CTDP
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3RD INTERNATIONAL TOP-DOWN PROTEOMICS SYMPOSIUM

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New Frontiers in Proteomics - Proteoforms, Proteoform Families, and the Human Proteoform Project

Author: Lloyd Smith[1]

[1] *Department of Chemistry, University of Wisconsin - Madison*

Corresponding Author: smith@chem.wisc.edu

Proteins are the primary effectors of function in biology, and thus complete knowledge of their structure and behavior is needed to decipher function. However the richness of protein structure and function goes far beyond the linear amino acid sequence dictated by the genetic code. Multigene families, alternative splicing, coding polymorphisms, and post-translational modifications, work together to create a rich variety of proteoforms, whose chemical diversity is the foundation of the biological complexes and networks that control biology. "Proteoforms" are the specific molecular forms in which proteins are present in biological systems; only direct analysis of the proteoforms themselves can reveal their structures, dynamics, and localizations in biological systems.

Remarkably, the dominant paradigm of proteomics research, "bottom-up"proteomics, does not identify proteoforms –rather, proteins are enzymatically digested into peptides, whose identification then indicates the likely presence of their parent proteins in the sample. This strategy destroys the information as to what form of the protein the peptide represents, and thus the critical information needed to identify proteoforms is lost. The entire field of Biology is thus attempting to understand life in the absence of the ability to understand the molecules that define life. This limitation of todays technology provides a "grand challenge"to the scientific community, to devise new strategies and approaches that are able to comprehensively and quantitatively reveal the full breadth of the proteome at the proteoform level.

In this presentation I will provide an overview of this interesting problem, along with a variety of new tools and approaches that we and others are developing to address it. Developing the technology to decipher proteoforms, building a comprehensive atlas of proteoforms present in human systems, and eventually deciphering the functional roles they play in normal and disease biology, comprise central elements in the quest to understand human biology.

User consent:

yes

New Frontiers in Life Sciences - Entering the Proteoform Era / 5

Digitizing Proteoform Biology with Single Molecule & Single Cell Mass Spectrometry

Author: Neil Kelleher[None]

Corresponding Author: kelleher-ofc@northwestern.edu

Since the completion of the Human Genome Project, much has been made of the need to bridge the gap from genes and traits. As a key nexus for the many interacting '-omes'(genome, transcriptome, proteome, metabolome, etc.), the proteome should offer a tight link between genotype and phenotype. Proteoforms, or all of the precise molecular forms of a protein, capture all sources of variability in protein composition (i.e., SNPs, isoforms, post-translational modifications), and thus provide crucial insights into regulation and function. Now, "single ion"mass spectrometry is poised to convert genes to proteoform signatures at a far faster rate. Recently we developed proteoform imaging mass spectrometry (PiMS), with individual ion mass spectrometry. This platform has been extended now to single-cell Proteoform imaging Mass Spectrometry (scPiMS), boosting cell processing rates by >20-fold in the field while detecting proteoforms from single cells.

User consent:

yes

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Revealing Functional Proteoforms by Native Top-Down Proteomics

Author: Joseph Loo[1]

[1] *UCLA*

Corresponding Author: jloo@chem.ucla.edu

Native mass spectrometry (nMS) measures proteins and complexes that are functionally relevant to biology. Top-down proteomics (TDP) reveals identification, sequence, and proteoform information. The combination of these platforms, native top-down proteomics (nTDP), could be ideal for understanding how proteins interact with other proteins (and ligands and cofactors), identifying unknown proteoforms, and gain information on their function at near physiological conditions. We are developing a nTDP workflow based on data independent acquisition (DIA) without on-line chromatographic separation to address two microbial-based projects. Understanding the host-pathogen interface is key to combating antimicrobial resistance. For unfractionated secretomes of model Gram+ pathogenic bacteria, Corynebacterium diphtheriae, a single direct infusion revealed more than 370 unique masses. We identify more than 70 proteoforms, including novel virulence factors and complexoforms reaching 300 kDa. A functional proteomics platform based on slow mixing mode (SLOMO) and DIA-proton charge reduction (PTCR)/higher-energy collisional dissociation (HCD) was developed to determine how they acquire iron during infection. For the second project, we apply nTDP to elucidate the structure and composition of the cellulosome, a massive (0.5-2 MDa), self-assembling, multi-enzyme complex with potential implications to the carbon cycle and sustainable biofuel production. Its function is to break down lignocellulose and other biopolymers. To date, little is known about the specific structure and composition of intact cellulosomes. Preliminary nMS of putative Clostridium thermocellum cellulosomes reveals highly complex spectra. By applying electron-capture charge reduction (ECCR), masses in the 100-300 kDa range were deconvolved. For a 184 kDa complex detected, HCD-based nTDP identified it as a homohexamer enoyl hydratase (29 kDa monomer) complex.

User consent:

yes

New Frontiers in Life Sciences - Entering the Proteoform Era / 65

Touching upon the millions of hidden treasures in the plasma proteome

Author: Albert Heck[1]

[1] *Utrecht University*

Corresponding Author: a.j.r.heck@uu.nl

I will describe how innovative techniques in mass spectrometry provide unique novel insights into our humoral immune response. In our body we produce every day huge amounts of antibodies, of which many end up in circulation. Humans can make about trillions of distinct antibody clones, all

exhibiting a different sequence, recognizing distinct antigens. We recently developed new LC-MS based antibody repertoire profiling methods for studying immunoglobulins in a quantitative manner. By now, we analysed a variety of samples (sera, milk and saliva) from both healthy as well as diseased donors, allowing us to make some paradigm-shifting observations of which several I will highlight in this talk. Moreover, I will describe how both peptide- and protein-centric approaches on new mass analyser facilitate de novo sequencing, a prerequisite for proper identification of circulating antibodies. Making use of such methods we are now able to identify patient specific antibody responses against diseases specific antigens, which may be considered leads for further therapeutic development. And yes, there are millions of different proteins in our blood.

User consent:

yes

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Advances in hardware design and function of the new timsOmni MS platform

Author: Dimitris Papanastasiou[1]

[1] *Fasmatech Science & Technology*

Corresponding Author: d.papanastasiou@bruker.com

Innovations in mass spectrometry (MS) instrumentation continue to emerge, driven primarily by the need to identify and characterize proteins with greater confidence. Simultaneously, fragmentation schemes are an essential performance component of any MS platform, delivering detailed structural and sequence data necessary for precise identifications. Here we report on the latest advances realized on the new timsOmni platform where novel offline as well as online data dependent acquisition (DDA) experimental workflows are applied for the analysis of different classes of analytes. The diverse operating modes described in this study highlight the exceptional versatility and broad applicability of this novel instrument configuration.

A series of hardware developments are reported including (a) a linear stacked-ring RF ion guide providing efficient desolvation, (b) an ion mobility gate for selecting ions separated in the tims device, (c) a new design of segment Q5 in the Omnitrap accommodating higher electron currents without comprising robustness and (d) an improved AC-ejection method to transfer a wide range of m/z ratios from the collision cell to the TOF analyzer. The range of m/z ratios recorded in single TOF spectra extends from <300 Th to >10,000 Th. An offline ESI source is developed and applied for analysis of protein complexes. Deep sequencing of IgG1 monoclonal antibodies fragmented by various MSn modes is reported. Gas phase reduction of intrachain disulfide bonds is demonstrated for intact mAbs and industrial enzymes using electron-based fragmentation while post translational modifications on histones are identified in MSn experiments. Collision activation followed by mobility separation and electron capture dissociation is performed to map the unfolding process of different protein systems. DDA experiments are performed on a protein standard and digested antibody mixtures. Dynamic control of the accumulation period for quad-selected charge states is shown to improve sequence coverage for all the protein systems and subunits examined.

User consent:

yes

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Top-down sequencing of intact, modified proteins by timsTOF technology with new multi-modal fragmentation capabilities

Author: Ole Nørregaard Jensen[1]

Co-authors: Athanasios Smyrnakis ; Christian Albers ; Dimitris Papanastasiou ; Francis Berthias ; Jasmin Mecinovic ; Mariangela Kosmopoulou ; Nurgül Bilgin

[1] *SDU Department of Biochemistry and Molecular Biology*

Corresponding Author: jenseno@bmb.sdu.dk

We demonstrate top-down sequencing and detailed PTM characterization of intact histone proteoforms using a novel timsOMNI mass spectrometry platform. We prepared well defined acetylated and methylated proteoforms of histones H3 and H4 that were analyzed and sequenced by nanoESI interfaced to a modified timsTOF Ultra mass spectrometer equipped with the omnitrap technology (FasmaTech, Bruker Daltonics). Intact protein sequencing by MS/MS was performed by CID, ECD and combinations thereof. Data analysis was expedited by the Omniscape software (Bruker Daltonics). The timsOMNI platform generated highly informative tandem mass spectra of intact proteins enabling comprehensive sequence analysis, localization of multiple PTM sites and assessment of proteoform heterogeneity.

User consent:

yes

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Sensitive Top-down Analysis using Spray-capillary-based CE-MS approaches

Author: Si Wu[None]

Corresponding Author: swu49@ua.edu

Quantitative analysis of intact proteoforms in mass-limited, complex samples remains challenging due to the low ion intensity in MS detection and peak overlap caused by insufficient separation. While targeted top-down proteomics methods such as parallel reaction monitoring (PRM) have been developed for LC-MS, they typically require microgram-level sample input, limiting their utility for scarce samples. To overcome this, we recently developed an ultrasensitive spray-capillary-based method that enables ultralow-volume sampling and online CE-MS quantitation of intact proteoforms from picogram-level complex samples such as single cell analysis. To further enhance throughput, we recently introduced a multisegmented injection strategy using this spray-capillary platform. By integrating multisegmented spray-capillary CE-MS with PRM, we achieved highly sensitive and targeted quantitation of intact proteoforms at the attomole level with high throughput (e.g., analyzing 7+ samples in less than one hour). This platform demonstrated high selectivity and specificity, enabling high-throughput characterization and quantification.

User consent:

yes

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Combining advanced fragmentation techniques and spectral simplification for deep proteoform interrogation

Author: Luca Fornelli[1]

Co-authors: Christopher Mullen ; Graeme McAlister ; Jake Kline [1]; Jingjing Huang [2]; John Syka [2]; Josh Hinkle [2]; Linda Lieu [1]; Rafael Melani

[1] *University of Oklahoma*

[2] *Thermo Fisher Scientific*

Corresponding Authors: graeme.mcalister@thermofisher.com, rafael.melani@thermofisher.com, jingjing.huang@thermofisher.com, luca.fornelli@ou.edu, jake.kline@ou.edu, josh.hinkle@thermofisher.com, christopher.mullen@thermofisher.com, john.syka@thermofisher.com, lindablieu@ou.edu

The "proteoform hypothesis"postulates that the proteome-to-phenotype connection is better explained through the characterization of the actual molecules present in a cell or tissue, or proteoforms, than by cataloguing "protein groups"that represent undistinguished molecule ensembles. The top-down (TD) approach to proteomics (i.e., the direct analysis of proteoforms) can theoretically ensure the access to the proteoform landscape of cells and tissues. However, TD is particularly challenging to properly implement, as both intact and fragmentation mass spectra of proteoforms suffer from problems such as signal dilution and ion signal overlap. Additionally, to remain true to its declared mission of molecularly defining proteoforms, top-down mass spectrometry should in principle analyze also typically neglected post-translational modifications such as Cys-linked ones, which remain present on proteins only if disulfide bond reduction is avoided.
We find that the use of ion-ion and photon-ion reactions in the gas-phase leads to extensive sequence coverage of a variety of proteoforms, natural or artificial. Case studies include 66 kDa human serum albumin, which comprises 13 disulfide bonds, and chemically modified proteoforms of antibody-drug conjugates.
We demonstrate that the use of advanced fragmentation methods such as activated ion electron transfer dissociation (AI-ETD), where low-energy IR photons are used to denature protein cations while ETD takes place, are beneficial for both increasing the number of identified backbone cleavages and sequencing disulfide-protected regions that remain otherwise uncharacterized. We also show how incorporating collisional activation of product ions post AI-ETD (referred to as AI-EThcD) further increases proteoform sequence coverage.
Finally, we show that reducing signal overlap of product ions via ion-ion reactions, specifically proton transfer charge reduction (PTCR), does not only give access to additional sequence information generated by these fragmentation methods, but it also dramatically facilitates the interpretation of complex mass spectra, substantially reducing the number of false positive matches.

User consent:

yes

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Mass-Invariant Log-Transformed Mass Spectra Enable De Novo Sequencing and Internal Calibration of Intact Proteins

Author: Lissa C. Anderson[1]

Co-authors: Krishna Saketh Kamadana [2]; Nathan K. Kaiser [1]; Xian Mallory [2]

[1] *National High Magnetic Field Laboratory, Florida State University*

[2] *Department of Computer Science, Florida State University*

Corresponding Authors: nkaiser@magnet.fsu.edu, kk23f@fsu.edu, anderson@magnet.fsu.edu, xfan2@fsu.edu

Most top-down proteomics workflows rely on deconvolution of intact and fragment ion m/z values using modeled isotope distributions, typically via an "averagine"approximation. This step often

limits accuracy: poor fits to distorted isotope patterns can lead to incorrect monoisotopic mass assignment, widened mass tolerances, and inflated false discovery rates. To address these limitations, we have developed a framework for *de novo* sequencing and internal calibration that operates entirely in natural log-transformed m/z space—eliminating the need for monoisotopic mass determination.

By transforming spectra to ln(m/z – q), where q is the charge carrier mass, peaks arising from the same analyte mass align along a predictable pattern defined solely by charge state—a principle formalized by Jeong et al. in the FLASHDeconv algorithm (2020). This mass-invariant spacing can be used to assign charge states, pair isotopologues, and perform internal calibration without averagine-based fitting. Calibration is achieved by optimizing the B coefficient in the Ledford equation until observed peaks align with the expected –ln(c) spacing. Sequence tag inference is performed by comparing log-transformed peak positions from consecutive fragment ions to expected values based on known residue mass differences. When observed ln(m/z – q) values match those predicted for a given residue across multiple isotopologues and charge states, the corresponding mass difference can be confidently assigned—even from a single scan.

This method was applied to 21 T FT-ICR MS/MS spectra of intact proteins, achieving sub-ppm agreement between predicted and observed values without spectral averaging. Internal calibration improved mass accuracy of myoglobin from 6.9 ppm RMSE to 0.8 ppm. Notably, near-isobaric residues such as lysine and glutamine were resolved at high charge state, and proteoform families were identified from MS^1 data using log-space mass differences alone. This database-independent, calibrant-free framework enables high-accuracy proteoform analysis and significantly improves the robustness and resolution of top-down *de novo* sequencing.

User consent:

yes

Lightning Talks / 58

Properties, Origin, and Reproducibility of Truncated Proteoforms Across Top-Down Proteomic Studies

Author: Philipp T. Kaulich[1]

Co-authors: Andreas Tholey [1]; James M. Fulcher [2]

[1] *Systematic Proteome Research & Bioanalytics, Institute for Experimental Medicine, Christian-Albrechts-Universität zu Kiel, 24105 Kiel, Germany*

[2] *Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA, USA*

Corresponding Author: p.kaulich@iem.uni-kiel.de

Top-down proteomics (TDP) offers a powerful approach to identifying intact proteoforms, inherently providing information about protein modifications. Truncated proteoforms are among the most frequently observed modifications in TDP studies. Here, we selected fifty datasets, including over 140,000 proteoforms, published over the past decade, spanning various organisms, sample preparation approaches, data acquisitions, and data analysis pipelines, and investigated the reported proteoforms. On average, across all studies, 70% of the proteoforms were truncated, and only 30% were identified as full-length proteoforms (including those with only N-terminal methionine excision). Only 16% of the exclusively N-terminally, 5% of the C-terminally, and 1% of the N- and C-terminally truncated proteoforms have been described in the UniProt database, highlighting that the biological function of most truncations is unknown. To understand cleavage patterns more clearly, we determined the amino acids N- and C-terminally located from the truncation sites. We found truncation sites to be very diverse, with specific datasets showing unique patterns. Several truncation sites indicated artifacts introduced during sample preparation (e.g., between the aspartate-proline bond due to sample heating), mass spectrometric analysis (e.g., N-terminal to proline residues due to in-source fragmentation), or data analysis (e.g., due to database and precursor tolerance settings). Moreover,

substantial differences between samples of the same organism but different tissues or growth conditions were also observed. Importantly, our analysis revealed specific protein termini not linked to artifactual cleavages that were consistently reported across numerous studies, implicating these proteoforms to have biological significance. This includes previously unannotated mitochondrial signal peptide sites and cleavages with protein domain or structural specificity. In conclusion, this study provides a comprehensive overview of truncated proteoforms identified in recent TDP studies, highlighting both methodological and biological influences. We believe these results can also serve as a resource for other scientists to investigate non-canonical termini from proteins of interest.

User consent:

yes

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Novel bridged hybrid monolithic columns combined with mass spectrometry for top-down proteomic analysis

Authors: Yu Liang[1]; Yue Sun[1]; Xue Yang[1]; Zhen Liang[1]; Lihua Zhang[1]; Yukui Zhang[1]

[1] *Dalian Institute of Chemical Physics, CAS, China*

Corresponding Author: yuliang@dicp.ac.cn

Top-down proteomics could offer comprehensive investigations of proteoforms. However, it is greatly challenged by the co-elution of intact proteins which results in overlapped mass spectra. Hence, highly effective protein separation to reduce the co-elution of intact proteins from complex samples is of vital importance.
The ethane-bridged hybrid monolithic column with homogeneous macropores of 1.1 μm and large mesopores of 24 nm was prepared for protein separation with high peak capacity of 646 within 240-min gradient. Based on MS/MS analysis, 959 proteoforms corresponding to 263 proteins could be unambiguously identified from E. coli lysates in a single 240-min run. Furthermore, 347 large proteoforms with Mw higher than 30 kDa were detected in the single 75-min run. Besides, 6264 proteoforms corresponding to 885 proteins were identified from THP-1 cells induced by LPS.
The amine-bridged hybrid monolithic column with unique macropores was prepared and coupled to MS for analysis of membrane proteoforms. Due to its unique macroporous structure and secondary amino groups in the framework, the column possessed fast mass transfer, low non-specific adsorption, and electrostatic repulsion to membrane proteins, thus greatly reducing peak broadening and outperforming traditional reversed-phase columns in top-down characterization of membrane proteoforms. With this column, a total of 3100 membrane proteoforms were identified in the mouse hippocampus. The proteoform information was integrated into the interaction network of membrane protein complexes involved in oxidative phosphorylation processing, uncovering more detailed molecular basis and interaction in the biological processes.
Furthermore, highly sensitive top-down proteomic analysis of LCM slices was developed based on the narrow-bore amine-bridged hybrid monolithic column with low non-specific adsorption. Integrated with MALDI MSI, high-throughput spatially resolved proteoform analysis were achieved, yielding 366 annotated proteoform images from the mouse brain and revealing 14 differential proteoforms in the subiculum region associations with amyloid-β pathology in AD.

User consent:

yes

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Spatial Phosphoproteomic Profiling of Murine Heart Reveals Region-Specific Functions via TiO_2 Enrichment Optimized for Laser-Capture Microdissected Samples

Author: Dan Zhao[None]

Corresponding Author: 13817801534@163.com

Phosphorylation-mediated signaling dynamics across spatially distinct cardiac regions remain poorly understood due to limited technical capacity for deep and sensitive analysis of minute samples. Here, we present an optimized TiO_2-based micropipette tip method for deep phosphoproteomics, achieving high sensitivity (12,117 class I phosphosites from only 10 μg HeLa peptides) and reproducibility. Applying this to laser-capture microdissected mice myocardial regions, i.e. left/right atria (LA, RA), left/right ventricles (LV, RV), interventricular septum (IVS), apex (APEX), and aortic valve (AV), we quantified 1,000–2,000 class I phosphosites per region (e.g., 1,050 in AV, which has an area of only 0.2 mm^2). Principal component analysis revealed distinct phosphoproteomic clustering aligned with anatomical positions, surpassing proteomic resolution. Functional enrichment uncovered region-specific functions: APEX and ventricles exhibited phosphorylation signatures linked to muscle contraction, while AV was enriched in cell junction and polarity. Metabolically, the LV demonstrated phos-phorylation patterns linked to energy metabolism, whereas LA showed enrichment in RNA processing. RA was pertinent to cellular component biogenesis and chromatin organization. This spatially resolved phosphoproteomic atlas elucidates func-tional specialization across cardiac sub-regions, establishing a molecular foundation for investing region-specific cardiac pa-thologies. Our approach addresses critical technical limitations in low-input phosphoproteomics while advancing under-standing of cardiac spatial heterogeneity at the post-translational level.

