2025 Advancing Mass Spectrometry for Biophysics and Structural Biology

Georgia Tech | Atlanta, GA USA
July 20-23, 2025

ABSTRACT BOOK

(in alphabetical order by presenter last name)

Native Charge Detection Mass Spectrometry Measurements of Messenger RNA

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The remarkable success of messenger RNA (mRNA) vaccines during the coronavirus pandemic highlighted the vast potential of nucleic acid-based therapeutics. Advancing this therapeutic modality requires analytical approaches capable of making rapid and accurate measurements to assess the identity and structural integrity of these large RNA molecules. However, most existing techniques of RNA characterization are limited by the size of RNA, necessitating the development of novel analytical tools for the analysis of large RNAs, defined broadly as RNAs in the 1000-10,000 base length range. In this work, we demonstrate the application of charge detection mass spectrometry (CDMS) to address this gap. This work establishes mRNA standards for method optimization and validates CDMS as a robust approach for native analysis of mRNA.

EGFP, FLuc, beta-galactosidase, and Cas9 mRNA were purchased from TriLink Biotechnologies. pUC19 DNA plasmid was purchased from Thermo Fisher Scientific and New England Biolabs. mRNA was directly diluted to 20 nM using 200 mM ammonium acetate prepared in DNAse/RNase-free water. pUC19 was buffer exchanged using centrifugal spin columns (Bio-Rad Micro Bio-Spin 6) and diluted to 20 nM using 10 mM ammonium acetate. Native charge detection mass spectrometry measurements were conducted on a Thermo Fisher Scientific Q Exactive™ UHMR Hybrid Quadrupole Orbitrap™ Mass Spectrometer equipped with Direct Mass Technology™. Samples were nanoelectrosprayed using in-house pulled Au/Pd-coated borosilicate emitters. Negative and positive polarity were evaluated. Data analysis was performed using STORIBoard and UniDec.

To develop a method for nucleic acid CDMS, we used a circular double-stranded DNA plasmid pUC19 (1690 kDa), a standard that has been previously studied by CDMS. We were able to measure the mass within ~6% of the sequence mass. This method is under development to obtain better mass accuracy; however, it laid the foundation for methods to be developed for mRNA analysis. Next, we systematically optimized CDMS methods and sample preparation protocols for the analysis of four commercially available 5'-capped, polyadenylated mRNAs (EGFP, FLuc, beta-galactosidase, and Cas9), representative of in vivo mRNA transcripts or mRNA biotherapeutics. mRNA samples were 1000 to 4500 nucleotides long with masses ranging from 300-1500 kDa. As nucleic acids are largely negative molecules, we developed methods and performed measurements in both positive and negative polarity, analyzing their cationic and anionic forms. All masses were measured within 6-10% of their sequence mass. Some of the additional mass in each case can be attributed to counterions (NH_4^+ and/or Na^+) as the negatively charged DNA/RNA backbone is typically coordinated by cations in solution. Compact charge state distributions have been observed in these measurements, confirming that mRNAs were structurally uniform and the ionization and analysis conditions were gentle. CDMS analysis was also performed at higher collision energies to improve mass accuracy via adduct removal. Under these conditions, the measured masses were within ~0.7-7% of the mRNA sequence mass. To evaluate the role of counterions in mass deviations, we measured mRNAs that were chemically denatured and those that were heat denatured and refolded in the presence of Mg^{2+}.

This work represents the first CDMS measurements of mRNAs in their native states and will contribute significant new tools for large RNA characterization. Also, this study will expand the landscape of mass spectrometric applications toward the characterization of large RNAs, which is currently a sparsely studied research area.

Studying Protein-ligand Interactions using Novel Ionization Methods and In-droplet Hydrogen Deuterium Exchange

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Presenting Author: Sohag Ahmed

The novel ionization technique capillary vibrating sharp-edge spray ionization (cVSSI) has been shown to more effectively preserve the native structures of challenging proteins for mass spectrometry (MS) experiments. For cVSSI, high ionization efficiency can even be obtained without the application of voltage. Additionally, decoupling microdroplet production from a coulombically driven process allows cVSSI to access three different pathways to progeny nanodroplet production. In this study, cVSSI is used to identify protein stability changes upon ligand binding for one of these pathways. Furthermore, protein structure stabilities and conformer heterogeneity in ligand-bound and free states are examined by in-droplet hydrogen deuterium exchange (HDX).

