a 1706-residue long toxin containing large intrinsically disordered segments.

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Keywords

HDX-MS; Integrative Structural Biology; CyaA toxin

Authors

O'brien Darragh [1], Durand Dominique [2], Vachette Patrice [2], Ladant Daniel [1], Chenal Alexandre [1], Brier Sebastien [1]

1. Institut Pasteur (France)
2. Institute of Integrative Biology of the Cell (France)

Towards the structural characterization of a retinoic acid (RAR/RXR) nuclear receptor complex [P176]

Introduction

Our project aims to use the potential of mass spectrometry in coupling with a cross-linking strategy (XLMS) in a context of structural analysis [1]. This technique consists of covalently link different subunits that are close enough by chemical reaction in order to obtain distance constraints within a biological complex. However, even if experimental approach becomes better controlled, we still face many difficulties in choosing the most appropriate cross-linker or in the choice of key criteria for a reliable interpretation of MS/MS fragmentation spectra of cross-linked peptides.

Methods

Classical (BS3) or MS-cleavable (CDI) cross-linkers have been tested on a retinoic acid (RAR/RXR) nuclear receptors complex, some of whose 3D structure elements are known at high/medium resolution. The XLMS strategy was applied in absence and in presence of a nucleotidic interacting partner in order to evaluate whether the spatial resolution obtained makes it possible to describe the possible conformational changes upon DNA binding.

Results

Monitoring and optimization of the cross-linking reaction was carried out using MALDI-TOF MS. The cross-linked amino acids were then characterized by proteomic analysis using dedicated bioinformatic tools. Both cross-linkers gave complementary information. A comparison of different XLMS dedicated software demonstrated that scoring cannot be directly relied to interpretation's confidence making thus manual validation of the cross-linked MS/MS spectra especially crucial. Discussion aiming to define a standard interpretation protocol is then proposed. A potential proximity map was drawn up and confronted to structural biology data obtained by Small Angle X-ray Scattering (SAXS). Distances constraints deduced from XLMS provided an empiric criterion to compare structural available models of our complex [2]. In a second step, XLMS was applied in presence of various oligonucleotides partners to assess whether the obtained spatial resolution makes it possible to describe an eventual conformational change upon DNA binding.

Conclusions

A XLMS protocol has been optimized after testing different cross-linkers and interpretation software in order to improve available structural models of a RAR/RXR complex. XLMS data were then compared with SAXS data to help refinement towards a more precise 3D model. Resolution of the technique is not easy to evaluate but it might be promising for the study of larger protein complexes even occurring with protein conformational changes.

Novel Aspect

- XLMS as new reliable approach to assess structural information on protein complexes

- Discussion on software scoring confidence

- How about conformational changes?

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Keywords

Cross-Linking Mass Spectrometry, Proteomics, Structural Analysis

Authors

Giorgiutti Christophe [1], Peluso-Iltis Carole, Osz Judit, Rochel-Guiberteau Natacha, Potier Noelle, Leize-Wagner Emmanuelle

1. LSMIS, UMR7140 (France)

An investigation into the use of cyclic ion mobility for the separation of biopharmaceutical peptide and protein modifications [P177]

Introduction

More sophisticated tools are being investigated to provide in-depth detailed molecular characterisation on biotherapeutics. Such studies focus on acquiring knowledge of the post-translational modifications (PTM's) including glycosylation, oxidation and deamidation present in the protein product, with control of these being paramount. Mass spectrometry (MS) is a central technique in biopharmaceutical characterization, however, the presence of isobaric PTM's can often be refractory to traditional LC-MS workflows. In this work we investigate cyclic ion mobility technology as a means to distinguish isomeric PTM's.

Methods

Studies were performed on a cyclic ion mobility-enabled quadrupole time-of-flight (Q-cIM-oaToF) mass spectrometer. Ions can cycle the path length multi-times, provides significantly higher resolution over a selected mobility range. The multifunctional T-WAVE ion entry/exit array allows mobility selectivity by ejecting species within a range of mobilities, to a pre-store array, enabling multiple stages of ion mobility selection and separation (IMSn). The native and iso-aspartic variants of a synthetic peptide were used to mimic deamidation products from protein biotherapeutics.