User consent:

yes

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FLASHApp: Interactive Data Analysis and Visualization for Top-Down Proteomics

Author: Tom David Müller[1]

Co-authors: Jihyung Kim [1]; Andrew Almaguer [2]; Ayesha Feroz [1]; Axel Walter [1]; Jaekwan Kim [3]; Wonhyeuk Jung [4]; Oliver Kohlbacher [1]; Kyowon Jeong [1]

[1] *University of Tübingen*

[2] *jambit GmbH*

[3] *Fullseeomics*

[4] *Astrazeneca*

Corresponding Authors: kyowon.jeong@uni-tuebingen.de, axel.walter@uni-tuebingen.de, oliver.kohlbacher@uni-tuebingen.de, tom.mueller@uni-tuebingen.de, jihyung.kim@uni-tuebingen.de, drewbudd@gmail.com, won.jung@astrazeneca.com, ayesha.feroz@uni-tuebingen.de, jkvision1101@fullseeomics.com

Introduction
Top-down proteomics (TDP) is increasingly being applied in proteoform-resolved biomedical and clinical research. The complexity of TDP data demands flexible visualization tools integrated with analysis workflows to streamline interpretation and validation. Existing tools often lack adaptability and interactivity, requiring complimentary manual analysis to generate publication-ready results and figures. This added layer of manual intervention impacts reproducibility, posing a significant challenge to consistent scientific outcomes.
FLASHApp addresses these challenges by providing an interactive solution for TDP data analysis and visualization. As a free, open-source, web-based application it is accessible on any modern computer at https://www.openms.org/FLASHApp/.

Methods
FLASHApp is based on the OpenMS Streamlit template. It supports methods involving TDP tools for spectral deconvolution, quantification, and characterization of proteoforms. In addition to being publicly accessible via the OpenMS website, FLASHApp can easily be hosted in-house (e.g. by core facilities) using a containerized image. A Windows installer eases offline execution for non-bioinformaticians.
Results
Upon entering FLASHApp, a new workspace is automatically created and embedded within the website URL, enabling users to bookmark their session, revisit analyses at a later time, and share results with collaborators.
The app's sidebar organizes common TDP tasks into dedicated sections, each guiding users through uploading input files, configuring parameters, executing tools, visualizing outputs, and downloading results. Tailored, publication-ready visualizations presented in a user-configurable layout address the specific requirements of each task, enhancing both interpretability and efficiency of data analysis. The interactive nature of these visualizations allows dynamic adjustments, such as modifying fragment ion matching tolerances.
Conclusion
FLASHApp provides modular, interactive visualizations specifically designed for TDP data analysis, improving usability, reproducibility, and accessibility through both web-based and local setup. These features position FLASHApp as a flexible, user-friendly platform that streamlines TDP workflows, facilitates collaboration through shareable URLs, and aids reproducible scientific discovery.

User consent:

yes

Lightning Talks / **32**

Metabolomics and proteomics reveal the inhibitory effect of Lactobacillus crispatus on cervical cancer

Author: Lingyan Zhong[None]

Corresponding Author: 21110220043@m.fudan.edu.cn

Cervical cancer remains a significant global health issue due to its high morbidity and mortality rates. Recently, Lactobacillus crispatus has been recognized for its crucial role in maintaining cervical health. While some studies have explored the use of L. crispatus to mitigate cervical cancer, the underlying mechanisms remain largely unknown. In this study, we employed non-targeted proteomics and metabolomics to investigate how L. crispatus affects the growth of cervical cancer cells (SiHa) and normal cervical cells (Ect1/E6E7). Our findings indicated that the inhibitory effect of L. crispatus on SiHa cells was associated with various biological processes, notably the ferroptosis pathway. Specifically, L. crispatus was found to regulate the expression of proteins such as HMOX1, SLC39A14, VDAC2, ACSL4, and LPCAT3 by SiHa cells, which are closely related to ferroptosis. Additionally, it activated the tricarboxylic acid (TCA) cycle in SiHa cells, leading to increased levels of reactive oxygen species (ROS) and lipid peroxides (LPO). These results revealed the therapeutic potential of L. crispatus in targeting the ferroptosis pathway for cervical cancer treatment, opening new avenues for research and therapy in cervical cancer.

User consent:

yes

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SEC-complex-down approaches with functional O2-affinity assay:

Correlation between the higher order structure of bird hemoglobin homologues and their function.

Author: Léa Letissier[1]

Co-authors: Christine Schaeffer [1]; Fabrice Bertile [2]; François Criscuolo [2]; Oscar HERNANDEZ ALBA [3]; Sarah Cianférani [1]; Turkan Nabiyeva [1]

[1] *LSMBO - CNRS (Université de Strasbourg)*

[2] *DEPE - CNRS (Université de Strasbourg)*

[3] *CNRS*

Corresponding Authors: sarah.cianferani@unistra.fr, ahernandez@unistra.fr, turkan.nabiyeva2@etu.unistra.fr, f.criscuolo@unistra.fr, christine.schaeffer@unistra.fr, lea.letissier@etu.unistra.fr, fbertile@unistra.fr

Hemoglobin is a tetrameric protein responsible for the blood oxygen transport. Any abnormalities in hemoglobin structure can lead to serious health outcomes. Although very well characterized in humans, it has been scarcely studied in the case of birds. Some studies have been carried out at the globin level without providing further evidences about the native structure of the complex. In this context, we propose for the first time the combination of size-exclusion chromatography with complex-down mass spectrometry for the straight correlation of binding stoichiometry of subunits, their primary structure, and the identity of different cofactors. Complex-up analysis were carried out to decipher the identity of the individual constituents of each tetramer population by providing increased energy in the mass spectrometer ion source. Thus, it was concluded that mass differences between the three tetramers were due to the substitution of alpha subunits. In addition, one cofactor was also identified for the first time linked to the tetramers.
Information about subunit sequence characterization and cofactor identification were afforded through SEC-complex-down approach (pMS3) to isolate and fragment the different constituents. Fragmentation of subunits was achieved by combining various fragmentation methods leading to an overall sequence coverage of 98, 94, and 96% for aA, aD, and b subunits, respectively. The pMS3 approach allowed to determine the presence of IP5 cofactor, which is known to regulate 02-hemoglobin affinity in birds.
Finally, the three tetramers were collected separately upon SEC separation to record 02-affinity data for each tetramer. The functional data clearly showed affinity differences between the three populations, clearly pinpointing for the first time that bird hemoglobin affinity correlated with the number of aD subunits in the tetrameric structure. Altogether, the results from the SEC complex-down analysis in combination with O2-affinity data could find application in evolution analysis, environmental adaptation, or different clinical contexts.

User consent:

yes

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Multi-dimensional High-Throughput Molecular Glue Screening via Gas Phase Affinity Selection Native Mass Spectrometry and Cryo-EM Analysis

Author: Weijing Liu[None]

Co-authors: Amanda Lee [1]; Christopher Mullen [1]; Erin Fischer [2]; Ken Durbin [3]; Kheewoong Baek [2]; Rosa Viner [1]; Shane Bechler [1]; Thomas Moehring [1]; Wenfei Song [1]; Yuan Xiong [2]

[1] *Thermo Fisher Scientific*

[2] *Harvard Medical School*

[3] *Proteinaceous*

Corresponding Authors: amanda.lee3@thermofisher.com, weijing.liu@thermofisher.com

Targeted protein degradation of undruggable proteins is transformative in drug discovery. Molecular glues (MGs) enhance weak interactions between targets and E3 ligase. Native mass spectrometry (nMS) identifies E3-MG-target complexes directly, but manual sample preparation limits throughput.

This study demonstrates high-throughput MG screening using nMS for WEE1 binding to CRBN-DDB1, enabling multiplex screening of over 2,500 compounds per day. Gas-phase ligand release and fragmentation help identify unknown binders, and cryo-electron microscopy (EM) analysis characterizes ligand-bound complexes.

We compressed 96 compounds into 24 mixtures and used a SEC column for rapid online buffer exchange. LC-MS screening of all 96 compounds took under an hour, achieving throughput of over 2,500 compounds per day. Strong ternary complex formation between CRBN-DDB1 and WEE1 was observed in 4 of 24 mixtures, with 2 additional samples showing moderate binding. Identifying individual binders within mixtures was challenged by compound multiplexing, native adduct interference, and non-specific interactions.

To resolve this, ternary complexes were isolated in the quadrupole and subjected to collision-induced dissociation. Released binders, potentially uncharged in the gas phase, were detected via polarity switching. MS2 analysis of low m/z ions enabled accurate mass determination and comparison with the compound library. Ligands with unmatched masses were classified as “Unknown” and further analyzed by MS3 fragmentation for structural elucidation.

This workflow uses MS1 to detect intact complexes, MS2 to identify bound ligands, and MS3 to characterize unknowns, increasing throughput and specificity in MG screening. This approach confirmed 16 of 96 compounds as potential MGs, with varying binding strengths.

Selected hits were further characterized using cryo-EM, yielding high-resolution structures of WEE1-MG-CRBN-DDB1 ternary complexes. These structures reveal how MGs mediate and stabilize protein-protein interactions, offering critical insights to guide drug design and optimization.

User consent:

yes

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Legionella effector AnkX puts the brakes on IMPDH2 filaments

Author: Marietta Sandkamp-Kaspers[1]

Co-authors: Aymelt Itzen ; Bente Siebels ; Christian Hedberg ; Hubert Hilbi ; Pia Stauffer

[1] *UniversitÃ¤t Hamburg*

Corresponding Author: marietta.kaspers@uni-hamburg.de

Bacterial pathogens often utilize post-translational modifications to manipulate host cells. Legionella pneumophila, the causative agent of Legionnaires’ disease, secretes the enzyme AnkX, which is known to use cytidine diphosphate-choline to post-translationally modify the human small G-protein Rab1 with a phosphocholine moiety. Later during the infection, the Legionella enzyme Lem3 acts as a dephosphocholinase, hydrolytically removing the phosphocholine. While the molecular mechanism of Rab1’s post-translational modification has been extensively studied by us, we have identified a previously unknown host target protein of AnkX: the metabolic enzyme IMPDH2. IMPDH2 catalyses the conversion of IMP to XMP, which is crucial for guanine nucleotide biosynthesis. Each IMPDH2 monomer features a catalytic domain and a regulatory Bateman domain, which binds ATP and GTP to regulate enzymatic activity and filament formation. IMPDH2 reversibly assembles into

filaments in cells, which is thought to provide an additional layer of regulation. Currently we investigate the molecular mechanism and consequences of IMPDH2 phosphocholination by AnkX. Mass spectrometry revealed the modification site within IMPDH2 and demonstrated that, in contrast to Rab1, Lem3 cannot reverse the modification of IMPDH2. While the modification does not alter the catalytic activity of IMPDH2, it disrupts filament formation, thereby impairing a key regulatory mechanism of enzyme function.

User consent:

yes

Lightning Talks / **42**

Top-Down Mass Spectrometry of a Clinical Antibody Light Chain Using the Omnitrap-Orbitrap-Booster Platform

Authors: Camille Garcia[1]; Nina Khristenko[None]; TingTing Fu[1]; Karen Druart[1]; Konstantin O. Nagornov[2]; Marie Yammine[3]; Athanasios Smyrnakis[4]; Anton N. Kozhinov[2]; Dimitris Papanastasiou[4]; Yury Tsybin[None]; Julia CHAMOT-ROOKE[3]

[1] *Institut Pasteur, Université Paris Cité*

[2] *Spectroswiss*

[3] *INSTITUT PASTEUR - CNRS*

[4] *Fasmatech Science & Technology, Lefkippos TESPA*

Corresponding Authors: julia.chamot-rooke@pasteur.fr, khristenko@spectrotech.fr, marie.yammine@pasteur.fr, tsybin@spectroswiss.ch

The Omnitrap-Orbitrap-Booster (OOB) mass spectrometry (MS) platform with enhanced high-resolution, fragmentation and data acquisition capabilities, was developed to advance top-down (TD) MS analysis of proteins. It integrates a multimodal tandem mass spectrometry (MS/MS) ion trap system (OmnitrapTM) offering a wide range of fragmentation methods, a high-resolution Orbitrap Fourier transform mass spectrometer (FTMS), and a high-performance data acquisition system (FTMS Booster) to improve fragmentation efficiency and spectral quality by increasing the signal-to-noise (S/N) ratio of product ions. In this study, we evaluate the OOB platform for electron capture dissociation (ECD)-based TD MS analysis of a clinical multiple myeloma antibody light chain extracted from the patient' s (P15) urine sample, benchmarking its performance against the "gold-standard"electron transfer dissociation (ETD)-based TD MS on an Orbitrap EclipseTM. These analyses were performed with online coupling to a LC system operating in nanoflow mode. Comparable sequence coverage was obtained for single precursor charge state analysis between ECD-based TD MS on OOB and ETD-based TD MS on EclipseTM (68.2% vs. 74.3% respectively). ECD showed a lower spectral peak density and reduced redundancy of product ions. Moreover, the analysis of multiple precursor charge states (15+ to 19+) sequentially across 5 runs of LC-MS/MS on the OOB platform improves the sequence coverage to 93%, indicating its suitability for comprehensive characterization of protein. Thus, this study uniquely establishes the OOB platform as a highly powerful and efficient system for TD MS of proteins.

User consent:

yes

Lightning Talks / **43**

X-ray spectroscopy meets native mass spectrometry: probing gas-phase protein complexes

Author: Jocky Chun Kui Kung[1]

Co-authors: Alan Kádek [2]; Tomislav Damjanović [1]; Sandeep Mishra [1]; Thomas Kierspel [1]; Charlotte Uetrecht [1]

[1] *Centre for Structural Systems Biology*

[2] *Institute of Microbiology of the Czech Academy of Sciences - BIOCEV*

Corresponding Authors: alan.kadek@biomed.cas.cz, tomislav.damjanovic@cssb-hamburg.de, sandeep.mishra@cssb-hamburg.de, thomas.kierspel@desy.de, jocky.kung@cssb-hamburg.de, charlotte.uetrecht@cssb-hamburg.de

X-ray activation and dissociation of proteins and their non-covalent assemblies may elucidate structural and functional details complementary to established top-down mass-spectrometry techniques. This is attributed to the rapid, site-specific ionization of atoms within biomolecules. Our research group conducted proof-of-concept experiments exploring X-ray activation of samples with masses ranging from small 17 kDa monomeric proteins to large 800 kDa non-covalent protein complexes at synchrotron (PETRA III) and free-electron laser (FLASH2) facilities. A quadrupole time-of-flight mass spectrometer, adapted for high-mass analysis, was further modified to enable photon-ion interactions. Native proteins and their complexes were introduced into the gas phase via nano-electrospray ionization and exposed to either extreme ultraviolet (FLASH2) or soft X-ray (PETRA III) radiation, in either their native folded state or following gas-phase collision-induced activation. The resulting effects—fragmentation, dissociation, or enhanced ionization—varied depending on biomolecule size and activation method. We also explored the integration of ion mobility to enhance structural separation prior to X-ray probing. Within the rapidly evolving domain of X-ray technologies, the activation of large proteins and their complexes via X-rays holds significant potential for advancing top-down analysis and structural biology research.

User consent:

yes

Lightning Talks / 55

A prototype TIMS-FT-ICR MS instrument capable of deep characterisation of complex samples and biomolecules

Author: Christopher Wootton[1]

Co-authors: Alina Theisen [1]; Gregory Brabeck [1]

[1] *Bruker Daltonics*

Corresponding Author: christopher.wootton@bruker.com

Trapped ion mobility spectrometry (TIMS) spatially separates ions when suspended in a high-pressure region between a retarding electric field gradient and constant gas flow from the atmospheric pressure inlet of the mass spectrometer. TIMS allows high resolution separation of ions/isomers/conformers with varying duty cycles in a relatively small device (<10cm). TIMS is also particularly well suited to slower scan speed instruments such as FT-ICR MS via the use of gated-TIMS. Herein we show initial data from a novel, fully integrated gTIMS-FT-ICR MS instrument, optimisation of this marriage, and its application to key areas such protein conformer-selective ExD MSMS.

The new gTIMS-MRMS system codenamed MATCH was built by combining commercial SolariX MRMS and TIMS-ToF Flex (Bruker Daltonics, Germany) instruments into a prototype. The front of the TIMS Tof Flex , including dual ESI+MALDI source, accumulation during separation dual-TIMS cartridge, mass-resolving quadrupole, and collision cell were combined via a custom transfer region

with the back-end elements of the SolariX MRMS system; UHV-isolating beam valve, ~1m transfer hexapole to inject ions through the fringe field of the 7T superconducting maxwell magnet, Paracell detector with electron dissociation (ExD) cathode, and 2-omega detection.

Native MS analysis of proteins was conducted on model isolated proteins to investigate the ability of gTIMS to separate and analyse proteins of interest without significantly affecting structure. Match was developed to use an ultra-low energy transfer and storage energy gradient throughout the TIMS separation, storage, and transfer processes, enabling batch-accumulation of conformer ensembles selected for enhanced dynamic range of downstream MS/MS. Collision-induced unfolding (CIU) was achieved using gradient potentials between the accumulation and analysis portions of the TIMS funnel. Subsequent IMS and quad selections of conformers of interest along the CIU profile followed by ECD MS/MS in the ICR cell revealed changes in fragmentation patterns depending on the conformer selected.

User consent:

yes

Lightning Talks / 57

Pin-pointing phosphorylation-dependent Pin1 binding to a cytoskeletal protein altered in Alzheimer's Disease using structural mass spectrometry

Authors: Danielle Kay[1]; Nikolas Brooks[1]; Simon Caulton[1]; Hiruni Jayasekera[1]; Andrew Lovering[1]; Aneika Leney[1]

[1] *School of Biosciences, University of Birmingham, Birmingham, B15 2TT*

Corresponding Author: nxb780@student.bham.ac.uk

Abnormal protein phosphorylation is a fundamental trigger in the pathogenesis of Alzheimer's Disease, leading to the formation of neurofibrillary tangles. Thus, molecular determination of the critical factors in controlling phosphorylation is in high demand. Pin1, a cis-trans prolyl isomerase has recently been implicated in Alzheimer's Disease progression. Moreover, Pin1 specifically targets phosphoproteins, regulating their function. Here, we utilise a combination of native MS and top-down MS to reveal a novel interaction between Pin1 and the Collapsin Response Mediator Protein-2 (CRMP2); a protein found hyperphosphorylated alongside tau within neurofibrillary tangles. Using native mass spectrometry, we show that Pin1 binds specifically to the disordered C-terminus of CRMP2 in a phosphorylation-dependent manner. Hydrogen-deuterium exchange mass spectrometry experiments further localised this binding site to the WW-domain of Pin1. Together, the data highlights how mass spectrometry has been utilised to provide novel insight into the regulatory role of Pin1 in a disease-relevant context.

User consent:

yes

Lightning Talks / 63

Enhancing Drug-Payload Localization in Antibody-Drug Conjugates with a Middle-Down Approach Utilizing Proton Transfer Charge Reduction on an Orbitrap Ascend BioPharma Tribrid Mass Spectrometer

Author: Linda B Lieu[1]

Co-authors: Christopher Mullen [2]; Cynthia Nagy [1]; David Bergen [2]; Graeme C. McAlister [2]; Jingjing Huang [2]; Kenneth R. Durbin [3]; Kristina Srzentić [2]; Luca Fornelli [1]; Rachel Grady [2]; Rafael Melani [2]; Vlad Zabrouskov [2]

[1] *University of Oklahoma*

[2] *Thermo Fisher Scientific*

[3] *Proteinaceous, Inc.*

Corresponding Authors: rachel.grady@thermofisher.com, rafael.melani@thermofisher.com

Monoclonal antibodies (mAbs) have revolutionized biotherapeutics, offering effective treatments for various diseases. Antibody-drug conjugates (ADCs) combine mAbs' specificity with potent cytotoxic drugs linked via Lys- or Cys-conjugation, targeting malignant cells. However, heterogeneity in payload attachment challenges ADCs' safety and efficacy. We employed a middle-down (MD) mass spectrometry (MS) approach to investigate a SiLu ADC mimic with variable drug-to-antibody ratio (DAR) using the Orbitrap Ascend BioPharma Tribrid mass spectrometer. By integrating native MS of 100 kDa F(ab')2 subunits with disulfide-reduced 25 kDa subunit analysis under denaturing conditions, we achieved thorough ADC characterization and precise localization of payload conjugation sites. Native MS analysis of F(ab')2 subunits isolated specific DARs with high purity, determining major payload occupancy combinations for DARs 2-8. Our disulfide-reduced subunit analysis aimed to localize payloads on Fd', which has up to three conjugation sites. While higher-energy collisional dissociation (HCD) and ultraviolet photodissociation (UVPD) provided limited diagnostic ions, Electron Transfer Dissociation (ETD) and ETD followed by supplemental HCD energy (EThcD) localized the conjugation site in the most abundant Fd'species with a single payload at the MS2 level. Minor single payload species and double payload species required spectral decongestion via proton transfer charge reduction (PTCR) at the MS3 level for higher ion count and sequence coverage. EThcD MS2-PTCR MS3 uniquely localized payloads for all species combinations, achieving ~60% sequence coverage. This approach provided unambiguous localization of drug-payload attachment sites through PTCR following EThcD on the Orbitrap Ascend BioPharma MS.