The cVSSI devices were constructed by affixing a piezoelectric transducer onto one end of a glass slide using epoxy glue. A laser-pulled fused-silica capillary, attached to the distal edge of the glass slide, was used as ion emitter tip (diameter 20-25 μm). A (~40 cm long) PTFE tubing was slip-fit onto the blunt end of the connected pulled-tip capillary, and a short platinum wire was inserted into the near end of the tubing to supply DC voltage to the infused solution. The opposite end of tubing was affixed to the needle luer lock attached to the syringe (BD 1ml), enabling direct infusion of samples by a syringe pump. Native mass spectrometry experiments were conducted using a Q-Exactive orbitrap mass spectrometer.

The native structure of carbonic anhydrase II (29kDa) was preserved successfully with this novel ionization method. Better preservation of the native state was achieved at 350° C temperature and an applied voltage of +1300 V. Notably, for the protein solution lacking the ligand (Acetazolamide), the CSD in the mass spectrum centered on the 10+ ions. Incubation of the protein with even the lowest concentration of the ligand (10 nM) resulted in a shift of the CSD (centered on the 8+ ions). This occurred even in the absence of the observation of protein-ligand ions. Ligand titration experiments revealed a Kd of 4±2 μM which is in good agreement with literature values. In-droplet HDX-MS studies revealed some conformational differences of the protein in ligand-bound and unbound states. Specifically, a significant reduction in deuterium uptake was observed in the ligand-bound state. Notably, ~50% reduction in HDX reactivity is obtained for the ligand-bound species. Considering the size of the ligand, it is unlikely that the reduced exchange is entirely related to protection at the ligand binding site. It is here suggested that ligand binding substantially reduces backbone flexibility which is necessary for rapid HDX in microdroplets. Presently, molecular dynamics (MD) trajectories are being scored to determine regions of the protein showing decreased flexibility upon ligand binding, revealing structural stabilization and conformational changes of the protein in bound and unbound states.

The native structure of carbonic anhydrase II was preserved by cVSSI. The binding affinity (Kd) of the ligand was determined. With the in-droplet HDX-MS study, the relative conformational flexibilities of the protein in ligand-bound and unbound states were determined.

Sometimes All You Need is a Little HeLP: Heme Labeling by Proximity Unveils Heme Regulation of the Insulin Receptor

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Heme is a necessary Janus-faced metabolite, involved in gas transport, catalysis, and signaling. However, when it is improperly regulated, it can have deleterious effects such as membrane disruption via intercalation and lipid oxidation, generation of reactive oxygen species, and activation of aberrant signaling pathways. This can lead to diseases such as colon cancer, Alzheimer's disease, and type two diabetes. Although much is known about the biosynthesis of heme, there is still much to discover about the transport, storage, and signaling pathways affected by heme, such as how high heme intake can lead to the onset of type 2 diabetes. We therefore sought to develop a hemoproteomics method that leverages the inherent redox capabilities of protein bound heme to catalyze self-biotinylation.

For live cell work, HEK293 or other cells were incubated with heme and/or heme analog in DMEM with biotin tyramide for 20 minutes, then hydrogen peroxide was added to catalyze the labeling reactions. Reactions were quenched with sodium azide and sodium ascorbate after 1 minute, then the cells were lysed. The extent of biotinylation was assessed via western blot and LC-MS/MS for hemoproteomics. To assess how heme impacts insulin signaling, HEK293 cells were incubated with heme or heme analog for 10 minutes. Insulin was added and cells were incubated for a further two minutes before cells were lysed with a phosphatase inhibitor lysis buffer. The phosphorylation state of the insulin receptor was assessed via western blot.

To develop our method, we started by assessing the peroxidase capabilities of known heme binding proteins human serum albumin (HSA) and bovine serum albumin (BSA). To do this, we used a dityrosine fluorescence assay, where we observed increased oxidative dityrosine formation only in the presence of heme with HSA or BSA. We next tested the ability of these proteins to catalyze their self biotinylation in the presence of heme, hydrogen peroxide, and biotin tyramide. We observed heme biotinylates these proteins in a dose dependent manner, with HSA having a higher biotinylation rate at lower heme concentrations. This result was reversed in the presence of non-redox active heme analog Zn (II) protoporphyrin IX (ZnPPIX), reiterating that heme needs to be bound to a protein in order to catalyze the labeling reaction. We next moved into live cells, wherein we demonstrated the ability of heme to label heme binding proteins in three different cell types. After confirming activity in live cells, we completed triplicate LC-MS/MS hemoproteomic analysis of HEK293 cells, finding 403 heme binding proteins, including known heme binders such as cytochromes and PGRMC 1/2. We also used this approach to visualize heme dynamics in cells over time via fluorescence microscopy. According to our hemoproteomic results, the insulin receptor (IR) binds heme, a previously unknown interaction. We then assessed how heme impacts insulin signaling, finding that heme alone activates the receptor and inhibits IR phosphorylation in the presence of insulin. To determine what aspects of heme and what oxidation state are necessary for signaling disruption, we used Ga (III) and Zn (II) protoporphyrin IX, metal free protoporphyrin IX, and iron (III) chloride to test their impact on insulin signaling, finding that heme in the 3+ state likely activates the receptor and heme in the 2+ state likely blocks insulin signaling.