Results

The two isomeric peptides T12-D and T12-isoD formed primarily the [M+3H]3+ ion at m/z 928. After a single pass of the cIM device it was observed from its arrival time distribution (ATD) that T12-D formed two conformers, one compact (1) and one more extended (2) with approximately 90 % of the signal present as conformer 1. By comparison T12-isoD was found to populate only conformer 2. These data suggest that the presence of the isoD variant biases the conformation of the T12 peptide to the more extended form under these conditions. The T12 peptide was selected by the resolving quadrupole and subjected to fragmentation in the trap ion guide situated prior to the cIM device. This enables separation and structural analysis of the resulting product ions by ion mobility followed by mass analysis. In order to probe the product ions in detail we subjected the peptides to both multi-pass and IMSn studies.

Conclusions

These preliminary data indicate that multifunction cIM could be used to identify and aid in sequencing peptide isomers. Further data will be collected on a range of additional modified peptides with on-line LC separation for chromatographic separation.

Novel Aspect

IMSn for distinguishing post-translational modifications in biopharmaceutical analysis

Keywords

IMSn, Cyclic IMS, Ion Mobility, Biotherapeutics characterisation

Authors

Carvalho Maryline [1], Langridge Jim, Shion Henry, Palmer Martin, Chen Weibin, Cooper-Shepherd Dale

1. Waters Corporation (France)

Application of cyclic ion mobility coupled to mass spectrometry for high peak capacity analysis of native and deuterated peptide mixtures [P178]

Introduction

A major bottleneck for the analysis of peptides originating from protein digests is the requirement for long chromatographic separation times. The introduction of hybrid ion mobility-TOF mass spectrometers has proven advantageous in retaining peak capacity when chromatographic times are shortened. Recent advances in IM instrumentation have led to the development of cyclic IM (cIM), a technology that provides variable IM separation by simply increasing the number of passes, before MS detection. We investigated the utility of cIM for peptide separation under fast chromatographic conditions and by direct infusion.

Methods

An enolase tryptic digest and peptide P1 (HHHHHHIIKIIK) were used as model systems in this study. Experiments were performed on a cyclic ion mobility-enabled quadrupole time-of-flight (Q-cIM-oa ToF) mass spectrometer. The cIM device has a circular path that provides a longer, higher mobility resolution separation path (1m) whilst a multi-pass capability provides significantly higher path length and hence resolution over a reduced (selected) mobility range. Data were acquired in both infusion and LC-MS modes. Increasing numbers of passes of the cyclic IM device were performed on the same sample.

Results

Separation of peptides with chromatography. The enolase digest was used as a model system for the separation of peptides by rapid LC-MS. 82 % sequence coverage was obtained with a gradient of less than 3 minutes, afforded by the enhanced mobility and TOF resolution.

Direct analysis of peptides. The same enolase digest was used to assess separation in the absence of chromatographic separation. Strikingly, using a single pass of the cIM device 91 % of the peptides observed with chromatography were observed by direct infusion with distinct *m/z* and arrival times. Choosing groups of similar mass peptides, the resolving power of the cIM device was compared with changes in the number of passes. Increasing the number of passes resolved peptide species in drift time such that their isotope distributions did not overlap in the *m/z* scale. *Deuterated peptides*. We used the model peptide P1 to also assess hydrogen/deuterium back exchange within the cIM device. The deuterium level in the peptide changed depending on the number of passes in the IM device: more passes resulted in decreased average deuterium. The time of each pass was approximately 15 ms, and after 5 passes (approx. 75 msec), it is unsurprising that some side chain deuterium could have back-exchanged to hydrogen.

Conclusions

Partially and fully co-eluting species were separated by a single pass through the cIM device, even with this short chromatographic gradient, showing promise for data-independent LC-MS

workflows. Increasing the number of passes resolved peptide species in drift time such that their isotope distributions did not overlap in the *m/z* scale.

Novel Aspect

Investigation of a cyclic ion mobility device as a means to increase peak capacity of peptide/ deuterated peptide separations

Keywords

Cyclic ion mobility, peptide, HDX, Peptide

Authors

Carvalho Maryline [1], Palmer Martin, Anderson Malcolm, Cooper-Shepherd Dale, Landgridge James, Tonge Robert

1. Waters Corporation (France)