User consent:

yes

Lightning Talks / 95

Digital Membrane Chromatography —A New Way to Rapid Antibody Purification for Top-Down MS

Author: Kilian Müller[1]

Co-authors: Julia Rößler [2]; Anna Klassen [2]; Hannes Passuth [2]; Florian Schmitt [2]; Bente Siebels [1]; Hartmut Schlüter [1]; Maria Riedner [3]

[1] *University Medical Center Hamburg-Eppendorf*

[2] *i3 Membrane*

[3] *University of Hamburg*

Corresponding Authors: b.siebels@uke.de, hschluet@uke.de

Antibody purification is a critical step in manufacturing and analytical workflows, yet conventional Protein A affinity chromatography often requires acidic elution, risking structural alterations and necessitating additional processing for mass spectrometry (MS). We present a Protein A-functionalized regenerated cellulose membrane adsorber with conductive gold-coated layers, enabling voltage-triggered elution—termed Digital Membrane Chromatography (DMC). Using Trastuzumab as a model, DMC achieved efficient antibody capture and mild, MS-compatible elution without desalting or buffer exchange. This approach preserves structural integrity, supports analysis from complex matrices, and reduces processing time compared to conventional methods.

User consent:

Native MS & Protein Complexes / 9

Uncovering the unique properties of circulating proteasomes: A mass spectrometry perspective

Authors: David Morgenstern[1]; Gili BeN-Nissan[2]; Katharina Zittlau[2]; Michal Sharon[2]; Yegor Leushkin[2]

[1] *The Nancy and Stephen Grand Israel NationalCenter for Personalized Medicine, Weizmann Institute of Science*

[2] *Department of Biomolecular Sciences, Weizmann Institute of Science*

Corresponding Authors: katharina.zittlau@weizmann.ac.il, david.morgenstern@weizmann.ac.il, michal.sharon@weizmann.ac.il, gili.ben-nissan@weizmann.ac.il, yegor.leushkin@weizmann.ac.il

Proteasomes are well-known mediators of intracellular proteostasis, yet their role in the extracellular space remains largely unexplored. Our recent study investigates the molecular architecture and functional specialization of freely circulating 20S proteasomes (c20S) in the bloodstream. Leveraging a CRISPR-engineered transgenic mouse model, we purified c20S complexes and applied a combination of native and top-down mass spectrometry to dissect their structural and compositional features. Our analyses revealed that serum proteasomes are predominantly uncapped 20S complexes, assembled intracellularly and exported to the blood. Native MS confirmed their intact assembly, while top-down MS identified a suite of post-translational modifications—including cysteinylation, glutathionylation, and truncations—that distinguish c20S from intracellular proteasomes. These extracellular complexes are enriched in immunoproteasome subunits and display enhanced caspase-like activity, indicating specialized roles in the blood environment. Together, these results showcase the power of integrative MS approaches in characterizing proteasome proteoforms and underscore the unique biology of circulating proteasomes, with potential implications for diagnostics and extracellular proteostasis.

User consent:

yes

Native MS & Protein Complexes / 16

Flying viruses –mass spectrometry meets X-rays

Author: Charlotte Uetrecht[1]

[1] *CSSB/DESY/University of Lübeck*

Corresponding Author: charlotte.uetrecht@cssb-hamburg.de

Viruses affect basically all organisms on earth. Some are detrimental to human development as we experienced during the COVID-19 pandemic, whereas those targeting pathogenic bacteria or crop pathogens can be beneficial for us. An integral part of icosahedral viruses is the capsid protein shell protecting the genome. Many copies of the capsid protein often self-assemble into shells of defined size. Low binding affinity of individual subunits allows efficient assembly and gives rise to highly stable particles. However, modifications can alter their size. Proteoforms also matter in viral replication, e.g. in polyprotein processing, or antigenicity of the glycoproteins.
Capsids and viral proteoforms can be studied by native mass spectrometry (MS), a single molecule like approach, in terms of stoichiometry, dynamics, assembly pathways and stability revealing coexisting states. However, the structural resolution provided is limited. Therefore, we built a prototype native mass spectrometer in the MS SPIDOC project to deliver select species to X-ray sources for

gas phase SAXS and single particle imaging. First experiments reveal good performance of the MS setup.

User consent:

yes

Native MS & Protein Complexes / **7**

Filling the Structural Knowledge Gap in Protein Design via Native Mass Spectrometry

Author: Mowei Zhou[1]

Co-authors: Shiwen Zhou [1]; Beiyao Fu [1]; Xinjie Yang [2]; Pengfei Ji [1]; Fangrui Zhong [2]; Yuanjiang Pan [1]

[1] *Zhejiang University*

[2] *Huazhong University of Science and Technology*

Corresponding Authors: moweizhou@zju.edu.cn, chemzfr@hust.edu.cn, jipengfei@zju.edu.cn, panyuanjiang@zju.edu.cn

Enzymes are powerful molecules for highly efficient and sustainable chemical synthesis. However, natural enzymes often have limitations and require optimization for large-scale industrial applications. Propelled by artificial intelligence, computational advances such as AlphaFold opened new venues for structure-based enzyme design. However, experimental validation still largely relies on high throughput screening (HTS) for functional and phenotypical data. HTS methods for structural screening only offer limited data and cannot readily resolve multi-component or heterogeneous systems. Only a few successful candidates will have the chance for in-depth structural biology analysis to interpret the mechanism due to the low throughput.
Furthermore, the majority of the "failed"designs can't be easily formulated into meaningful knowledge to improve the computational models, leading to significant losses of research resources. Native mass spectrometry (native MS) can characterize molecular structures and interactions with fast speed, potentially filling the missing mechanistic knowledge in HTS methods. When combined with top-down MS techniques (native top-down, complex-up, etc.), subunit and residue level information can also be extracted. Recently, native MS have been integrated with computational drug design workflows for finding drug candidates. But more fundamental studies and method developments are still needed to accurately define the correlation of subtle structure features with native MS data, especially for weak interactions.
Herein we selected an artificial triplet photoenzyme RamR with two of its tailored triplet quenchers, and investigated their interactions. The two molecules has different potency in enzyme inhibition, but only differ by the presence/absence of a carboxyl group. Our preliminary data suggested the enzyme-inhibitor interaction was significant but likely non-selective on protein surface. Further experiments are ongoing to examine the enzyme-inhibitor interaction in presence of the native substrate. In summary, the native MS data improve our understanding of the biophysical mechanism of the molecular interactions, and help inform rationale design for enzymes.

User consent:

yes

Databases & Bioinformatics / **48**

Advancing Top- and Middle-Down Antibody Analysis Using Simulated FTMS Datasets

Authors: Nina Khristenko[1]; Konstantin Nagornov[2]; Marie Yammine[3]; Natalia Gasilova[4]; Anton Kozhinov[2]; Laure Menin[4]; Julia Chamot-Rooke[3]; Yury Tsybin[None]

[1] *Spectrotech*

[2] *Spectroswiss*

[3] *Institut Pasteur*

[4] *EPFL*

Corresponding Authors: julia.chamot-rooke@pasteur.fr, marie.yammine@pasteur.fr, kozhinov@spectroswiss.ch, tsybin@spectroswiss.ch, nagornov@spectroswiss.ch, khristenko@spectrotech.fr, laure.menin@epfl.ch, natalia.gasilova@epfl.ch

The structural complexity and heterogeneity of monoclonal antibodies (mAbs) continue to pose analytical challenges. Over the past three decades, top-down (TD) and middle-down (MD) MS approaches have become powerful tools for characterizing intact antibodies and their subunits [1]. These methods are routinely applied in our CRO operations to complement intact mass and bottom-up proteomics, particularly for resolving complex structural questions in real-world mAb samples. However, despite major advances in instrumentation and data analysis, the structural detail gained from current TD/MD MS workflows remains limited.

A key obstacle to improving TD/MD MS bioinformatics is the lack of standardized, annotated benchmark datasets. To address this, we launched the ProteoGold initiative. It is focused on generating high-quality, in silico FTMS datasets that mimic the complexity of real experimental spectra. Using the proprietary FTMS Simulator (Spectroswiss), we produce isotopically resolved datasets based on user-defined instrument settings and known protein sequences [2]. The simulator is available as a desktop application for full-spectrum simulations and as a web-based platform at www.peakbypeak.com for real-time isotopic modeling, profile-mode spectrum generation, and hybrid server-side processing.

As a proof of concept, we generated a simulated ETD TD MS dataset of carbonic anhydrase II, modeled after data from a 21 T FT-ICR MS at the MagLab [3]. Additional simulations include TD/MD MS datasets of mAbs and subunits acquired on various FTMS platforms, such as Orbitraps. For example, we simulated data from an antibody light chain analyzed on the Omnitrap-Orbitrap-Booster (OOB) platform at the Institute Pasteur, Paris [4].

These datasets form the foundation of the ProteoGold repository, supporting benchmarking, deconvolution evaluation, improved ion assignment, and driving innovation and education in TD/MD MS analysis.

1. Khristenko, et al., Mol. Cell Prot., in press
2. Nagornov, et al., JASMS (2022) 1113-1125
3. Weisbrod et al., JASMS (2017) 1787–1795
4. Garcia et al., submitted

User consent:

yes

Databases & Bioinformatics / 11

Computational methods in top-down proteomics to address challenges in proteoform analysis

Author: Kyowon Jeong[1]

[1] *University of Tübingen*

Corresponding Author: kyowon.jeong@uni-tuebingen.de

Top-Down Proteomics (TDP) has emerged as the dominant method for elucidating the intricacies of proteoform diversity, providing insights crucial for understanding biological processes. With development ranging from sample preparation to instrumentation, there has been a notable increase in research endeavors adopting and developing different TDP protocols that suit the objectives of the studies. Moreover, the information density within TDP data sets have grown dramatically, and more TDP data sets are being deposited in the public repository like PRIDE.

Fully realizing the potential of TDP for proteoform resolved analysis requires robust, flexible, and reproducible computational methods capable of handling the complexity of analytes (proteoforms) and data (spectra), while also accommodating the different requirements inherent in each experimental protocol. Due to distinct characteristics (e.g., complexity of ion signals), the computational tools used in the well-established field of bottom-up proteomics (BUP) cannot be readily adopted for TDP; dedicated methods are still demanded for the data analysis, data acquisition, and signal processing.

In this talk, I will introduce our contribution to the field of computational TDP, which include various computational methods such as deconvolution and quantification. I will provide a concise overview of the main concepts and core results of each method, and outline the future directions our group intends to pursue. Finally, the current project for the proteoform identification and characterization method that push the boundaries of the existing search engines will be discussed.

User consent:

yes

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TDAuditor assesses deconvolution quality for the Blood Proteoform Atlas

Author: David Tabb[1]

Co-authors: Julia St.Germain [2]; Kyowon Jeong [3]; Mathias Walzer [4]

[1] *University Medical Center of Groningen*

[2] *University College Groningen*

[3] *University of Tuebingen*

[4] *European Bioinformatics Institute*

Corresponding Authors: mathias.walzer@gmail.com, kyowon.jeong@uni-tuebingen.de, d.l.tabb@rug.nl, j.f.st.germain@student.rug.nl

The new TDAuditor software generated quality metrics across the 1551 RAW files of the CTDP Blood Proteoform Atlas (BPA). The algorithm incorporates both spectral clustering and de novo sequence tagging. Multi-threading makes it possible to evaluate 100 mzMLs per minute on a standard desktop PC. The software produces reports in tab-delimited text and mzQC (JSON) formats.

The metrics reported by TDAuditor from the BPA illustrate substantial differences in deconvolution among ProSight PD's "Xtract", TopPIC Suite's "TopFD", and OpenMS' "FLASHDeconv." The precursor charge states and number of masses produced from MS/MS scans have only superficial agreement. A re-identification of all 1551 BPA experiments via TopPIC Suite shows that TDPortal and TopPIC are using very different search spaces, making identifications even more diverse among pipelines than deconvolution differences would suggest.

The advanced signal processing in TDAuditor seeks redundancy among MS/MS scans and attempts to predict identifiability on the basis of deconvolution outputs. Spectral clustering compares deconvolved mass lists for every pair of MS/MS scans in a given mzML file. The resulting graphs of MS/MS relationships illustrate the redundancy of top-down MS/MS measurement and reveal the high-level

structure of a top-down experiment. Sequence tagging attempts to infer contiguous amino acid sequences from deconvolved MS/MS scans. The length of the longest tag from an MS/MS scan can predict its identifiability; researchers can use these values to find the "best MS/MS that our search didn't identify."

TDAuditor offers top-down researchers a much more complete appraisal of the LC-MS/MS experiments they generate, and the software is free to use and to modify.

User consent:

yes

Databases & Bioinformatics / 22

The Implementation of Open Science Practices Can Enable A Faster Development Of Top-Down Proteomics

Author: Juan Antonio Vizcaino[1]

[1] *European Bioinformatics Institute (EMBL-EBI)*

Corresponding Author: juan@ebi.ac.uk

Open data and science practices, including e.g. the FAIR (Findable, Accessible, Interoperable and Reusable) data principles, are widely implemented in the life sciences. Although this process started years later in proteomics than for other more established omics approaches (e.g. genomics and transcriptomics), their implementation in the field have enabled spectacular advances e.g. in analytical and computational data workflows, including the integration of large amounts of proteomics data in bioinformatics data resources. In the concrete case of top-down proteomics (TDP) and proteoform-centric data, the implementation of open science practices has been more limited due to different reasons, and there is the need for some key new developments. In my view, one of the main priorities of the TDP field should be to fully endorse and implement them to enable new approaches that would help to develop the field faster and a wider dissemination of the outputs of the field. In my talk, I will describe some concrete needs and ideas in this context for TDP and proteoform data

User consent:

yes

Sample Preparation & Separation Technologies / 27

Enabling High-Throughput Proteoform Analysis via Gel-Based Sample Pre-Fractionation with PEPPI-SP3

Author: Nobuaki Takemori[1]

[1] *Ehime University*

Corresponding Author: takemori@m.ehime-u.ac.jp

Achieving deep proteoform coverage in top-down proteomics critically depends on effective sample pre-fractionation. To address this, we developed PEPPI-MS (Passively Eluting Proteins from Polyacrylamide gels as Intact species for MS) in 2020, leveraging the widely used SDS-PAGE method in biochemistry as a tool for pre-fractionation. PEPPI enables efficient passive extraction of intact proteins from gels, offering a simple, cost-effective, and highly reproducible workflow. Since its

introduction, PEPPI has been adopted in various top-down proteomics studies, and more recently, its importance is becoming increasingly more recognized in middle-down proteomics as well. Its potential applications include disease biomarker discovery in top-down/middle-down proteomics although high-throughput processing of large sample cohorts remains a major challenge at present. Automation of the workflow will therefore become essential. In this presentation, we introduce "PEPPI-SP3," our latest workflow combining PEPPI with the magnetic bead-based SP3 method, an established platform for automated sample preparation in bottom-up proteomics, as a promising step forward toward future automation in proteoform-level proteomics.

User consent:

yes

Sample Preparation & Separation Technologies / 37

Characterization of proteoforms of intact proteins by CE-MS and LC-CE-MS

Author: Christian Neusüß[1]

Co-authors: Tobias Waldmann [1]; Alisa Höchsmann [1]

[1] *Hochschule Aalen*

Corresponding Author: christian.neusuess@hs-aalen.de

Electromigrative techniques are powerful tools for the separation of intact proteins and their proteoforms. However, CE-MS is still restricted by the sensitivity and ease-of-use of the interface in conjunction with low injection volumes limiting its application for biological samples. Various solutions will be presented here overcoming these shortcomings.
Initially, the power of CE-MS for the characterization of proteoforms will be presented applying the nanoCEasy interface [1]. Efficient separation of proteins and proteoforms depends strongly on the applied capillary coating. Very recently we developed efficient coatings enabling finetuning the EOF [2] and, thus, increase the separation efficiency for proteins of certain mobility. Results on the application for protein separation from biological samples will be presented.
nanoLC-CE-MS is a promising tool for targeted protein and proteoform analysis in biological samples. Initially a heart-cut nanoLC-CE-MS was setup and the performance regarding improved sensitivity as well as separation of proteoforms was demonstrated [3]. Due to the increased loadability, the nanoLC-CZE-MS setup exhibits a strongly improved increased concentration sensitivity compared to CZE-MS. The combination of high sensitivity and orthogonal selectivity enables the detailed characterisation of intact proteoforms at physiologically relevant concentrations. A novel selective comprehensive online nanoLC-CE-MS configuration will be presented and discussed in the context of targeted proteoform analysis in biological samples using proteoforms of histone as an example.

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User consent:

yes

Sample Preparation & Separation Technologies / 69

Impact of sample preparation methods on proteoform identification by top-down proteomics

Author: Andreas Tholey[None]

Co-authors: Kyowon Jeong ; Oliver Kohlbacher ; Philipp T. Kaulich

Corresponding Author: a.tholey@iem.uni-kiel.de

Numerous workflows have been developed for top-down proteomics (TDP). We systematically investigated the influence of different sample preparation steps on proteoform and protein identifications, including cell lysis, reduction and alkylation, proteoform enrichment, purification, and fractionation [1]. We found that all steps in sample preparation influence the subset of proteoforms identified (e.g., their number, confidence, physicochemical properties, and artificially generated modifications). The various sample preparation strategies resulted in complementary identifications, significantly increasing the proteome coverage. Overall, more than 13,000 proteoforms from more than 2,700 proteins of human Caco-2 cells were identified.
The results presented can serve as suggestions for designing and adapting TDP sample preparation strategies to particular research questions. Moreover, the sampling bias and modifications identified at the intact protein level will also be useful in improving bottom-up proteomics approaches.

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User consent:

yes

Sample Preparation & Separation Technologies / 94

Proteoforms in Tissues –Approaching Their Native Composition with Nano- and Pico-Second Infrared Laser Systems

Author: Hartmut Schlüter[1]

[1] *Institute of Biochemistry*

Corresponding Author: hschluet@uke.de

To analyze tissue molecules accurately, they must be first solubilized. Conventional sampling and homogenization methods disrupt cell compartments, releasing enzymes that can alter proteoforms through proteolysis and modifications of post-translational modifications (PTMs), thereby changing their original composition.
Using nano- or pico-second infrared laser systems (NIRL or PIRL) for tissue sampling minimizes this issue significantly. Their ultrafast sampling and homogenization processes prevent enzymatic activity from altering proteoforms, preserving their native state. Moreover, the gentle, rapid approach reduces fragmentation of proteoforms during sample preparation.
The current NIRL and PIRL systems achieve a spatial resolution of approximately 20 x 20 x 20 µm voxels, approaching single-cell resolution. In summary, NIRL and PIRL-based sampling and homogenization techniques enable a more accurate representation of proteoforms in tissues, bringing analysis closer to their original biological state.

User consent:

yes

Sample Preparation & Separation Technologies / 45

Comparison of RP-LC with CE for histone analysis

Author: Ansgar Poetsch[1]

Co-author: Can Xu [1]

[1] *Nanchang University*

Corresponding Author: ansgarpoetsch@ncu.edu.cn

Introduction

Histones are heavily and variably decorated by PTMs, thereby affecting their binding to chromosomal regions. Top-down proteomics of histones is advantageous in capturing the PTM combination to obtain epigenetic status. Being rich in lysine residues hampers histone separation by RP chromatography. Capillary electrophoresis (CE) –MS has emerged as powerful alternative for histone analysis. Thus, ZipChip(CE) was benchmarked against nanoRP-LC with a C4 column.

Methods
Calf thymus histone preparation was dissolved in water to 1 mg/ml. For CE 5 ng (5 nl) were injected per analysis and separated in 6 min on a HS CE chip at a field strength of 500V / cm2 in intact antibody BGE buffer. MS and MS/MS spectra were recorded on an orbitrap Ascend set to intact protein mode using instrument templates (<30 kDa intact protein). Protein XML files of Bos Taurus histones were imported into Prosight 4.2/Proteome Discoverer 3.0 for proteoform identification. For LC, 2 µg protein were separated on C4 column in 50 min.