We have developed a novel hemoproteomics approach that can be used in live cells and has been used to identify 403 heme binding proteins. We also found that heme binds to the insulin receptor and can activate it on its own and inhibit activation in the presence of insulin.

Enhanced Detection of Targeted Phospholipids by Native Ion Mobility–mass Spectrometry with Selective Lipid Binding and Collisional Ligand Ejection

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Presenting Author: Michael Armbruster

Targeted metabolomics is a proven tool used to understand biological systems, yet many metabolites remain elusive due to low abundance, difficult isomers, or inefficient ionization. Here, we leverage protein-ligand interactions and native ion mobility-mass spectrometry (nIM-MS) to selectively capture difficult metabolites. The cyclic ion mobility (cIM) platform is used for successive rounds of mobility isolation and activation to release and purify metabolites in the gas phase. Phospholipase A_2 (PLA_2) is used to selectively bind phospholipids from E. coli lipid extracts, reducing sodium adduction and nonspecific binding. We then show high-resolution phosphatidylinositol phosphate (PIP) profiling by applying mobility-aligned fragmentation to brain-derived PIP_2 lipids incubated with glucose-6-phosphate isomerase (GPI). Overall, we demonstrate the utility of nIM-MS as a tool for targeted metabolomics.

PLA_2 was incubated with a polar lipid extract from E. coli (Avanti Research) in 25 mM ammonium acetate at neutral pH, followed by buffer exchange into 25 mM ammonium acetate to remove unbound lipids and adducts. For PIP_2 experiments, brain-derived PIP_2 (porcine, Avanti Research) was incubated with glucose-6-phosphate isomerase under similar conditions. Both sample sets were analyzed using a Waters Select Series cyclic IM–MS (Milford, MA), enabling mobility selection and sequential fragmentation, including mobility-aligned fragmentation in the transfer region for detailed fatty acid profiling. Protein-metabolite complexes were generated by static nanoelectrospray ionization (nESI) using gold-coated borosilicate capillary needles and were isolated in both m/z and mobility space prior ligand ejection in the pre-array of the Cyclic IMS.

The cIM-MS platform was leveraged for gas phase fractionation, activation, and separation of noncovalent complexes. Complexes were mobility selected and activated upon re-injection from the pre-array into the cyclic IMS to liberate the bound ligands. The lipids were then mobility selected and released for a final separation before detection. Collision cross-section (CCS) comparisons between gas-phase liberated lipids and both database and experimentally determined drift tube values of standards varied by less than 2%.PLA_2-assisted extraction of PG lipids from an E. coli polar lipid extract resulted in a highly selective enrichment of phospholipids. This selective extraction and gas-phase release also resulted in approximately 50% lower levels of salt adduction for PG lipids compared to an equivalent solution analyzed directly by nESI. Quantitative catch-and-release experiments were performed with an isotope-labeled standard and demonstrated excellent linearity in the quantitation of PG lipids, achieving an average R^2 of 0.995 across an order of magnitude of concentrations. A separate series of experiments leveraged glucose-6-phosphate isomerase (GPI) for the selective extraction of brain-derived PIP_2 lipids. PIP 2 lipids were liberated from the complex in the gas phase, separated by multiple passes around the cyclic IM separator, and fragmented in the transfer region to produce mobility-aligned fragments which enabled detailed fatty acid chain profiling of PIP 2 isomers. Collectively, the data sets from the PLA_2 and PIP_2 experiments highlight the versatility of our native IM–MS and collisional ligand ejection strategy for the detection of difficult metabolites. The enzyme-assisted selective extraction significantly reduces spectral complexity, even in the presence of mM levels of interfering salts and polar metabolites. Further work expands the scope of targeted lipid species and integrates additional isotope standards to refine quantitation of PIP 2 lipids. These results show the utility of native MS and cIM as a powerful tool for targeted metabolite analysis.

This native IM–MS method enables quantitative, selective metabolite extraction with enhanced phospholipid targeting and fatty acid profiling by mobility-aligned fragmentation.