Results and Discussion

The HS chip separated the histones into two major peaks, whereas LC displayed broad elution without baseline separation. The preparation contained histones H1, H2a, H2b, H3, and H4, all of which were identified in the merged MS data (CID, HCD, ETD, EThCD, UVPD). In total, 216 proteoforms and 6291 PrSMs (proteoform spectral matches) were identified at high confidence with CE compared to 569 proteoforms and 35091 PrSMs with RP-LC. PrSMs displayed a most frequent cleavage efficiency of 10%. PrSM count increased from UVPD, EThCD, ETD, HCD to CID. Even though the longer analysis time and higher protein load increased identification, separation performance and speed favored CE. The fast analysis time in combination with very low sample consumption qualify CE as a convenient tool for optimization and data acquisition over a wide range of MS settings.

User consent:

yes

New approaches for proteoform analysis / 49

Advances in Orbitrap mass spectrometry for top-down analysis

Author: Alexander Makarov[1]

[1] *Thermo Fisher Scientific (Bremen) GmbH*

Corresponding Author: alexander.makarov@thermofisher.com

The analysis of intact proteins and protein complexes presents unique challenges to mass spectrometry (MS), prompting a fundamental re-evaluation of principles previously validated for small molecules and peptides. As the initial limitations of fragmentation have largely been overcome in the past two decades—culminating in near-complete freedom of fragmentation in the latest instruments—severe spectral congestion has emerged as a major obstacle, persisting despite advances in modern liquid separation techniques.
Until recently, this issue could only be addressed by high-resolution MS, preferably featuring isotopic resolution and accurate mass. Orbitrap mass spectrometry has proven particularly effective in such analyses, but further progress has been hampered by inherently limited dynamic range for species that overlap in m/z space.
Nowadays, this limitation can be addressed using two distinct approaches: charge reduction and direct charge detection. This presentation explores the practical implementation of both techniques on the latest generation of Orbitrap instruments.
Charge reduction is achieved through high-speed ion–ion reactions, allowing proteins to shed a number of protons and shift to significantly higher and less congested m/z values. By stepping a narrow m/z isolation window in a data-independent manner, this method enables detection of up to ten times more proteoforms—not only for intact proteins, but also for protein complexes. It is also compatible with spectra produced by a broad range of fragmentation techniques.
For direct charge detection, Direct Mass Technology (DMT) allows the charge states of individual ions to be determined in parallel for hundreds to thousands of ions. While typically suited to low-intensity ion beams, DMT is shown to offer intriguing possibilities for top-down analysis as well.
In conclusion, recent rapid advances all aspects of mass spectrometry instrumentation and analysis are shown to open up exciting new possibilities for top-down proteomic analysis.

User consent:

yes

New approaches for proteoform analysis / 91

Exploring Spatial Top-Down Proteomics

Author: Ljiljana Paša-Tolić[1]

[1] *Pacific Northwest National Laboratory, Richland, WA 99354, USA*

Corresponding Author: ljiljana.pasatolic@pnnl.gov

Understanding proteins within their functional contexts, whether in tissue units, cellular neighborhoods, small clusters of cells, or even at the single-cell level, remains a significant scientific challenge that pushes the boundaries of analytical methods. Traditional proteomic approaches largely rely on antibody-based techniques, which limit multiplexing and require prior knowledge of target proteins. While advancements like nanoPOTS-based bottom-up proteomics offer promising tools for analyzing small tissue sections, even single cells, these methods fall short in capturing proteoform-specific information. Proteoforms, representing distinct variations of proteins, are fundamental to cellular roles and functions. To address this gap, we have developed an approach that combines laser capture microdissection (LCM) nanoPOTS with mass spectrometry imaging (MSI), enabling both bottom-up and top-down proteomics. This integrated strategy has been applied across diverse systems, including human, murine, plant, and microbial tissues. For example, in studies of human pancreatic tissue, our methods provided an extensive proteoform landscape, identifying 500-1000 proteoforms and revealing unique variations of endocrine proteins that are often overlooked by conventional methods. MSI further enabled the spatial profiling of hundreds of islets, allowing clustering based on their proteoform signatures. LCM was instrumental for isolating both pooled and individual pancreatic islets, which were then analyzed using nanoPOTS for label-free quantitative top-down proteomics. Additionally, single islets and single cells were dissected to perform comprehensive bottom-up proteomic. These tools are now being applied to investigate chronic pancreatic diseases, T1D progression and therapeutic intervention.

User consent:

New approaches for proteoform analysis / 14

Exploring the effects of isotope depletion on proteins by native mass spectrometry and cryogenic electron microscopy

Author: Sneha Hakke[None]

Co-authors: Carmen Iglesias López ; David Clarke ; David Kilgour ; Kèvin Knoops ; Ron Heeren ; Anjusha Mathew ; Ye Gao

Corresponding Author: anjusha.mathew@maastrichtuniversity.nl

Isotope depleted protein samples have successfully addressed various challenges in native mass spectrometry (MS), notably by enhancing the signal-to-noise (S/N) ratio- an advantage particularly beneficial for high molecular weight protein analysis. In this study, we explore the broader impact of isotope depletion on reducing sample heterogeneity, and enhancing mass spectral quality in MS, as well as in improving imaging resolution in cryogenic electron microscopy (cryo-EM). Together, these two techniques offer a more detailed visualization of higher-order molecular structure of proteins with high spatial and mass resolutions.
We successfully expressed and purified test proteins of varied MW, in isotope depleted media. Native mass spectra of these samples showed a distinctive shift towards monoisotopic peaks and a reduction in mass window as compared to the proteins grown in normal media, confirming effective isotope depletion. The improved S/N ratio also enhanced sequence coverage in top-down proteomic analysis in isotope depleted proteins compared to the normal ones. The study extends to cryo-EM to assess the potential improvements in imaging resolution and any structural alterations induced by isotope depletion.

User consent:

yes

New approaches for proteoform analysis / 60

Discovering the ʻnegativeʼside of the proteomic landscape with top-down mass spectrometry

Authors: Cynthia Nagy[1]; Luca Fornelli[1]

Co-authors: John Syka [2]; Joseph Greer [3]; Josh Hinkle [2]; Ken Durbin [4]; Rafael Melani [5]; Ryan Fellers [3]

[1] *University of Oklahoma*

[2] *Thermo Fisher Scientific*

[3] *Proteinaceous, Inc*

[4] *Proteinaceous*

[5] *Thermo*

Corresponding Authors: josh.hinkle@thermofisher.com, luca.fornelli@ou.edu, john.syka@thermofisher.com, cynthia.nagy@ou.edu, rafael.melani@thermofisher.com

Conventional mass spectrometry-based proteomics use positive polarity and provide a wealth of qualitative and quantitative information; however, these methods may not capture the true complexity of the proteome. Our work aims at alleviating this issue by resorting to ionization in negative polarity to account for the acidic portion of the intact proteome that preferentially ionizes as anions. Negative polarity bottom-up strategies have already shown utility in boosting coverage of acidic peptides and our objective was to extend this strategy to the top-down (i.e., intact protein) domain to discover acidic species at the proteoform level, as well. Standard polypeptides with molecular weights between 1-48 kDa were interrogated by HCD, CID and UVPD using both polarities, whereby cleavage propensities and the effect of charge density were determined. A unique feature of negative mode HCD and CID is their ability to cleave disulfide-linkages more readily than in positive

polarity. While cleaving disulfides is appealing, using negative mode HCD and CID comes at the cost of producing a high ratio (>50% of the total ion current) of neutral losses, including series of consecutive ammonia, water and CO2 losses. These additional peaks make spectral interpretation challenging and negatively impact the p-score of proteoform anions. Measurements conducted on the LC timescale for a standard protein mixture (Pierce standard) using high-pH separation on a polymeric resin seem promising and we are working on introducing new bioinformatic tools for conducting database searches for negative mode data.

User consent:

yes

New approaches for proteoform analysis / 31

Glycoproteomics Based on Deep Learning and Data Independent Acquisition

Author: Liang Qiao[1]

[1] *Fudan University*

Corresponding Author: liang_qiao@fudan.edu.cn

Large-scale profiling of intact glycopeptides is critical but challenging in glycoproteomics. In 2021, we propose GproDIA [1], a framework for the proteome-wide characterization of intact glycopeptides from DIA data with comprehensive statistical control by a 2-dimentional false discovery rate approach and a glycoform inference algorithm, enabling accurate identification of intact glycopeptides using wide isolation windows. We benchmark our method for N-glycopeptide profiling on DIA data of yeast and human serum samples, demonstrating that DIA with GproDIA outperforms the data-dependent acquisition-based methods for glycoproteomics in terms of capacity and data completeness of identification, as well as accuracy and precision of quantification.
In 2024, we further present DeepGP [2], a hybrid deep learning framework based on Transformer and graph neural network (GNN), for the prediction of MS/MS spectra and retention time of glycopeptides. Testing on multiple biological datasets, we demonstrate that DeepGP can predict MS/MS spectra and retention time of glycopeptides closely aligning with the experimental results. Comprehensive benchmarking of DeepGP on synthetic and biological datasets validates its effectiveness in distinguishing similar glycans. Remarkably, DeepGP can differentiate isomeric glycopeptides using MS/MS spectra without diagnostic ions.
More recently, we present a method using the ZenoTOF instrument with optimized fragmentation for intact glycopeptide identification and demonstrate its ability to analyze large-cohort glycoproteomes[3]. From 124 clinical serum samples of breast cancer, non-cancerous diseases, and non-disease controls, a total of 6901 unique site-specific glycans on 807 glycosites of proteins were detected. Much more differences of glycoproteome were observed in breast diseases than the proteome. By employing machine learning, 15 site-specific glycans were deter-mined as potential glycosignatures in detecting breast cancer.
[1] Nature Communications, 2021, 12, 6073
[2] Nature Machine Intelligence, 2024, 6, 950-961
[3] Analytical Chemistry, 2025, 97, 114-121

User consent:

yes

New approaches for proteoform analysis / 46

High resolving power meets proton transfer charge reduction: unlocking new depths in intact protein characterization

Author: Rafael D. Melani[1]

Co-authors: Linda B. Lieu [2]; Jingjing Huang [1]; Jake T. Kline [2]; Ryan N. Oates [2]; Cynthia Nagy [2]; David Bergen [1]; Graeme C. McAlister [1]; Christopher Mullen [1]; Luca Fornelli [2]

[1] *Thermo Fisher Scientific, San Jose*

[2] *University of Oklahoma*

Corresponding Author: cynthia.nagy@ou.edu

Although the characterization of intact proteins remains a demanding endeavour –particularly as protein size increases - ongoing technological/bioinformatic advancements are steadily enhancing the capabilities of top-down mass spectrometry (TDMS) for deep sequencing. Different ion activation techniques –such as electron transfer dissociation (ETD), electron transfer higher-energy collisional dissociation (EThcD), and ultraviolet photodissociation (UVPD) available on the Thermo Scientific Orbitrap Tribrid MS platform - can provide complementary fragmentation patterns, and when combined, offer elevated sequence coverage. Despite the advancements in fragmentation techniques, data interpretation remains challenging due to extensive overlapping of ion signals, which compromises product ion matching. Herein, we present strategies for resolving spectral ambiguities, implemented on the Orbitrap Ascend BioPharma MS.
Proteins in the 8-46 kDa Mw range were interrogated with higher-energy collisional dissociation (HCD), ETD, EThcD, and UVPD. Data collection was carried out using a range of resolving powers (RP). A gradual increase in coverage was observed moving from an RP of 60,000 towards 480,000 in all cases - for enolase reaching 53% from the initial 14% yielded by lower resolution. However, high RP alone could not effectively resolve spectral congestion due to fragment ions signals being restricted to m/z < 2000. Additional increase in coverage (~8-15%) could only be achieved with proton transfer charge reduction (PTCR), due to its ability to distribute the product ion population over a broader m/z range (up to m/z 8000). The PTCR MS3 workflow yielded a coverage of 98% for carbonic anhydrase (~29 kDa) when results from all tested fragmentation techniques were combined.
To evaluate the approach under chromatographic time constraints, we applied the workflow for the middle-down analysis of mAb and antibody-drug conjugate (ADC) ~25 kDa subunits. PTCR MS3 enabled not only increased coverage (87% for Fd'subunit) but also the unambiguous localization of payload conjugation sites for the ADC.

User consent:

yes

Biomedical Applications / 30

Top-Down Proteomics of the Heart: Decoding Cardiac Proteoforms for Precision Medicine

Author: Ying Ge[1]

[1] *University of Wisconsin-Madison*

Corresponding Author: ying.ge@wisc.edu

Proteoforms - encompassing the diverse protein products arising from alternative splice isoforms, genetic variations, and posttranslational modifications (PTMs) originating from a single gene - are fundamental drivers in biology. Top-down mass spectrometry (MS)-based proteomics (TDP), analyzing whole proteins without digestion, offers a comprehensive perspective of proteoforms, which is invaluable in deciphering proteoform function, uncovering disease mechanisms, and advancing precision medicine. We have been developing novel technologies to address the challenges in top-down

proteomics in a multi-pronged approach including new cleavable surfactants for protein solubilization, new strategies for multi-dimensional chromatography separation of proteins, novel nanomaterials for enrichment of low-abundance proteins. In this presentation, I will highlight the application of TDP to enable proteoform-resolved analysis of cardiac proteins directly from human heart tissues and human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs). Notably, we have identified altered cardiac proteoforms associated with contractile dysfunction. Through case studies in hypertrophic, ischemic, and dilated cardiomyopathy, we demonstrate how TDP uncovers disease mechanisms, reveals novel biomarkers, and informs therapeutic strategies. By mapping the proteoform landscape of the human heart, top-down proteomics has the potential to transform cardiovascular research and enable more precise, individualized interventions.

User consent:

yes

Biomedical Applications / 21

Dissecting the Proteoform Landscape of Prostate-Specific Antigen: Intact, Bottom-Up, and Glycomic Perspectives

Author: Guinevere Lageveen-Kammeijer[1]

Co-authors: Alan Moran [2]; Elena Dominguez Vega [2]; Jan Nouta [2]; Manfred Wuhrer [2]; Noortje de Haan [2]; Peter A. van Veelen [2]; Tamás Pongrácz [2]; Tao Zhang [2]; Theo M. de Reijke [3]; Wei Wang [2]

[1] *University of Groningen*

[2] *Leiden University Medical Center*

[3] *Amsterdam UMC*

Corresponding Authors: g.s.m.kammeijer@rug.nl, m.wuhrer@lumc.nl, e.dominguez_vega@lumc.nl

Early detection of prostate cancer (PCa) using prostate-specific antigen (PSA) serum levels suffers from poor specificity and sensitivity, frequently causing unnecessary treatment or missed diagnoses. To overcome this, detailed characterization of PSA proteoforms, especially regarding glycosylation, is essential. Here, we employed an integrated analytical strategy using capillary electrophoresis coupled with mass spectrometry (CE-MS), progressively scaling from micro-level glycan details to macro-level intact protein analysis.

At the micro-level, released *N*-glycan profiling provided validation of glycosylation patterns identified by peptide-level (bottom-up) and intact analyses. The bottom-up approach offered detailed differentiation of glycopeptides, particularly distinguishing α2,3- from α2,6-linked sialic acid isomers through differences in electrophoretic mobility correlated with subtle pKa variations (relative pKa difference: 3.4×10^{-2}). This high-resolution separation uniquely revealed precise structural features, including the first identification of ketodeoxynononic acid (Kdn) on PSA glycans derived from seminal plasma and urine.

Moving to the macro-level, intact protein analysis delivered a global view of PSA proteoforms, capturing six proteolytic cleavage variants alongside diverse glycosylation states—including tri-, di-, mono-, and non-sialylated glycans—and, for in one of the patients urinary samples, uncovered a second glycosylation site resulting from genetic mutation.

Together, these complementary methodologies overcome individual analytical limitations, offering an extensive characterization of PSA proteoforms and their glycomic complexity. Future research will evaluate whether the proteoform diversity can be utilized to enhance discrimination between aggressive PCa, indolent PCa, and benign prostate hyperplasia. Additionally, further studies will investigate glycomic variations in plasma, preserve native PSA molecular complexes, and assess the clinical relevance of proteoform diversity in relation to disease severity and progression in larger patient cohorts.

User consent:

yes

Biomedical Applications / **52**

Spatial Phosphoproteomic Profiling Reveals Regional Functional Heterogeneity in the Murine Heart

Authors: DAN ZHAO[None]; Hartmut Schlüter[1]; Liang Qiao[2]; Ling Lin[2]

[1] *Institute of Biochemistry*

[2] *Fudan University*

Corresponding Authors: linglin@fudan.edu.cn, hschluet@uke.de, liang_qiao@fudan.edu.cn, 13817801534@163.com

Phosphorylation mediated signaling is fundamental to cardiac function. However, the dynamic signaling patterns across different spatial regions of the heart have been inadequately explored. This is mainly because of the technical difficulties in analyzing tiny tissue samples with the required depth and sensitivity. To tackle these challenges, we developed an optimized TiO_2 based micropipette tip method for in-depth phosphoproteomics. This methodology showcases outstanding sensitivity, identifying 12,173 class I phosphosites from 10 μg HeLa peptides, and also offers high reproducibility.

We then utilized this advanced technique to study spatially defined regions of the mouse heart. Through laser-capture microdissection, we isolated seven specific anatomical areas: the left atrium (LA), right atrium (RA), left ventricle (LV), right ventricle (RV), interventricular septum (IVS), apex (APEX), and aortic valve (AV). For each region, we were able to quantify 1,000-2,000 phosphosites. Principal component analysis revealed distinct phosphoproteomic signatures that cluster according to anatomical positions, providing higher resolution differentiation than proteomic profiling. Functional enrichment analysis further unveiled region-specific phosphorylation patterns. The APEX and ventricular regions were characterized by phosphorylation signatures associated with the contractile machinery. In the AV tissue, proteins related to cell junctions and polarity were significantly enriched. From a metabolic perspective, the LV exhibited phosphorylation patterns closely tied to energy metabolism, while the LA showed enrichment in RNA processing pathways. Phosphoproteins in the RA were predominantly involved in cellular component biogenesis and chromatin organization.

This spatially resolved atlas establishes a molecular foundation for investigating region-specific cardiac pathologies and advances understanding of post-translational cardiac heterogeneity.

User consent:

yes

Biomedical Applications / **4**

Spatially Resolved Proteoform Mapping in Alzheimer's Disease Brain Tissues

Author: Yue Sun[1]

Co-authors: Dan Liu [1]; Yu Liang [1]; Xue Yang [1]; Xinxin Liu [1]; Baofeng Zhao [1]; Zhen Liang [1]; Yukui Zhang [1]; Lihua Zhang [1]

[1] *Dalian Institute of Chemical Physics, Chinese Academy of Sciences*

Corresponding Authors: yuesun@dicp.ac.cn, yuliang@dicp.ac.cn, yangxue@dicp.ac.cn, liudan@dicp.ac.cn, lihuazhang@dicp.ac.cn, baofzhao@dicp.ac.cn, liuxinxin@dicp.ac.cn, liangzhen@dicp.ac.cn, ykzhang@dicp.ac.cn

Understanding spatial heterogeneity of proteoforms has great potential to unravel physiological and disease mechanisms. However, conventional proteomics often lacks spatial information or focuses on the protein-coding gene level, while existing methods for spatial proteoform analysis suffer from low throughput or limited coverage. These gaps hinder the exploration of spatially resolved proteoform-function relationships

In this work, we developed high-throughput proteoform imaging (HTPi), an integrative workflow combining matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI MSI) with deep annotation via region-specific top-down proteomics, using a custom-designed narrow-bore monolithic column to enhance sensitivity. HTPi achieved proteoform visualization at 20–100 μm spatial resolution and annotated 366 proteoform images in mouse brain tissues, revealing distributions of individual proteoforms across different brain regions and distinct spatial patterns of proteoforms from a single gene (e.g., six Pcp4 proteoforms).

Applied to 5×FAD mice, HTPi was used to explore proteoform perturbations in the hippocampus, cortex, thalamus, and striatum. Notably, HTPi uncovered Aβ proteoforms (1–38, 1–40, 1–42) localized to subiculum plaques and identified 14 differential proteoforms, including truncated Ubb and mitochondrial Ndufv3. The co-localization of truncated Ubb proteoforms with Aβ plaques suggested a link between Aβ accumulation and ubiquitin-proteasome dysfunction. These results highlighted HTPi's ability to resolve proteoform-level spatial dynamics in Alzheimer's disease pathogenesis.

By bridging high-throughput MALDI MSI with region-specific top-down proteomics, HTPi advances spatial proteomics, offering insights into molecular mechanisms and potential biomarkers for neurodegenerative diseases. Future applications may expand to 3D brain-wide proteoform mapping and clinical translation for disease diagnosis.

User consent:

yes

Biomedical Applications / 28

Top-down Proteomics Deciphers Cardiac Proteoform Landscape in Phospholamban R14del Cardiomyopathy for Precision Medicine

Author: Holden Rogers[1]

Co-authors: Frederik Deiman [2]; Zhan Gao [1]; Neils Grote Beverborg [2]; Rachel Baum [3]; Peter van der Meer [2]; Pieter Glijnis [4]; Ioannis Karakikes [3]; YING GE [1]

[1] *University of Wisconsin-Madison*

[2] *University Medical Center Groningen*

[3] *Stanford University*

[4] *PLN Foundation*

Corresponding Authors: ying.ge@wisc.edu, htrogers@wisc.edu

Phospholamban (PLN) is a transmembrane protein that regulates cardiomyocyte calcium handling and contraction. PLN function is dynamically regulated by post-translational modifications (PTMs), most notably through phosphorylation. A pathogenic deletion of arginine-14 (PLN-R14del), is associated with dilated cardiomyopathy (DCM), and defined by a high mortality rate with minimal treatment options. Moreover, the molecular mechanism of pathogenesis remains unclear, as some R14del carriers are asymptomatic. We use mass spectrometry (MS)-based top-down proteomics to investigate proteoform alterations in two systems: 1) human cardiac tissue from healthy donors and late-stage DCM patients (R14del carriers and noncarriers); 2) human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) from symptomatic and asymptomatic patients. Proteins were extracted from cryopulverized tissue or hiPSC-CMs using a two-stage extraction. Cytosolic proteins

are depleted, while PLN and additional cardiac proteins were extracted with Azo (MS-compatible surfactant). Proteins were buffer exchanged and analyzed with reverse phase liquid chromatography coupled to tandem MS. Characterization of the proteoform landscape in R14del patients revealed multiple PLN proteoforms. Notably, we observed a significant decrease in the total phosphorylation levels of R14del samples compared to both donor samples and DCM patients without the mutation, providing a novel insight into the endogenous phosphorylation potential of PLN-R14del. Additionally, we observed dysregulation of phosphorylation in key sarcomere/Z-disk proteins, further associating contractile dysfunction with proteoform alterations. In the hiPSC-CM model, preliminary analysis revealed that the asymptomatic patient line contained PLN phosphorylation levels comparable to the isogenic control line; importantly, there was a significant increase in phosphorylation in both when compared to the symptomatic carrier. These results suggest that proteoforms may factor into variable disease expressivity and that our method is capable of delineating distinct phenotypes between patients that share the same mutation, which can aid in the pursuit of therapeutic strategies for precision medicine and bridge the gap between genotype and phenotype.

User consent:

yes

Biomedical Applications / 50

MALDI MS-Based Rapid Antimicrobial Susceptibility Prediction

Author: Jia Yi[1]

Co-author: Liang Qiao [2]

[1] *Minhang Hospital, Fudan University*

[2] *Department of Chemistry, Fudan University*

Corresponding Authors: jia_yi@fudan.edu.cn, liang_qiao@fudan.edu.cn

Bacterial infections are among the diseases with high morbidity and mortality rates worldwide, posing a significant threat to global public health. There is an urgent need to develop precise and rapid diagnostic methods for bacterial infections to enable personalized medication and treatment for infected patients, promptly save lives, and reduce the spread of antimicrobial resistance. Bacterial infection diagnosis encompasses two key aspects: bacterial identification and antibiotic susceptibility testing (AST). Current clinical methods for bacterial identification and AST are limited by the time-consuming process of bacterial culture. Bacterial identification is typically performed using MALDI-TOF MS, while AST is conducted with automated biochemical analyzers, requiring an additional step of proliferation testing under antibiotic stimulation, resulting in a delay of 6~24 hours compared to bacterial identification.

To accelerate antibiotic susceptibility testing (AST) and reduce costs, we have developed two rapid AST methods based on MALDI-TOF MS. The first method detects deuterium incorporation into newly synthesized proteins under antibiotic stimulation, allowing for monitoring of protein synthesis and using machine learning to predict bacterial susceptibility. This approach introduces a series of discriminative features, resulting from mass shifts induced by deuterium incorporation, which significantly enhances the performance of machine learning models, especially on small datasets. Additionally, when transferring training results from public datasets to smaller datasets, this method improves the accuracy of antibiotic susceptibility predictions. The second method monitors changes in bacterial metabolites under short-term antibiotic stimulation and, when combined with machine learning, also predicts antimicrobial susceptibility. Both methods reduce AST time to just 0.5 to 1 hour after bacterial identification by MALDI MS. Furthermore, these approaches integrate both AST and bacterial identification into a single mass spectrometer, facilitating faster diagnoses, reducing equipment and labor costs, and demonstrating great potential for broader clinical applications of mass spectrometry.

User consent:

yes

Biopharmaceutical & Therapeutic Proteins / 88

Deciphering Biotherapeutic Biotransformations with Top-Down Mass Spectrometry

Author: Julia CHAMOT-ROOKE[1]

[1] *INSTITUT PASTEUR - CNRS*

Corresponding Author: julia.chamot-rooke@pasteur.fr

Today, within the R&D pipelines of pharmaceutical companies, monoclonal antibodies are gradually being replaced by new-generation biotherapeutics, including engineered hybrid and multispecific constructs. While these innovative formats hold great therapeutic promise, their structural complexity often leads to unexpected in vivo instabilities that may compromise efficacy or alter pharmacokinetics. Understanding their metabolic fate is therefore crucial to guide rational design and ensure functional performance.

However, current analytical workflows—originally designed for small molecules or peptide-level proteomics—struggle to capture the full heterogeneity of these large, hybrid biomolecules. Proteolytic digestion breaks apart structurally distinct proteoforms into overlapping peptide mixtures, leading to a loss of connectivity and incomplete characterization.

To overcome these limitations, we have developed and applied complementary middle-down and top-down mass spectrometry strategies capable of analyzing intact proteins and large subunits (25–100 kDa). These approaches were implemented on the timsOmni multimodal platform (Bruker) and the Orbitrap Eclipse Tribrid system (ThermoFisher), allowing high-resolution analysis with advanced dissociation methods (ECD, EID, CID, ETD, HCD).

We demonstrate that these techniques can detect and partially sequence low-abundance mAb proteoforms directly from in vivo plasma samples following administration in mice. A key part of this work involved the automated immunoenrichment of biotherapeutic metabolites from complex biological matrices, enabling their isolation and characterization despite low concentrations and high background.

Together, these innovative analytical workflows provide new insight into biotherapeutic metabolism, including the detection of truncated forms resulting from rapid in vivo cleavage—such as the loss of target-binding domains—which directly impacts the therapeutic mechanism of action. These results highlight the importance of proteoform-level analysis for next-generation biologics and open the path toward more robust, predictive tools in biotherapeutic development.

User consent:

yes

Biopharmaceutical & Therapeutic Proteins / 15

Functional and structural characterization of antibodies by native-mode affinity separation-, middle-up, and top-down mass spectrometry

Authors: Constantin Blöchl[1]; Christoph Gstöttner[1]; Yue Li[1]; Bianca van Tol[1]; Despoina Mavridou[1]; Eva Maria Stork[1]; David Falck[1]; Markus Haberger[2]; Dietmar Reusch[2]; Rene E. M. Toes[1]; Manfred Wuhrer[1]; Elena Dominguez Vega[1]

[1] *Leiden University Medical Center*

[2] *Roche Diagnostics GmbH*

Corresponding Authors: d.falck@lumc.nl, b.d.m.van_tol@lumc.nl, d.reusch@roche.com, y.li7@lumc.nl, e.dominguez_vega@lumc.nl, c.j.gstoettner@lumc.nl, c.blochl@lumc.nl, e.m.stork@lumc.nl, m.haberger@roche.com, r.e.m.toes@lumc.nl, d.mavridou@lumc.nl, m.wuhrer@lumc.nl

Antibodies show tremendous structural diversity shaping their biological functions in immunity and immune pathologies. While IgG1 Fc domains have been extensively characterized by MS approaches, more complex antibody structures (including Fab glycosylated IgGs or isotypes such as IgA or IgM) are less well understood. This presentation showcases the integration of miniaturized bottom-up, middle-up and intact mode workflows for dissecting the Fab and Fc hetereogeneity of endogenous antibodies, with a particular focus on defining glycoform profiles. By employing specific capturing approaches in combination with selective hinge-region cleavage strategies, specific antibody populations and fragments are isolated and characterized revealing intrinsic differences. Native-mode affinity-capillary electrophoresis with mass spectrometry allows to assess how antibody proteoforms including Fc and Fab glycosylation define Fc-receptor interactions. This work provides insights into molecular details of antibody functionalities in health and disease.

We applied the middle-up approach to characterize IgG Fc portions of anti-citrullinated protein antibodies (ACPA) in rheumatoid arthritis (RA) from both synovial fluid and plasma samples, and compared them to the proteoform profiles of total or bulk IgG from the same samples. We observed differences in isotype and allotype usage as well as Fc glycosylation between the different antibody populations. Remarkably, both IgG1 agalactosylation and IgG4 subclass usage appeared to associate with disease activity as well as erythrocyte sedimentation rate, providing indications of a possible contribution of these antibody variants to RA etiology.

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User consent:

yes

Biopharmaceutical & Therapeutic Proteins / 25

AiDA Accelerates Top-Down and Middle-Down MS Data Analysis Across Multiple Antibody Variants

Author: Francois Griaud[1]

[1] *Novartis Pharma AG*

Corresponding Author: francois.griaud@novartis.com

Mass spectrometry (MS) is essential for characterizing biotherapeutics, with Top-Down (TD) and Middle-Down (MD) approaches offering faster alternatives to peptide mapping. Despite achieving

high sequence coverage using advanced fragmentation techniques like HCD, ETD, and UVPD, traditional data analysis presents challenges. These include time-consuming fragmentation map creation, difficulty localizing post-translational modifications (PTMs) in non-fragmented regions, and missed diagnostic ions due to monoisotopic peak detection errors in complex spectra.
To overcome these issues, the All ion Differential Analysis (AiDA) method was developed for online antibody variant characterization. (1) AiDA enables rapid identification of diagnostic spectral differences across multiple MS spectra before fragment assignment, significantly accelerating data analysis. It introduces a quantitative layer to TD and MD workflows by analyzing preferential fragmentation patterns, particularly near aspartic and iso-aspartic acid residues, to localize PTMs with statistical confidence.(1, 2) AiDA also helps detect interactions between neighboring residues and has proven effective in characterizing deamidation, sequence variant and sequence positional isomers of oxidation and iso-aspartic acids in antibodies. Finally, AiDA supports multi-level validation of internal fragments N-terminal to Proline which can be used to increase MD sequence coverage by up to 34% and access otherwise non-fragmented regions.(1) Both 1D and 2D AiDA applications will be discussed.

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User consent:

yes

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Enhanced usage of top-down data for de novo sequencing of antibodies

Author: Kira Vyatkina[1]

[1] *Sechenov University*

Corresponding Author: kira.vyatkina@gmail.com

The Twister algorithm (Vyatkina et al., 2015, 2016, 2017), initially intended for de novo sequencing of antibodies from top-down MS/MS data supported with high-resolution bottom-up MS/MS spectra, is being developed further –currently aiming, at particular, at taking the maximum profit from internal fragment ions. In this talk, we will present the latest version of the Twister algorithm, along with the most recent results obtained.

User consent:

yes

Biopharmaceutical & Therapeutic Proteins / 20

Mass spectrometric ITEM-FOUR analysis reveals coding single nucleotide polymorphisms in human cardiac troponin T that evade detection by sandwich ELISAs which use monoclonal antibodies M7 and M11.7 from the Elecsys Troponin T® assay

Author: Michael Glocker[None]

Co-authors: Claudia Röwer [1]; Cornelia Koy [1]; Kristian Kormann [1]; Manuela Russ [1]

[1] *Proteome Center Rostock*

Corresponding Author: michael.glocker@uni-rostock.de

Immunoassays for cardiac troponin, such as the Elecsys® hs-TnT, have become the gold standard for myocardial infarction diagnostics. While various protein/chemical factors affecting the troponin complex and, thus, its diagnostic accuracy have been investigated the role of coding single nucleotide polymorphisms remains underexplored. To evaluate potential cSNP-induced interference with antibody binding in the Elecsys® hs-TnT immunoassay, we applied ITEM-FOUR, a mass spectrometry-based method that quantifies changes in antibody binding upon amino acid substitutions in epitope pep-tides. Candidate cSNPs were selected from the dbSNP database and were mapped to human cardiac troponin T by molecular modeling. Consuming micromolar antibody concentrations and microliter sample volumes, two wild-type and 17 cSNP-derived variant epitope peptides—six for monoclonal antibody M7 and eleven for monoclonal antibody M11.7—were investigated to reveal the binding motifs 'V131-K134-E138-A142' for M7 and 'E146-I150-R154-E157' for M11.7. Loss of binding to M11.7 was observed for substitutions Q148R (rs730880232), R154W (rs483352832), and R154Q (rs745632066), whereas the E138K (rs730881100) exchange disrupted binding of M7. Except for cSNP Q148R they are associated with cardiomyopathies, placing affected individuals at risk for both, underlying heart disease and false-negative hs-TnT assay results in case of myocardial infarction. Our results highlight the need to account for cSNP-related interferences in antibody-based diagnostics. ITEM-FOUR offers a powerful approach for tackling this challenge, fostering next-generation assay development.

User consent:

yes

Poster Session 1 / 29

Decoy spectrum for accurate proteoform level FDR estimation in top-down proteomics

Authors: Ayesha Feroz[1]; Kyowon Jeong[2]

Co-author: Oliver Kohlbacher [3]

[1] *Eberhard Karls Univeristy of Tuebingen Germany*

[2] *University of Tübingen*

[3] *Applied Bioinformatics, Department for Computer Science, University of Tübingen*

Corresponding Authors: kyowon.jeong@uni-tuebingen.de, ayesha.feroz@uni-tuebingen.de, oliver.kohlbacher@uni-tuebingen.de

False Discovery Rate (FDR) estimation is critical in proteomics to minimize false positive (FP) identifications. Traditional FDR estimation via the Target-Decoy Approach (TDA) mainly accounts for FPs due to fragment mass coincidences but overlooks those introduced by errors in precursor mass determination. In top-down proteomics (TDP), where spectral deconvolution assigns precursor masses, such errors are frequent and can significantly distort FDR estimates.
We introduce the concept of a decoy spectrum, which simulates false positives resulting from precursor mass errors during deconvolution. This is implemented within FLASHDeconv, which generates decoy masses mimicking typical deconvolution errors. MS2 spectra assigned to these decoy precursor masses constitute the decoy spectra. Combined with traditional target-decoy database search, this allows us to compute a more accurate refinedFDR, accounting for both types of false positives.
We evaluated this approach using an E. coli K-12 LC-MS/MS dataset. Deconvolution was performed using FLASHDeconv, and proteoform identification was conducted using TopPIC. At 5% TDA-FDR,

TopPIC identified 79 decoy hits. However, an additional 86 decoy spectrum hits were found, increasing the refinedFDR to 8%. Similarly, at 1% TDA-FDR, refinedFDR was 3.25%, nearly doubling the estimated false positives.
To validate these findings, we analyzed mass shifts in identifications at 1% TDA-FDR. About 80% of shifts in the <1% refinedFDR group matched known modifications in UniMod, compared to only 58% in the >1% refinedFDR group. This suggests that many hits with high refinedFDR are likely due to incorrect precursor masses. Our results demonstrate that decoy spectra provide a more complete model of FPs in TDP and significantly improve FDR estimation, enhancing the reliability of proteoform identification and paving the way for more sensitive TDP workflows.

User consent:

yes

Poster Session 1 / 73

Protein Characterization with CIU and MSn eXd on the timsOmni platform

Author: Stuart Pengelley[1]

Co-authors: Amalia Apalategui [1]; Anastasios Grigoriadis [2]; Athanasios Smyrnakis [2]; Christoph Gebhardt [1]; Dimitrios Papanastasiou [2]; Eduardo Carrascosa [1]; Florian Busch [3]; Maria-Aggeliki Kosmopoulou [2]

[1] *Bruker Daltonics GmbH & Co. KG, Bremen, Germany*

[2] *Fasmatech Science & Technology, Chalandri, Greece*

[3] *Bruker Switzerland AG, Fällanden, Switzerland*

Corresponding Author: stuart.pengelley@bruker.com

Protein characterization is a very wide-reaching discipline which can encompass a broad range of techniques and approaches. Mass spectrometry is making an increasingly significant contribution to this field as the technology matures and top-down workflows are improved upon with new hardware and software innovations. The new timsOmniTM platform combines trapped ion mobility spectrometry (TIMS) with the Omnitrap® multidimensional MSn ion processor, providing the necessary analytical flexibility required for the increasingly complex studies aimed at sequencing and structural characterisation of biopharmaceuticals.
The configuration of the timsOmni instrument contributes greatly to the experimental options that are available, including a variety of ion activation methods which can be applied in multiple different locations along the ion path. Firstly, the protein desolvation unit (PDU) is positioned upstream of the TIMS cell and can be used for desolvation or unfolding of protein ions. Here, we demonstrate collision induced unfolding (CIU) of proteins in the PDU prior to mobility analysis in the TIMS analyzer. This was then combined with electron capture dissociation (ECD), a soft fragmentation technique, which was applied to proteins at differing levels of unfolding in the Omnitrap section Q5 to reveal which sequence regions become more flexible as the protein unfolds.
In-source collision induced dissociation (isCID) can be applied directly downstream of the TIMS analyzer and is typically used for desolvation or all-ion CID. For MS3, fragment ions produced by isCID can be isolated in the quadrupole ion filter, accumulated in the Omnitrap section Q2 and fragmented again by electron activated dissociation (eXd) in Q5. For pseudo MS4 experiments, eXd products can be isolated and accumulated prior to fragmentation by CID in the collision cell. The MSn eXd workflow is demonstrated for NISTmAb, producing up to 88% sequence coverage for N-terminus light and heavy chain fragments produced by isCID.

User consent:

yes

Poster Session 1 / 17

Co-interaction of Tau and S100A9 Proteins

Author: Lukas Krasauskas[1]

Co-authors: Andrius Sakalauskas [1]; Mantas Žiaunys [1]; Rūta Sniečkutė [1]; Vytautas Smirnovas [1]

[1] *Amyloid Research Sector, Institute of Biotechnology, Life Sciences Center, Vilnius University*

Corresponding Author: lukas.krasauskas@gmc.vu.lt

Neurodegenerative diseases are one of the most common disorders in the world, and despite intensive research, the understanding of these diseases is limited. Alzheimer's disease is the most common neurodegenerative disease, affecting about 50 million people worldwide. In addition to amyloid plaques composed of amyloid-β, neurofibrillary tangles formed from the protein Tau are a hallmark of this disease and other tauopathies. Amyloid beta aggregates (and alpha-synuclein aggregates in Parkinson's disease) have been shown to promote Tau aggregation. It has also been observed that the aggregation of these two peptides involves the pro-inflammatory protein S100A9, whose elevated levels in the brain are recorded after various head injuries.
Furthermore, Chronic traumatic encephalopathy registers high levels of Tau aggregates, and the exact reasons for their formation are unknown. Researchers observed that this disease is quite prominent in contact sports players who experience chronic head concussions. There has been some speculation that neuroinflammation could induce Tau pathology; thus, it's feasible that S100A9, as a pro-inflammatory protein, could be at least in part responsible. However, it is strange that little information is available to confirm or rule out the potential of the S100A9 protein or its aggregates to participate directly in Tau aggregation. Therefore, we examined the ability of the S100A9 protein to promote Tau aggregation. We observed that Tau aggregation is dependent on S100A9 aggregate formation. Aggregation kinetics were recorded by fluorescence spectroscopy using the amyloidophilic dye thioflavin T. Atomic force microscopy was performed to analyze the morphology of the formed aggregates, and FTIR spectroscopy was done to analyze secondary structures in the formed aggregates. Cell viability studies have also tentatively shown that higher concentrations of S100A9 aggregates are associated with lower toxicity, which may indicate that the formation of S100A9 aggregates and co-interaction with Tau protein acts as a protective mechanism.

User consent:

yes

Poster Session 1 / 36

Revealing the architecture and dynamics of SARS-CoV-2 RTC

Author: Assel Berikkara[1]

Co-authors: Alice Grün [1]; Charlotte Uetrecht [2]; Fatema-Aqila Said [1]; Kira Schamoni-Kast [1]

[1] *CSSB, DESY, LIV, UzL*

[2] *CSSB/DESY/University of Lübeck*

Corresponding Authors: fatema-aqila.said@cssb-hamburg.de, kira.schamoni-kast@cssb-hamburg.de, assel.berikkara@cssb-hamburg.de, alice.gruen@cssb-hamburg.de, charlotte.uetrecht@cssb-hamburg.de

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a single-stranded RNA virus that caused an outbreak of coronavirus disease in 2019 (COVID-19). The replication of the viral genome is facilitated by the replication/transcription complex (RTC), in which nonstructural proteins (nsps) are the principal contributors. Therefore, unraveling the functional properties and structural organization of nsps will deepen our understanding of the evolutionary success of the virus and aid in developing preventative and curative drug strategies against SARS-CoV-2.

The primary aim of this research project is to reveal the spatial configuration and topology of non-structural proteins (nsps) and reconstruct a complete RTC while preserving its native conformation. This will be achieved by transforming bacterial and mammalian cell lines, expressing and purifying nsps, and analyzing them using native top-down mass spectrometry (nTDMS). nTDMS enables structural investigation of proteins and protein complexes while preserving their native, folded conformation. This methodology allows for the detection of transient protein-protein interactions, assembly and disassembly of nsps within the RTC. Additionally, nTDMS can identify post-translational modifications (PTMs) of proteins. Comparing PTMs across these expression systems will provide valuable insights into the role of PTMs in SARS-CoV-2 pathogenesis.

We have developed a protocol for performing affinity purification directly in an nMS-compatible buffer, significantly reducing purification time while maintaining optimal protein yield. Using this optimized methodology, we successfully purified multiple nsps, including nsp10, nsp14, nsp15, and nsp16. Our results revealed protein-protein interactions between nsp10, nsp14, and nsp16, as well as the homohexamer formation of nsp15.

As a future direction, we plan to analyze the remaining nsps in both bacterial and mammalian expression systems and examine their PTMs using nTDMS. This approach will provide deeper insights into the dynamic nature of the RTC and the functional roles of nsps in viral replication and transcription.

User consent:

yes

Poster Session 1 / 71

Implementation of in-source pH modulation for flexible top-down analysis

Authors: Rachel Franklin[1]; Yury Vasilev[1]

Co-author: Christian Klein [1]

[1] *Agilent*

Corresponding Authors: rachel.franklin@agilent.com, christian_klein@agilent.com, yury.vasilev@agilent.com

Top-down analysis of denatured, highly charged proteins often yields greater sequence coverage than native charge states. However, the abundance of individual precursors under denaturing conditions is limited because of signal dilution amongst a wide charge envelope. Further, the resulting MS/MS spectra are often highly congested, making it difficult to confidently assign fragmentation signals above noise levels. To address this, we utilized the Agilent dual AJS electrospray ion source to perform in-spray acid/base reactions, enabling precise control over charge distribution. We also explore how acidified nebulizer gas can be utilized to expand top-down capabilities for native proteins containing cofactors.
Charge reduction was achieved by infusing base into the reference nebulizer of the dual spray AJS source. Adjusting gas flow or base infusion rate enabled controlled shifts in charge state distributions. For example, denatured carbonic anhydrase shifted from a dominant 32+ to 22+ precursor, with a two-fold increase in intensity after reduction. Similar shifts were observed for aldolase (39kDa) and enolase (46kDa). These charge-reduced precursors were fragmented using electron capture dissociation in the Agilent ExD cell, resulting in improved sequence coverage. Aldolase increased from 38% to 46% and enolase increased from 23% to 39%. Ion mobility studies showed that charge-reduced precursors retained extended structures, however, folding/unfolding behavior was found to be protein size and charge-dependent. CIU was also used to study dynamic structural changes.
In-spray acidification involved introducing acidified gas into the main nebulizer, enabling online hydrolysis of cofactors such as heme in native myoglobin. This allowed simultaneous top-down fragmentation of both holo and apo forms. Fragment evidence for heme binding was visualized using ExDViewer, with top-scoring sequences aligning with known binding sites. Ion mobility revealed structural differences between holo and apo myoglobin. Combining these in-spray acid/base

reactions with top-down mass spectrometry enhances protein characterization by providing complementary structural and sequence information.

User consent:

yes

Poster Session 2 / 72

Applying cDDA-ExD on a timsOmni to the analysis of the Fab regions of antibodies originating from human serum

Author: Jean-Francois Greisch[1]

Co-authors: Iuliia Stroganova [2]; Athanasios Smyrnakis [3]; Mariangela Kosmopoulou [3]; Detlev Suckau [4]; Dimitris Papanastasiou [3]; Albert Heck [2]

[1] *Bruker Switzerland AG*

[2] *Utrecht University*

[3] *Fasmatech Science & Technology*

[4] *Bruker Daltonics*

Corresponding Authors: a.j.r.heck@uu.nl, i.stroganova@uu.nl, j.greisch@bruker.com

Introduction
Protein-centric proteomics is an emerging mass spectrometric field where to answer a biological question related to cells or tissues, one analyzes proteins directly, without relying on their digestion into smaller peptides. The unique ability of protein-centric proteomics to disentangle isoforms and proteoforms is directly beneficial to the complex functional analysis usually needed to answer a given biological question.
Methods
We cleaved the fragment antigen-binding (Fabs) regions from immunoglobulins using a hinge-cleaving protease. We then used reverse phase liquid chromatography (LC) to separate Fabs according to their hydrophobicity. MS analysis was performed on the new timsOmniTM instrument (Bruker) combining the capabilities of a timsTOF with the fragmentation techniques and flexibility of an OmnitrapTM platform. Practically, we analyzed the ~50 kDa Fabs inline relying on MS1 scans to identify precursors belonging to the charge state distribution of a given clone or proteoform. A given charge state was then automatically selected, accumulated, and fragmented using reagent-free electron-based fragmentation techniques. The resulting data was processed using OmniScape.
Preliminary data
We demonstrate that the timsOmni can overcome the limitations of current LC-MS methods in which many species coelute, potentially resulting into congested spectra unsuitable to the analysis of complex samples. Specifically, we use cDDA-ExD method to identify the charge states of a given-mass species characterized, select relevant precursors, and selectively fragment them. The parameters used by the cDDA-ExD method were adapted to sample complexity and chromatographic separation. Under these conditions, we show accurate determination of the masses of the light and heavy chains and good sequence coverage of the hypervariable CDR3 regions of therapeutic recombinant Fabs.
We demonstrate that, focusing on the CDR3 regions, we can generate easy-to-read sequence ladders that provide a unique insight into the complexity of the serum antibody repertoires.

User consent:

yes

Replacing "OR"Logic and Mass Accuracy with "AND/OR"Logic and Mass Resolving Power, as the Basis for Peak Assignment in Top-Down Mass Spectrometry Data.

Author: Jeffrey Agar[1]

[1] *Northeastern University*

Corresponding Author: j.agar@neu.edu

This talk concerns how to assign a peak in a fragment ion mass spectrum to a polypeptide sequence. This task is recognized to be problematic for some internal fragment ions, and as we will show, is problematic for all ion types (e.g., terminal fragments). Most experimental mass (peak) to theoretical database entry (molecule) correlations are delivered as a biproduct of automated peptide/proteoform-spectral (PSM) matches. Unfortunately, peak assignments are not what PSMs were designed to do. There are conditions where it is fair to assume—as many do—that PSM peak assignments are correct. We show these conditions involve sparsely populated experimental data generated, for example, with prior MS technology. We propose and demonstrate that assigning fragments ions from rich, e.g., modern, mass spectra, including from TDMS, requires changing the search paradigm from "OR"to "AND/OR"logic for assigning a given peak to related database entries, as well as moving from the use of mass accuracy-related search space to a mass resolution-related search space.

User consent:

yes

Poster Session 1 / 41

Enhanced proteoform identification in top-down proteomic workflows using packed emitter columns.

Authors: Amy K. Carfagno[1]; Jake Kline[1]; Erik Verschuuren[2]; Andrea Almeida[None]; Jarrod Sandow[2]; Lucca Fornelli[1]

[1] *University of Oklahoma, Norman, OK*

[2] *IonOpticks, Melbourne, Australia*

Corresponding Authors: erik@ionopticks.com, andreia.almeida@ionopticks.com, jarrod@ionopticks.com

Top-down proteomics has emerged as a powerful analytical approach for characterizing intact proteins and their proteoforms offering unique insights into protein structure, post-translational modifications (PTMs), and biological function. Proteoform identification is generally achieved by separation of intact proteins using reversed-phase liquid chromatography (LC) followed by fragmentation and detection on a mass spectrometer. One of the biggest challenges in the field is the inability to identify different proteoforms due to over-lapping separation profiles created by limited peak capacity. To address this, we developed a novel packed emitter chromatography column containing a C4 stationary phase. We demonstrate that this column achieves narrow peak widths and sensitive ionization enabling the efficient separation of complex intact proteins mixtures.

User consent:

yes

Poster Session 2 / 76

ETD / EThcD functionality on the novel Orbitrap Excedion Pro mass spectrometer

Authors: Andreas Kuehn[None]; Cong Wang[None]; Heiner Koch[None]; Min Du[None]; Nicole Zehethofer[None]; Peter Krüger[None]; Reiko Kyoani[None]; Roberto Gamez[None]; Rosa Viner[None]

Corresponding Author: cong.wang2@thermofisher.com

Electron-transfer dissociation (ETD) is a well-established technique, where fragmentation is caused in cationic precursors by electron transfer from a donor molecule. While ETD/EThcD is already present in the family of Orbitrap Tribrid mass spectrometers, using Fluoranthene as an electron donor, this functionality is missing on the Orbitrap Hybrid mass spectrometers. With the Orbitrap Excedion Pro mass spectrometers, we introduce a Orbitrap Hybrid mass spectrometer with ETD and EThcD capabilities. Using the same electron donor as the Orbitrap Tribrid systems, ETD /EThcD reactions can be performed in the ion routing multipole (IRM).
Here, we want to present the performance of the ETD / EThcD functionality of the Orbitrap Excedion Pro mass spectrometer on the most common user cases of this fragmentation technique:
1) Detection of post translational modifications (PTM): Higher-energy Collision Dissociation (HCD) fragmentation is usually too harsh and causes the dissociation of the PTM from the connected peptide in the instrument. This can be avoided using ETD/EThcD as fragmentation technique
2) Differentiation between isomeric amino acids: ETD/EThcD can cause alternate fragmentations in isomeric amino acids like Asp/Iso-Asp and Leu/Ile. These alternate fragments (e.g. w-ions) can be used to differentiate between isomeric amino acids.
3) Top/Middle down analysis of proteins: Using ETD and especially EThcD fragmentation, a much higher sequence coverage can be achieved for the Top/Middle down analysis of proteins compared to HCD fragmentation
4) Analysis of disulfide bonds: Disulfide bonds can be cleaved in the mass spectrometer using ETD/EThcD fragmentation. This allows disulfide linked peptides to be analyzed as MS cleavable crosslinks.

User consent:

yes

Poster Session 1 / 75

Comparison of Standard vs High-field Orbitrap™ mass analyzer for charge detection mass spectrometry applications

Authors: Tobias Wörner[1]; Dmitry Grinfeld[1]; Alexander Makarov[1]

[1] *Thermo Fisher Scientific*

Corresponding Authors: alexander.makarov@thermofisher.com, tobias.woerner@thermofisher.com, dmitry.grinfeld@thermofisher.com

Top-down proteomics is a powerful tool for investigating complex biological processes in living organisms, both in health and disease. Unlike the more common bottom-up approach, top-down proteomics does not involve digesting proteins into peptides, providing a more comprehensive view of the proteome landscape. However, using intact proteins instead of peptides presents experimental challenges such as reduced sensitivity, low fragmentation efficiency, and difficulties in generating isotopically resolved precursor and product ion spectra.
Charge Detection Mass Spectrometry (CD-MS) is a single-ion technique where the ion's charge is directly inferred from its image current for each individual ion, eliminating the need for resolvable m/z features in ensemble mass spectra. While CD-MS has primarily been used for high-mass native applications, where individual charge states are not resolvable due to the heterogeneous nature of large protein complexes, its benefits can also be applied to (native) top-down proteomics.
CD-MS offers increased sensitivity and the ability to deconvolve congested spectra, ensuring that each peak corresponds to a single protein or peptide ion. This is particularly advantageous for top-down applications, where spectra are typically complex and congested. Thermo Scientific™ Direct Mass Technology™ mode, one of the few commercially available CD-MS solutions, has demonstrated

its capability in improving the detection of low-abundant proteoforms (DOI:0.1021/acs.jproteome.9b00797) and achieving isotopic resolution for high-m/z native complexes (DOI: 10.1021/acs.analchem.0c03282). In this study, we compare the performance of the standard and high-field Orbitrap™ mass analyzers using the Direct Mass Technology mode on native protein complexes. We explore their differences at longer transient times (4 seconds) and demonstrate the increased mass and charge accuracy of the more compact high-field Orbitrap mass analyzers.

User consent:

yes

Poster Session 2 / **80**

Quantification of monoclonal antibodies by middle-up capillary electrophoresis-mass spectrometry

Author: Jana Havlikova[1]

Co-authors: Katarina Marakova [1]; Peter Mikus [1]

[1] *Comenius University Bratislava*

Corresponding Authors: mikus@fpharm.uniba.sk, marakova@fpharm.uniba.sk, jana.havlikova@uniba.sk

Monoclonal antibodies (mAbs) are biopharmaceuticals widely used in treatment of various diseases. Due to their intrinsic complexity and post-translational modifications, therapeutic mAbs are highly heterogeneous molecules requiring extensive quality control. Quantitative analysis typically relies on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and the gold-standard bottom-up (BU) proteomic approach. This study presents an alternative middle-up (MU) approach combined with capillary zone electrophoresis–mass spectrometry (CZE–MS). CZE has previously demonstrated suitability for mAb analysis and is recognised as a quality control technique in the pharmaceutical industry. Here, we focus on the quantitative MU CZE–MS analysis of the immunoglobulin G (IgG) mAb infliximab (IFX) in the pharmaceutical product Remicade and two other biopharmaceuticals Avastin and MabThera.
IFX calibration standards were prepared at six concentrations, and mAb standard solution of adalimumab (ADA) (Merck, Darmstadt, Germany) as internal standard was added to each sample. Next, samples were reduced with TCEP reducing agent at 37 ℃ for 1 hour, then analysed using an Agilent 7100 CE system coupled to an Agilent 6410 triple quadrupole mass spectrometer (both Agilent, Santa Clara, USA). Pharmaceutical formulations of Remicade, Avastin, and MabThera were diluted to 20 µg/mL and analysed using the same MU CZE–MS workflow.
The developed method was validated in accordance with the ICH Q2(R1) guidelines for analytical method validation. It demonstrated good accuracy and precision for IFX quantification in Remicade, and was further applied to quantify bevacizumab and rituximab in their respective drug formulations. The results indicate that the optimised MU CZE–MS method is suitable for the quantitative analysis of various IgG therapeutic mAbs.

User consent:

yes

Poster Session 2 / **68**

Detection of intact bovine milk protein after gastrointestinal digestion using UHPLC-HRMS

Author: Hannah Zenker[None]

Co-author: Sabrina Gensberger-Reigl

Corresponding Author: hannah.zenker@fau.de

The presence and relative abundance of intact protein after gastrointestinal digestion is an important aspect of the evaluation of digestion efficiency and the assessment of protein allergenicity. The detection of intact proteins after gastrointestinal digestion is commonly done using gel electrophoretic applications (SDS-PAGE). These are highly sensitive, however in some cases SDS-PAGE does not allow for an unambiguously identification of the food proteins due to 1.) overlap of the bands of the digestive enzymes with the proteins of interest and 2.) the overlap of larger protein break-down products with lower molecular weight proteins. Therefore, this project aimed to explore whether UHPLC-HRMS can be used as a fast, sensitive, and unambiguous tool to monitor intact protein after gastrointestinal digestion. This was done on the example of bovine milk proteins. Skimmed raw milk was applied to a static in vitro gastrointestinal model to simulate infant digestion [1]. Samples were taken after 60 min in the gastric phase (GP60) and after 10 min intestinal digestion (IP10) and measured on an UHPLC-HRMS system (timsTOF Pro 2, Bruker Daltonics, Billerica, Massachusetts, USA). Additionally, samples were applied to SDS-PAGE to compare the two methods. Detection of β-lactoglobulin variant A was possible in the soluble phase of the digests GP60 and IP10 using UHPLC HRMS, while caseins were not detectable. This was in line with the analysis by SDS PAGE. In contrast, the band of α-lactalbumin (ALA) was visible on SDS PAGE at both digestion times points however with UHPLC-HRMS it could only be detected in GP60 but not in IP10. This could indicate that the band for ALA that is visible on SDS-PAGE in IP10 corresponds to a partially hydrolysed ALA with a hydrolysis degree that is too low to resolve with SDS-PAGE. However, this requires further confirmation.

User consent:

yes

Poster Session 2 / 62

Selective comprehensive online nanoLCxCZE-MS platform for top-down proteoform analysis

Author: Tobias Waldmann[1]

Co-authors: Alexander Stolz [1]; Christian Neusüß [1]

[1] *Aalen University*

Capillary zone electrophoresis-mass spectrometry (CZE-MS) has been used to separate proteoforms on intact protein levels, however, sensitivity and selectivity are often not sufficient for complex biological samples. We therefore developed a two-dimensional (2D) heart-cut nanoLC-CZE-MS platform and showed that this allows the pre-separation of intact proteins from a complex matrix and a 280-fold increased sensitivity compared to a one-dimensional CZE-MS approach[1]. While the transfer of a peak from the first to the second dimension is efficient in this setup, the characterization of multiple chromatographic peaks or partly separated proteoforms is time-consuming, requires high sample amounts, and might lead to incomplete proteoform characterization. Therefore, we expanded the platform to perform selective comprehensive nanoLCxCZE-MS. Here, the RPLC column is connected to a storage capillary by a 10-port valve to decouple the storage capillary from the first dimension. The volume of the stored fraction can easily be adjusted by changing the inner diameter or length of the capillary. The storage capillary is also connected to an 8-port nanoliter valve with 20 nL internal loops. Hence, 20 nL fractions can be transferred from the storage capillary to the second dimension. Here, we show the essential parameters of our selective comprehensive nanoLCxCZE-MS platform and demonstrate the high selectivity and sensitivity for the analysis of proteoforms in a human cell lysate (Caucasian colon adenocarcinoma cells). Using our selective comprehensive nanoLCxCZE-MS platform, we were able to detect up to four times more proteoforms in the stored

fraction compared to 1D nanoLC-MS. In addition, we show for Histone H4 how using CZE in the second dimension provides an additional layer of information for proteoform identification and validation in the cell lysate.

[1] A. Stolz, C. Neusüß, Characterisation of a new online nanoLC-CZE-MS platform and application for the glycosylation profiling of alpha-1-acid glycoprotein, Anal. Bioanal. Chem. 414 (2022) 1745–1757.

User consent:

yes

Poster Session 2 / 66

Illuminating Proteins: Dual UV/IR Photodissociation in a custom FT-ICR system

Authors: Ezgi Gürler[1]; Alan Kádek[2]

[1] *BIOCEV - Institute of Microbiology, Czech Academy of Sciences; Faculty of Science, Charles University in Prague*

[2] *BIOCEV - Institute of Microbiology, Czech Academy of Sciences*

Corresponding Authors: ezgi.gurler@biomed.cas.cz, alan.kadek@biomed.cas.cz

Top-down mass spectrometry (TDMS) elucidates the molecular weight, characterizes proteoforms, and, in its native form, can even inform binding stoichiometry and higher-order structure of proteins and their complexes. The information gained is, however, largely dependent on the protein system in question and the dissociation technique used. Therefore, there is a great need for more efficient and informative fragmentation methods to complement continued improvements in mass analyzers. These are together essential for extending the top-down MS to larger and more diverse assemblies. Photodissociation approaches, such as infrared multiple photon dissociation (IRMPD) and ultraviolet photodissociation (UVPD), coupled with ultra-high-resolution MS, offer interesting avenues for multi-modal fragmentation schemes with high information content.

In this contribution, following up on the pioneering work by Halim et al. (JASMS 2016) we explore parallel 193 nm UVPD and 10.6 μm IR laser activation applied to proteins using a custom laser-coupled 15-Tesla FT-ICR mass spectrometer. A rich and balanced fragmentation array of a/x, b/y, and z ions is produced when intact ubiquitin is exposed to UV and IR lasers simultaneously or sequentially in a single MS/MS experiment. Internal fragment inclusion in data processing further increases identified fragment numbers and improves average sequence coverage for denatured proteins. Our initial results demonstrate that the use of mixed photodissociation provides benefits for comprehensive protein characterization of denatured proteoforms and offers great promise for future native TDMS, especially when combined into multi-modal fragmentation schemes with other dissociation techniques available in an FT-ICR.

User consent:

yes

Poster Session 2 / 64

Top-Down Mass Spectrometry in a Service Environment: Insights from User Projects and Workflow Implementation

Authors: Laure Menin[None]; Natalia Gasilova[1]

Co-authors: Alexander Hilditch [1]; Konstantin Nagornov [2]; Yury Tsybin

[1] *EPFL*

[2] *Spectroswiss*

Corresponding Authors: tsybin@spectroswiss.ch, laure.menin@epfl.ch, natalia.gasilova@epfl.ch, alexander.hilditch@epfl.ch, nagornov@spectroswiss.ch

Top-down mass spectrometry (TD MS) is a powerful approach for the in-depth analysis of protein modifications, offering detailed, and sometimes unique, insights into intact proteoform structures. As the demand for proteoform-level information grows in biomarker discovery, drug development, and structural biology, there is increasing need for specialized fee-for-service (core) facilities equipped with TD MS instrumentation, advanced data analysis tools, and the expertise required to execute these complex experiments reliably, rapidly, and efficiently.
We present the TD MS workflow implemented in our MS service facility for proteoform-level characterization. As a representative example, we analyzed a set of proteins (18 - 40 kDa) chemically modified on lysine residues with a retinal group, aiming to localize the modification sites. All experiments were performed on an Orbitrap Exploris™ 240 instrument equipped with the Intact Protein Mode and HCD fragmentation.
After intact mass determination, precursor ions across multiple charge states were subjected to multiplexed fragmentation using various normalized collision energies (NCEs). Data were processed using the Peak-by-Peak software (Spectroswiss), enabling comprehensive analysis from RAW files to annotated sequence maps with putative modification localization. The workflow includes isotopically resolved deconvolution (Hardklör algorithm with ±1 Da correction), charge state grouping, spectral recalibration, peak similarity scoring, user-defined modification input, multiplexed result fusion, and final interactive visualization of sequence maps.
We will share our experience and highlight selected user-submitted projects successfully completed using this workflow, demonstrating its value for proteoform analysis in a fee-for-service context. Despite its promise, broader adoption of TD MS in Switzerland remains limited, primarily due to restricted access to TD MS-grade platforms combining high-resolution mass spectrometers with suitable LC configurations, particularly in core facilities focused on peptides or small molecules.

User consent:

yes

Poster Session 1 / 51

Deuterium-Induced Mass Shifts in MALDI Protein Fingerprints for Enhanced Antibiotic Susceptibility Prediction

Author: Jia Yi[1]

Co-author: Liang Qiao [2]

[1] *Minhang Hospital, Fudan University*

[2] *Department of Chemistry, Fudan University*

Corresponding Authors: jia_yi@fudan.edu.cn, liang_qiao@fudan.edu.cn

Bacterial infections are among the leading causes of morbidity and mortality worldwide, posing a significant threat to public health. The rapid emergence of antimicrobial resistance has further exacerbated the challenges in treating infections, making timely and accurate diagnosis crucial for effective patient management. Current methods for bacterial infection diagnosis typically involve two key processes: bacterial identification and antibiotic susceptibility testing (AST). Bacterial identification is usually performed using MALDI-TOF MS, but AST remains limited by the time-consuming bacterial culture process, which can delay results by 6–24 hours. The need for faster, more efficient AST methods is urgent to guide the appropriate use of antibiotics, minimize the spread of resistance, and improve patient outcomes.

To address the limitations of traditional AST methods, we developed a rapid, cost-effective approach that integrates deuterium labeling, MALDI-TOF MS, and machine learning. This method leverages deuterium incorporation into newly synthesized bacterial proteins during antibiotic exposure. The resulting deuterium-induced mass shifts in protein profiles create unique patterns that can be used to distinguish resistant from sensitive strains. These mass shift features are extracted and analyzed using machine learning algorithms, enhancing the accuracy of antibiotic susceptibility predictions. By eliminating the need for extended bacterial culture and sample preparation, this method reduces AST time to just 0.5 to 1 hour after bacterial identification. This streamlined approach not only accelerates AST but also integrates both bacterial identification and susceptibility testing into a single mass spectrometer platform, making it highly compatible with existing MALDI-TOF MS systems used in clinical settings. This innovation offers a scalable solution for rapid AST, improving diagnostic efficiency and enabling faster, more accurate patient care while reducing healthcare costs.

User consent:

yes

Biomedical Applications / 47

A Top-down Hybrid MS Approach Captures Extent and Dynamics of Simultaneous Phosphorylation Events in AMP- activated kinase Complex

Author: Boris Krichel[1]

Co-authors: Hsin-Ju Chan [2]; Liam Bandura [2]; Zhan Gao [2]; Holden Tyler Rogers [2]; Marius Natsch [3]; Alina Theisen [4]; Susanne Alfken [4]; Detlev Suckau [4]; Uetrecht Charlotte [5]; Ying Ge [2]

[1] *CSSB Centre for Structural Systems Biology, Deutsches Elektronen Synchroton DESY, Leibniz Institute of Virology, Notkestraße 85, 22607 Hamburg, Germany & University of Wisconsin, WI, USA*

[2] *University of Wisconsin-Madison, WI, USA*

[3] *Universität zu Lübeck, SH, Germany*

[4] *Bruker Daltonics*

[5] *CSSB Centre for Structural Systems Biology, Deutsches Elektronen Synchroton DESY, Leibniz Institute of Virology, Notkestraße 85, 22607 Hamburg, Germany*

Corresponding Author: boris.krichel@uni-luebeck.de

Introduction:
AMP-activated kinase (AMPK) complex is the regulatory hub of cell energy metabolism, linked to heart disease and longevity, making it an attractive drug target. AMPK activity involves sophisticated subunit isoforms, conformational changes, and post-translational modifications. Phosphorylation controls AMPK through upstream kinases, generating diverse proteoforms whose extent and dynamics remain unexplored. We studied AMPK phosphorylation using a novel hybrid top-down and bottom-up MS approach to reveal simultaneous phosphorylation events and capture biochemical reaction dynamics.
Methods:
Full-length heterotrimeric AMPK complexes (α1β1γ1/α2β2γ2) were expressed in E.coli and modified in vitro with CaMKK, GSK3-beta, and PP2A in presence of nucleotides, inhibitors, and activator Pfizer-739. Total subunit phosphorylation was determined using intact mass RPLC-MS on C4 columns with Bruker Impact II Q-ToF. Site-specific phosphorylation kinetics used DDA/PRM on timsToFpro. Top-down MS employed TriVersa Nanomate coupled with solariX XR6 12-Tesla FTICR-MS. Data analysis used FragPipe v.22.0, Skyline, MASH Native v.1.1, and Bruker OmniScape 2025.
Results:
Biochemical assays with CaMKK, PP2A, and Pfizer-739 revealed extensive AMPK phosphorylation dynamics. Top-down LC-MS showed unique plateauing growth curves for three AMPK subunits, revealing distinct proteoform pool dynamics. Bottom-up peptide quantification followed site-specific kinetics using a phosphorylated/unphosphorylated peptide database, quantifying >10 phosphopeptide pairs at MS1 level. Mapping these timsToF kinetic curves onto structural models provides novel

insights into AMPK's activation cycle. Top-down MS characterized simultaneous phosphorylation sites missed by bottom-up approaches. Current work focuses on GSK3-β phosphorylation in cardiac isoform AMPK α2 C-terminus and high-resolution top-down MS on ScimaX 7T MRMS. Our complementary results facilitate dysregulation studies and pharmacological interventions.
Novel Aspect:
Hybrid MS approach reveals simultaneous AMPK phosphorylation events and captures biochemical reaction dynamics with unprecedented precision.

User consent:

yes

Poster Session 1 / 87

Identification of deamidation sites in intact insulin via internal fragments produced by collision-induced dissociation

Author: Ruben Szabo[1]

Co-authors: Cynthia Nagy [1]; Attila Gaspar [1]

[1] *University of Debrecen*

Corresponding Author: szabo.david.ruben@science.unideb.hu

The prevalence of diabetes is steadily increasing, prompting the widespread use of various commercially available insulin analogs products in clinical practice. Assessing the chemical and structural stability of these pharmaceuticals should be carefully addressed, as their long-term efficacy and safety are intrinsically linked to chemical degradation pathways, such as deamidation [1]. Currently, it is unknown exactly which positions and to what extent deamidation occurs in the various insulin analogues. Therefore, identifying the most frequently occurring deamidated forms and thoroughly investigating their potential physiological effects is of particular importance [2].
Our research group successfully separated the deamidated forms of various insulin analogs, as well as distinguishing them from the native active pharmaceutical ingredient, using capillary zone electrophoresis [3]. We employed collision-induced dissociation (CID) to fragment the deamidated forms of human insulin during their separation. Although CID is a widely used technique, its efficiency is limited in this context: fragmentation events occurring in the region enclosed by the disulfide bridges do not result in a change in m/z, as the disulfide bonds hold the structure together. Terminal fragments outside of this region allow the straightforward identification of some deamidation sites. Appropriate low-abundant internal fragments had to be selected to identify the rest of the deamidation sites. Overall, we were able to separate human insulin from four singly deamidated forms and identified the site of deamidation for each component.

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User consent:

yes

Poster Session 2 / 26

Identification and quantification of methionine oxidation in monoclonal antibody using middle-down proteomics with electron ac-

tivated dissociation

Authors: Abdulafeez Akinloye[1]; Alok Shah[2]; Clifford Young[1]; Leigh Donnellan[1]; Mariam Nassiri[2]; Mark Condina[3]; Nathan Edwards[2]; Peter Hoffmann[1]

[1] *University of South Australia, Adelaide, Australia*

[2] *CSL Ltd, Melbourne, Australia*

[3] *Mass Dynamics, Melbourne, Australia*

Corresponding Authors: clifford.young@unisa.edu.au, abdulafeez.akinloye@mymail.unisa.edu.au, alok.shah@csl.com.au, nathan.edwards@csl.com.au, mark@massdynamics.com, peter.hoffmann@unisa.edu.au, mariam.nassiri@csl.com.au, leigh.donnellan@unisa.edu.au

Methionine oxidation is one of the most frequently observed post-translational modifications of monoclonal antibody (mAb) products during their development and production that can affect the drug' s safety and efficacy. Such modifications are typically assessed using bottom-up peptide mapping techniques. However, this methodology is often tedious and prone to the generation of artefacts through the sample preparation process. In this study, we present a middle-down method that is complementary to a bottom-up approach to characterise methionine oxidation in mAbs, utilising electron activated dissociation (EAD) using a ZenoTOF 7600 (Sciex).
Oxidised mAb and non-oxidised mAb samples were mixed to create different oxidation levels. These samples were digested to subunits (Lc, Fd'and Fc/2) with IdeS protease and EndoS. Digested samples were denatured and reduced in guanidine hydrochloride/DTT. The samples were injected on to a Waters Acquity BEH C4 column and separated with 0.1% FA in H2O and 0.1% FA at 7 µL/min. MS analysis was carried out using an optimised MRMHR EAD experiment on ZenoTOF7600. Data obtained were processed for subunit mass analysis, sequencing, oxidation identification and quantification using Sciex OS and Biologics Explorer software packages.
The optimised middle-down EAD method allowed us to measure different oxidised species of the antibody subunits with 15 PPM or better mass accuracy. The MS/SM spectra showed the fragments mass shifts due to various oxidation on the subunits. EAD fragmentation allowed site-specific identification of methionine oxidation sites on Lc at M4; Fd'at M34 and M101; Fc/2 at M16, M122 and M192. Quantification of oxidised mAb, using Fc/2, was achieved down to 1% oxidation level with a strong correlation between expected and measured oxidation percentage at both MS1 and MS2.
A middle-down EAD method has been developed that can be applied for complimentary characterisation of site-specific methionine oxidation of mAb products during formulation process.

User consent:

yes

Poster Session 2 / 70

Mapping transient, short linear motif-mediated protein-protein interactions using photo-crosslinking top-down mass spectrometry

Author: Yee-Man Michelle Pang[1]

Co-authors: Maria Mateos-Jimenez [2]; Peter Fox [1]; Shannon Misplon [1]; Adam Schofield [1]; Caroline Morris [3]; Christopher Coxon [1]; Clinton Veale [4]; David Clarke [1]

[1] *EaStCHEM, School of Chemistry, University of Edinburgh*

[2] *Navarrabiomed, centro de investigación biomédica*

[3] *AstraZeneca*

[4] *Department of Chemistry, University of Cape Town*

Corresponding Authors: p.fox@ed.ac.uk, s.k.misplon@sms.ed.ac.uk, clinton.veale@uct.ac.za, caroline.morris@astrazeneca.com, dave.clarke@ed.ac.uk, mariamateos.mmj@hotmail.com, y.m.pang@sms.ed.ac.uk, chris.coxon@ed.ac.uk, adam.schofield@ed.ac.uk

Protein–protein interactions (PPIs) govern countless biological processes and range from stable, permanent complexes to transient, short-lived ones. Among the latter, short linear motifs (SLiMs) play a critical role by mediating low-affinity, transient interactions. These are essential for cellular functions like directing protein localization through targeting signals. However, due to their small, transient interfaces, SLiM-mediated interactions are challenging to study using traditional structural biology techniques or current machine learning tools. Despite their importance, methods to specifically discover and characterize SLiM-mediated interactions remain limited. Herein, we developed a new photo-crosslinking strategy to map SLiM-mediated interactions in the HSP90-HOP model system, where binding is known to be mediated by the C-terminal MEEVD motif of HSP90 and the TPR2A domain in HOP. Using chemical biology techniques and solid-phase peptide synthesis, we substituted specific residues within the MEEVD sequence with diazirine-containing unnatural amino acids. Diazirine generates highly reactive carbene species upon UV-irradiation, which covalently insert into nearby bonds within the TPR2A domain, thereby stabilizing the transient interaction for downstream analysis. We initially verified that diazirine containing peptides retained the ability to bind to TPR2A by native mass spectrometry. After UV-irradiation crosslinking was verified by LC-MS before crosslinked complexes were subsequently examined by top-down mass spectrometry (TD-MS). TD-MS allowed us to locate crosslinking site of MEEVD with the TRP2A domain with single amino acid spatial resolution. Interestingly, different diazirine-containing peptide displayed different crosslinking efficiencies and patterns.

The results allowed us to build a picture of binding orientation in the peptide-protein complex and our findings aligned with the binding interface observed in the crystal structure (PDB: 1ELR). These findings demonstrate a new powerful strategy for capturing and mapping SLiM-mediated interactions. In the future, this approach could be extended to map SLiMs across broader PPI networks, advancing our understanding of transient signaling interactions at the proteome level.

User consent:

yes

Poster Session 2 / 40

Multi-scale mass analysis for the characterization of engineered DNA assemblies

Author: Elisabetta Boeri Erba[1]

Co-authors: Vincent Agache4 Agache [2]; Mathilde Manceau [3]; Didier Gasparutto [3]; Christophe Masselon [4]; Christine Saint-Pierre [3]; Chloee Tymen [1]

[1] *Univ. Grenoble Alpes, CEA, CNRS, IBS, F-38000 Grenoble, France*

[2] *Univ. Grenoble Alpes, CEA, LETI, F-38054 Grenoble, France*

[3] *Univ. Grenoble Alpes, CEA, CNRS, SyMMES-UMR 5819, F-38000 Grenoble*

[4] *Univ. Grenoble Alpes, CEA, Inserm UA13, BGE, F-38054 Grenoble, France*

Corresponding Author: elisabetta.boeri-erba@ibs.fr

Nucleic acids (DNA, RNA, artificial analogues such as TNA) can form remarkable three-dimensional structures, which play key roles in biology and nanobiotechnology. In the field of DNA nanotechnology, static or dynamic nucleic acid assemblies have applications in biotechnology, nanomedicine, nanophotonics and nanoelectronics. Determining the size, shape and assembly route of oligomeric nucleic acids (like cages, Legos or DNA origami) is essential to understand their structure and to improve their rational design. Current techniques, notably gel electrophoresis, chromatography and dynamic light scattering, determine the average size of nanostructures, while atomic force microscopy and electron microscopy can assess individual particle morphology with nanometre resolution. However, these methods do not provide accurate details on the stoichiometry of folding products, intermediates in assembly pathways, nor homogeneity and stability of biomolecular assemblies in different buffers.

To fill this gap, we aim to develop a multi-scale analytical approach for characterising DNA assemblies using mass spectrometry (MS) in native mode. In particular, we propose to utilise different modalities of MS: native nano-ESI-MS, Suspended NanoResonator (SNR) and NanoElectroMechanical Sensor (NEMS). These approaches allow the investigation of a wide mass range from a few hundred Dalton to hundreds of MegaDalton. Indeed, we intend to determine masses of a variety of DNA assemblies and hybrid architectures (DNA-organic molecules, DNA-particles, DNA-proteins, etc.). In addition, we aim to synthesize functionalized architectures that can be used as standards to improve the accuracy of mass measurement in the multi-megadalton mass range. Overall, our multi-scale analytical approach will enable us to elucidate the structural composition of diverse DNA architectures, their oligomeric state, size and morphology.

User consent:

yes

Poster Session 2 / 74

Assessment of the small protein proteomes of Methanosarcina mazei under different stress conditions using a combination of top-down and bottom-up proteomics.

Authors: Liam Cassidy[1]; Eva Herdering[1]

Co-authors: Philipp T. Kaulich [2]; Ruth Schmitz [1]; Andreas Tholey [1]

[1] *Kiel University*

[2] *University of Kiel*

Corresponding Authors: p.kaulich@iem.uni-kiel.de, l.cassidy@iem.uni-kiel.de, a.tholey@iem.uni-kiel.de

Short open reading frame (sORF) encoded proteins fulfil important roles in many cellular processes. In the methanoarchaeon *Methanosarcina mazei*, numerous small proteins have previously been identified under different nitrogen availabilities, however, few have been functionally characterised. Consequently, a detailed analysis of small proteins translated under a range of growth conditions may reveal interesting candidates for future downstream analyses.
We investigated the small proteome of *M. mazei* under five growth conditions (sufficient nitrogen, insufficient nitrogen, viral stress, heat stress, and salt stress). Here, we utilised a previously developed, top-down and bottom-up compatible, solid phase extraction methodology that enabled enrichment of the low molecular weight proteome. Subsequently, we applied both an optimised LC-FAIMS-MS top-down workflow and a standard LC-MS-based bottom-up proteomic analysis. The approach allowed for the inferred BUP identification of 234 small proteins. Additionally, top-down proteomic analysis identified 408 proteoforms for 130 protein accessions with canonical sequences less than 101 residues in length. In total, 251 small proteins could be identified via the combined approach, of which 16 were only detected by top-down analysis.
Aiming to unravel the functions of the uncharacterized small proteins, we performed sequence-based clustering with emphasis on the presence of characteristic motifs. This led to the identification of 51 small proteins containing CxxC motifs, which are potential ferredoxin-like small proteins with putative iron-sulfur (Fe-S) cluster binding-sites, or zinc-binding proteins.
Overall, the comprehensive analysis of the *M. mazei* small proteome under various growth conditions, using a combination of top-down and bottom-up proteomic approaches, in addition to sequence-based analyses, represents a key step in systematically uncovering the functions of small proteins in *M. mazei*.

User consent:

yes

Poster Session 1 / 67

Increasing sensitivity and reducing carryover for IgG glycoform characterization with monolithic hydrophilic interaction liquid chromatography-mass spectrometry

Author: Thomas Holmark[1]

Co-authors: Andrea Gargano [1]; Annika van der Zon [1]

[1] *University of Amsterdam*

Corresponding Authors: t.holmark@uva.nl, a.gargano@uva.nl, a.a.m.vanderzon@uva.nl

Immunoglobulins are a class of proteins that are crucial for the immune response to fight diseases. They contain many different post-translational modifications, and in particular, changes in the glycosylation patterns of immunoglobulins subclass-G (IgGs) can be linked to disease progression. In our project we aim to develop capillary-based LC-MS methods to characterize intact IgG secreted by in-vitro cultivated B-cells aiming to profile the changes in IgG glycosylation profile as response to the exposure to different stimuli.

Traditional RPLC-MS based methods focusing on intact analysis fail to capture the full glycoform distribution as isomeric species and low abundance glycoforms cannot be characterized due to co-elution. HILIC separations represent a good alternative to separate the different glycans on intact IgGs and allow for a more in-depth analysis of the glycoproteoform expressed.

Previous research has focused on synthesis optimization of monolithic HILIC columns to improve separation of monoclonal antibody (mAb) samples. The current methods suffers from carryover requiring several blank injections between samples, and requires 50 ng to be loaded on-column.

This study systematically investigates methods to mitigate the carryover affects by employing self-packed PLRP-S stationary phase trap columns and changing washing solvents and modes. Additionally, we also explore the decrease in column I.D. as a method to decrease the flowrate and gain sensitivity, potentially allowing for analysis of sample loading of a few ng on-column. Our preliminary results show that the PLRP-S trap columns can reduce the carryover from the trap column due to a decrease in secondary interactions. We also observed increases in sensitivity when decreasing the I.D. of the HILIC columns and flowrate. The developed workflow allows for analysis of IgG glycan distributions with reduced carryover and increased sensitivity. In the next steps, we aim to apply this optimized method to the in-depth IgG glycoform characterization of exposed B-cells.

User consent:

yes

Poster Session 2 / 82

Probing Human Oocyte Heterogeneity by Single Cell Proteoform Imaging Mass Spectrometry

Authors: Chelsea Lock[1]; Diane C. Saunders[2]; Elizabeth L. Tsui[2]; Hannah B. McDowell[2]; Indira Pla Parada[3]; Jared O. Kafader[4]; Monica M. Laronda[5]; Neil L. Kelleher[6]; Nickolas Fisher[1]; Tian Xu[3]; Vijaya Kanchustabahm[7]

[1] *Department of Chemistry and Molecular Biosciences, Northwestern University, Evanston, IL, USA.*

[2] *Stanley Manne Children's Research Institute, Ann & Robert H. Lurie Children's Hospital of Chicago and Department of Pediatrics, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA*

[3] *Proteomics Center of Excellence, Northwestern University, Evanston, IL, USA*

[4] *Department of Chemistry and Molecular Biosciences, Proteomics Center of Excellence, Northwestern University, Evanston, IL, USA*

[5] *Stanley Manne Children's Research Institute, Ann & Robert H. Lurie Children's Hospital of Chicago, Department of Pediatrics, Feinberg School of Medicine, Department of Obstetrics and Gynecology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA*

[6] *1Department of Chemistry and Molecular Biosciences, 2Proteomics Center of Excellence, Northwestern University, Evanston, IL, USA*

[7] *Proteomics Center of Excellence, Northwestern University*

Corresponding Authors: chelsealock2026@u.northwestern.edu, diane.saunders@northwestern.edu, n-kelleher@northwestern.edu, indira.plaparada@northwestern.edu, hannah.mcdowell@northwestern.edu, nickolasfisher2027@u.northwestern.edu, vijayalakshmi.kanchustambh@northwestern.edu, mlaronda@luriechildrens.org, jared.kafader@northwestern.edu, tian.xu@northwestern.edu

Ovarian tissue cryopreservation (OTC) is utilized by clinics to preserve oocytes and immature eggs for pediatric patients who are at increased risk of becoming infertile due to a diagnosis or treatment. Late-stage oocytes, that are not yet mature, are often released during OTC. We are interested in identifying proteoforms that may improve in vitro maturation of oocytes into eggs for these patients as an additional fertility preservation strategy. We are utilizing single cell proteoform imaging mass spectrometry (scPiMS), which is a nanoDESI direct sampling approach coupled to individual ion mass spectrometry (I2MS) detection, to profile human oocytes to identify and assess the intact proteoform population between single oocytes. We have used this scPiMS approach to analyze 24 denuded oocytes from 6 different participants/donors aged 1.71 years old to 33. We have also utilized the high spatial resolution afforded by the scPiMS probe (~100 μM) to selectively sample oocytes and cumulus granulosa cells within 4 cumulus oocyte complexes (COCs) from two participants to identify proteoforms that are cell-type specific. We have identified ~78 proteoforms on average across the 28 oocytes sampled, with an average identification rate of THRASH proteoform features of ~55%. We have identified several proteoforms that are highly specific to oocytes rather than cumulus granulosa cells, including oocyte expressing protein homolog (OOEP), KH-domain containing protein 3 (KHDC3), and ferritin light chain. We utilized this data to further understand the proteomic heterogeneity of single oocytes between patients and between multiple oocytes from the same donor. Finally, we demonstrate the utility of scPiMS for analyzing patient specific proteoform landscapes by identifying OOEP and KHDC3 proteoforms with SNPs and variable modifications across the cohort. We ultimately aim to expand our analysis to quantify the abundance of these proteoforms and understand how proteoform landscapes change in the context of age.

User consent:

yes

Poster Session 1 / 81

Tau proteoform assay performance using a single-molecule analysis platform

Author: Zhang Zhengjian[1]

Co-authors: Ana Forton-Juarez [2]; Andreas Huhmer [1]; Brittany Nortman [1]; Emily Sartori [3]; James Joly [1]; Jarrett Egertson [1]; Jessica Schwarz [2]; Joel Blanchard [2]; Kara Juneau [1]; Maryam Jouzi [1]; Parag Mallick [1]; Raj Bhatnagar [1]; Vivek Budamagunta [1]

[1] *Nautilus Biotechnology*

[2] *The Ronald Loeb Center for Alzheimer's Disease at Mt.Sinai*

[3] *The Ronald Loeb Center for Alzheimer's Disease at Mt.Sinai,*

Corresponding Authors: vivekb@nautilus.bio, rajb@nautilus.bio, karaj@nautilus.bio, zhengjianz@nautilus.bio, emily.sartori@mssm.edu, andreash@nautilus.bio, joel.blanchard@mssm.edu, maryamj@nautilus.bio, jarrette@nautilus.bio, paragm@nautilus.bio, jschwarz1030@gmail.com, brittanyn@nautilus.bio, jamesj@nautilus.bio, ana.forton-juarez@icahn.mssm.edu

The microtubule binding protein Tau (MAPT) has been implicated as a driver of diverse progressive neurodegenerative diseases such as Alzheimer's disease. Six splicing isoforms of the Tau protein

and over 70 different post-translational modifications (PTMs) have been identified on Tau that are believed to impact fibril formation and ultimately disease progression. Though the general prevalence of these alterations has been studied, there remains a significant knowledge gap with regards to which proteoforms (defined by splice variants and one or more PTMs) are most prevalent.

We have developed a single-molecule protein analysis platform. The platform immobilizes individual protein molecules on a hyper-dense flow cell and then iteratively probes those molecules with splice-variant specific or PTM-specific affinity reagents. Each protein molecule on the flow cell is assigned an estimated proteoform based the probes that were detected to have bound that protein.

For platform validation we first created reference proteoforms with recombinantly expressed proteins and mass spectrometric analysis was used to determine which sites and percent of Tau were modified. Several mixtures of these control proteoforms were then generated, with different ratios of components and analyzed on the platform. These studies showed both that mixtures of proteoforms could be accurately quantified. We additionally estimate the platform reproducibility. Finally, the quantitative sensitivity was measured for individual proteoforms over 3 logs of dynamic range. To demonstrate the biological relevance of the assay, we applied the assay to a number of neuronal tissue such as iNeurons, miBrains and human brain.

These studies demonstrate that the Nautilus platform is able to accurately and reproducibly quantify proteoform molecular heterogeneity in cell-based samples. We anticipate that assays like this will ultimately enable a deeper investigation into the role that proteoform heterogeneity plays in diseases like Alzheimers and may also potentially enable proteoform-based diagnostics with greater sensitivity than existing approaches.

User consent:

yes

Sample Preparation & Separation Technologies / 92

Homogenization of tissues via picosecond-infrared laser (PIRL) ablation

Corresponding Author: hschluet@uke.de

User consent:

Poster Session 2 / 86

nQuant Enables Precise Quantitative N-Glycomics

Author: Yudong Guan[None]

Co-authors: Shanshan Zhao [1]; Chunjin Fu ; Junzhe Zhang ; Fan Yang ; Jiankai Luo ; Lingyun Dai ; Xihai Li ; Hartmut Schlüter ; Jigang Wang ; Chengchao Xu

[1] *University Hamburg*

Corresponding Author: shanshan.zhao@studium.uni-hamburg.de

Abstract: N-glycosylation, as the most complex post-translational modification of proteins, involves crucial biological functions. It has been demonstrated that aberrant N-glycosylation is directly linked to various human diseases, while mass spectrometry-based N-glycomics still lags behind, limiting the in-depth mining of glycobiological information. Aiming to rectify the bias of the previous quantitative N-glycomics strategies, we developed nQuant, a glycoinformatics tool that enables label-free

and isotopic labeling quantification of N-glycomics data obtained via LC-MS/MS, ensuring a low false quantitation rate. nQuant integrates the total signal intensities of N-glycans by considering their mass spectrometric behaviors, including different adducts, charge states, and isotopic distributions, which enables precise quantitative N-glycomics.
Conclusion: Due to the emerging roles of N-glycans in fundamental biological processes and human health, it is important to accurately quantify N-glycans in a systematic manner, a prerequisite for functional studies. In this study, we developed nQuant, a glycoinformatics tool that enables label-free and isotopic labeling quantification of N-glycomics with a low FQR. The key to our design is to automatically integrate different adducts, charge states, and isotopic signals of N-glycans detected by MS. Compared to previous glycoinformatics tools, our design exhibits two major strengths: 1) it enables more precise quantitative N-glycomics through newly developed quantification algorithms, and 2) it includes a calibration algorithm for isotopic labeling quantification. Thus, nQuant proves to be a powerful glycoinformatics tool to understand N-glycome landscapes and dynamics, and it could be used in large-scale glycomics studies.

User consent:

yes

Poster Session 1 / 85

Advancing Sustainability in Liquid Chromatography: Eco-Friendly Solvents and Innovative Column Technologies

Author: Egidijus Machtejevas[1]

Co-authors: Benjamin Peters [1]; Petra Lewits

[1] *Merck Life Science KGaA*

Corresponding Authors: benjamin.peters@merckgroup.com, petra.lewits@merckgroup.com, egidijus.machtejevas@merckgroup.co

Liquid chromatography is a vital analytical technique widely employed in pharmaceuticals, biotechnology, food and beverage industries, and environmental monitoring. This presentation will explore sustainable alternatives to traditional reversed-phase chromatography, which accounts for over 75% of applications and typically relies on environmentally harmful solvents like acetonitrile.
The role of eco-friendly solvents, including water, hot water, ethanol, supercritical CO2, and bio-based solvents, will be highlighted, emphasizing their importance in reducing the environmental impact of liquid chromatography. Method optimization through smaller internal diameter columns, shorter column lengths, and energy-efficient systems will also be discussed, focusing on strategies to minimize solvent consumption and waste.
Part 1 will present Capillary High-Performance Liquid Chromatography (HPLC) as a means to enhance sensitivity and sustainability, featuring complementary phases such as RPLC, HILIC, and porous graphitized carbon (PGC) columns for various analytical challenges, including nucleoside separations and glycan profiling.
Part 2 will cover advancements in superficially porous particles, showcasing new additions to the Ascentis® Express and BIOshell™ portfolios. These innovations, including columns designed for improved peak shapes and stability across pH ranges, will be presented in the context of applications in OMICS fields, such as metabolomics and glycomics, underscoring their potential to enhance analytical capabilities while promoting sustainability.

User consent:

yes

Poster Session 2 / 84

MS-based characterization of clinically relevant glycoproteoforms

Author: Yuri van der Burgt[1]

Co-authors: Elena Domínguez-Vega [1]; Manfred Wuhrer [1]

[1] *Leiden University Medical Center*

Corresponding Authors: y.e.m.van_der_burgt@lumc.nl, e.dominguez_vega@lumc.nl, m.wuhrer@lumc.nl

Dynamically altering biomolecules are the true actors in studies on health and disease rather than genotypes or phenotypes that refer to a certain risk or an end-point observation. Unfortunately, clinically effective disease-specific tests that support diagnoses at an early and curable stage are still lacking for a wide variety of diseases. Proteoform-resolved data provides a layer of information additional to transcription, translation, and posttranslational events that underlie complex phenotypes. Of these, glycoproteoforms experience an increasing interest in providing personal baselines for diagnostic and disease monitoring purposes. It is hypothesized that determining a limited number of different species is sufficient to achieve clinical relevance and provide new means of patient stratification. In glyco-analytical strategies, mass spectrometry (MS) combined with different types of chromatography has dominated innovations, and glycoproteins such as transferrin and immunoglobulins are commonly identified and glyco-profiled in an intact or semi-intact manner [MCP 22(6) 100565 (2023)]. Top-down approaches are feasible, albeit that such methods report relative quantifications. As a rule, clinical implementation however requires precise characterization and quantification of glycoproteoforms with complementary strategies, namely released glycan- and glycopeptide-centric analysis, such as for antithrombin and prostate-specific antigen. It is foreseen that glycoproteoform analysis will provide unique phenotypes for each individual that combined with pattern recognition tools exhibit great potential in guaranteeing safe and accurate test results for patients in the era of precision medicine.

User consent:

yes

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Top-down characterization of intact adeno-associated virus (AAV) capsid protein deamidation

Authors: Corentin Beaumal[1]; Jonathan Bones[2]; Josh Smith[1]; Sara Carillo[1]

[1] *National Institute for Bioprocessing Research and Training, Dublin, Ireland*

[2] *National Institute for Bioprocessing Research and Training, Dublin, Ireland; University College Dublin, Dublin, Ireland*

Corresponding Authors: jonathan.bones@nibrt.ie, corentin.beaumal@nibrt.ie, josh.smith@nibrt.ie, sara.carillo@nibrt.ie

Recombinant AAV (rAAV) vectors are a leading gene delivery system due to their low immunogenicity, tissue specificity, and high efficiency. Monitoring intact AAV capsid proteins (VPs) and their post-translational modifications (PTMs) is critical for ensuring product quality and efficacy. A crucial PTM to monitor is deamidation as it can alter product potency, stability, and function1. Reversed-phase intact LC-MS methods have proven effective in separating VPs from their deamidated counterparts2 while top-down MS approaches have been shown to be effective in characterizing individual proteoforms. Combining the two approaches can improve AAV VP characterization by allowing for detection of modifications on individual isoforms.
Commercial and in-house produced AAV products were used. Samples were analysed using a Vanquish Horizon ultra-high pressure liquid chromatograph hyphenated to an Orbitrap Eclipse mass spectrometer equipped with ETD, HCD, EThcD and UVPD fragmentation, as well as PTCR capabilities. Separation was performed using an ACQUITY Premier Protein BEH C4 (2.1 x 150 mm) column. Data was processed using Thermo Scientific Biopharma Finder software.

Along with the expected VP1, VP2, and VP3 proteins, deamidated proteoforms of each respective VP were separated. Unstressed and heat-stressed samples were analysed to examine whether changes in deamidation could be detected when samples were exposed to stressed conditions. Samples exposed to heat exhibited increased levels of deamidation, particularly in on VP3. A variety of different top-down (TD) fragmentation strategies were explored for characterizing the VPs of AAV9. Sequence coverage up to 5% was obtained by combining multiple fragmentation techniques. Additionally, 10% and 5% sequence coverage of VP2 and VP1 was achieved, respectively. The increased sequence coverage for VP3 is due to both its increased abundance and smaller size. Finally, thanks to the combination of multiple fragmentation techniques, TD-MS approach provided insights into the location of deamidation events, particularly for the stressed samples.

User consent:

yes

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Spatial and Single Cell Proteoform Analysis Using Direct Sampling Single Molecule Mass Spectrometry

Author: Pei Su[1]

[1] *Northwestern University*

Corresponding Author: pei.su@northwestern.edu

"Proteoform"is a term describing the actual molecular forms of proteins including sequence variations and posttranslational modifications, bringing us one step closer to phenotypes and dynamic states. In addition, direct proteoform measurement bypasses steps in conventional proteomics experiment (e.g., proteolytic digestion, chromatographic separation), providing a path to proteomics in spatial tissue compartments and single cells at unprecedented sensitivity and throughput.
Mass spectrometry (MS) is superior for proteoform measurement by accurate determination of molecular masses and connectivity of amino acid sequences and modifications. To achieve sensitive MS detection of proteoforms, we employed individual ion mass spectrometry (I2MS), an Orbitrap-based charge detection MS technique in conjunction with direct sampling MS approaches. We demonstrate proteoform imaging mass spectrometry (PiMS) using nanospray desorption electrospray ionization (nano-DESI) has enabled highly-multiplexed imaging and identification of tissue proteoforms up to 70 kDa. These proteoforms not only help discern tumor margins in ovarian cancer tissues at ~20 μm spatial resolution, but also report anatomical regions and cellular neighborhood of human kidney, contributing to our knowledge in reference tissue atlases. Such approach has been extended to high-throughput single cell profiling at a speed of >1000 cells per day, enabling proteoform-based cell typing in complex cell mixture from rat brain hippocampus. We discovered small populations of glial cells and resident immune cells using novel proteoform signatures. Finally, nanosecond infrared laser ablation was coupled to Orbitrap MS that allowed for molecular analysis of intact proteins and their complexes in their native physiological environment. Together these tools unveiled previously unknown molecular channels that may be employed for future fundamental biological research, biomarker discovery and disease diagnostics.

User consent:

yes

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A Comprehensive Approach for Top-Down Biopharmaceutical Characterization, PTM Assessment, and Unknown Identification

Author: Detlev Suckau[1]

Co-authors: Stuart Pengelley [1]; Georgia Orfanoudaki [2]; George Alevizos [2]; Waltraud Evers [1]; Eckhard Belau [1]; Susanne Alfken [1]; Alina Theisen [1]; Marius JC Natsch [3]; Boris Krichel [4]; Mariangela Kosmopoulou [2]

[1] *Bruker Daltonics GmbH & Co. KG*

[2] *Fasmatech Science & Technology*

[3] *University Lübeck*

[4] *CSSB, DESY & Leibniz Institute of Virology, Hamburg & University of Lübeck, Germany*

Corresponding Author: detlev.suckau@bruker.com

This study discusses the advanced software-supported Top-Down characterization of biopharmaceutical proteins, addressing the limitations of traditional bottom-up analyses. Despite historical challenges in Top-Down sequence analysis due to inadequate software and instrumentation, this research presents a comprehensive method using modern software tools and fragmentation techniques for high sequence coverage. We examined proteins such as monoclonal reference antibody NISTmAb fragments, including LC, Fd, and Fc/2, utilizing a script to identify chromatographic peaks and generate average MS1 and MS2 spectra. These spectra were matched to expected sequences, assessing putative PTMs.
A novel workflow allowed method optimization and analysis via direct-infusion setups with various acquisition methods, including charge state and fragmentation chemistries (such as CID, EAD, ECD), and fragmentation strengths (reaction time, collision energy). The OmniScape software facilitated direct spectrum comparisons and combined sequence maps for optimal SC and PTM/proteoform assignments.
Additionally, workflows for identifying unknown proteins and side products through de novo sequence tag generation and homology-based database searches using MS-BLAST were developed.
The challenge of simultaneous identification and localization of multiple PTMs was addressed using heuristic algorithms in OmniScape, screening billions of proteoforms to retrieve top-scoring candidates for confirmation. Using this PTM Screening approach, we analyzed the poly-phosphorylated protein AMPK, identifying a proteoform phosphorylated at multiple specific residues. This comprehensive software-supported method enhances the accuracy and efficiency of biopharmaceutical characterization, facilitating improved PTM assessment and unknown identification.

User consent:

yes

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Advanced IgG subunit characterization employing nanoRPLC-MS

Author: Despoina Mavridou[1]

Co-authors: Constantin Blöchl [2]; Christoph Gstöttner [2]; Manfred Wuhrer [2]; Elena Domínguez-Vega [2]

[1] *Leiden University Medical Center (LUMC)*

[2] *Leiden University Medical Center*

Corresponding Authors: d.mavridou@lumc.nl, c.blochl@lumc.nl, m.wuhrer@lumc.nl, c.j.gstoettner@lumc.nl, e.dominguez_vega@lumc.nl

IgGs mediate diverse immunological responses by binding antigens via their Fab and interacting with downstream receptors (e.g., Fcγ) via their Fc region, activating different pathways. Their remarkable variability originates from the B cell differentiation and activation in response to antigens,

resulting in an immense repertoire of different antigen-specific clones. In addition, the immune system can fine-tune the immune response by using an armamentarium of different Fc characteristics. This highlights the necessity to characterize IgGs. The large heterogeneity of endogenous antibodies requires highly sensitive methods to tackle critical clinical samples. Therefore, we developed two miniaturized methods focusing on the intact subunit level with enhanced sensitivity: one for Fc/2 and one for IgG1 Fab analysis.
Polyclonal IgGs are purified from the serum of single donors using Fc-specific beads. Subsequently, depending on the focus of the analysis, IgGs are subjected to either below-hinge cleavage or above-hinge cleavage for intact Fc/2 or Fab characterization, respectively. For the analysis of IgG subunits - Fc/2 and Fab - separation is conducted by employing a miniaturized RPLC-MS-based method, with a flow rate of 1 µL/min. Furthermore, a CaptiveSpray source with dopant gas is used to enhance the sensitivity and the ionization efficiency. This strategy enables us to achieve high sensitivity while we can analyze intact Fc/2 from only 1 µL of serum and intact Fabs from 10 µL.
When applied to clinical samples, this method provides a comprehensive analysis of the Fc/2 at the proteoform level, providing information on subclasses, allotypes, and glycoforms. On the other hand, Fab profiling offers insights into clonality and potential post-translational modifications (PTMs).

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User consent:

yes