Influence of Methylating the Serine Side-chain on the Decomposition of Protonated Asparaginyl Serine using GIBMS, IRMPD, and Theoretical Modeling

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In previous work on the protonated dipeptide, [AsnSer+H]+, dehydration occurs primarily to form an oxazoline (OxaI) losing the O1 oxygen, along with a minor contribution of a diketopiperazine (DKP), which loses the more usual O3 oxygen. To help confirm this unusual behavior, we have examined decomposition of protonated AsnSer methylated on the serine side chain, [Asn(OMe)Ser+H]+.

We utilize threshold collision-induced dissociation (TCID) conducted on a guided ion beam tandem mass spectrometer (GIBMS) to examine deamidation and dehydration from [Asn(OMe)Ser+H]+. We also use infrared multiple photon dissociation spectroscopy (IRMPD) to verify the reactant and product structures. These experimental analyses are reported in parallel with complementary quantum-chemical calculations, where key reaction energies are determined at the B3LYP, ωB97XD, and MP2(full) levels of theory.

Comparison of IRMPD and theoretical spectra identifies the deamidation product as a furanone. Dehydration occurs through the formation of multiple products, but we assign the primary product to be a DKP. The data were modeled and show that deamidation begins at 142 ± 9 kJ/mol and dehydration at 123 ± 17 kJ/mol. Compared to the unmethylated analogue, the deamidation threshold is comparable whereas that for dehydration is elevated, consistent with methylation shutting down the lowest-energy OxaI pathway for dehydration. Thus, methylation of the Ser side-chain shuts down the OxaI pathway, whereas the DKP and oxazolone pathways that eliminate the O3 oxygen are still available. Consistent with this is the observation that dehydration of [Asn(OMe)Ser+H]+ has a higher threshold energy than that determined for [AsnSer+H]+, 104 ± 10 kJ/mol. This shift in thermodynamics and the IRMPD spectra are consistent with our previous conclusions that the backbone O1 oxygen is lost to form the OxaI product from [AsnSer+H]+. Here, loss of the O3 oxygen is the primary contributor for dehydration.

Experimental validation of an unusual dehydration mechanism for protonated asparaginyl serine.

Rapid Online Buffer Exchange with the DynaChip Platform for Complex Biological Analysis

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Top-down and native analysis of complex biomolecules by mass spectrometry (MS) offers new insights into their higher-order structures (HOS). To preserve HOS, protein samples must be stored in specialized buffer solutions often incompatible with MS due to required high concentrations of non-volatile salts. Buffer exchange into MS-compatible solutions is therefore essential prior to MS analysis. This transfer typically utilizes manual offline spin-column washing protocols or online, lengthy size-exclusion chromatography methods. In contrast, the novel DynaChip platform uses tangential flow filtration/microdialysis to enable rapid online buffer exchange with sample-to-sample analysis time under 15 minutes. This vendor-agnostic platform, optimized for nanoESI applications, simplifies sample preparation by enhancing existing workflows while providing flexibility and throughput to enable MS integration of new workflows.

The DynaChip features a microfluidic chip containing a biochemically inert membrane with tunable pore size, allowing for retention and analysis of diverse analytes while effectively removing various interferents. Samples were manually loaded into the DynaChip (Andson Biotech) sample loop and injected at 0.5 µL/min. Online buffer exchange was performed via tangential flow filtration/microdialysis with the conditioning solvent flowing at 50 mL/hr. The conditioner flow can be customized to incorporate MS-compatible solutions (e.g., ammonium acetate), supercharging molecules (e.g., mNBA), or protonation sources (e.g., formic acid) based on analytical requirements. The DynaChip was directly coupled to downstream instruments for analysis, including a Xevo G2-S Q-Tof (Waters), a SELECT SERIES Cyclic IMS (Waters), a Q Exactive (Thermo), and a CD-MS instrument (Megadalton Solutions).

Through rapid online buffer exchange, the DynaChip streamlines MS workflows across diverse protein samples and complex macromolecules while preserving native structural integrity. This versatility was demonstrated in applications ranging from native MS to charge detection MS (CD-MS). For native MS, the DynaChip successfully characterized mAb glycoforms, protein-ligand complexes, and membrane proteins, while demonstrating unique benefits for specialized techniques like collision-induced unfolding (CIU). For example, we tested AqpZ, an E. coli aquaporin membrane protein with sample buffer containing C8E4 detergent. AqpZ was successfully ionized despite having no detergent in the exchanging fluid, which is hypothesized to be due to the rapid buffer exchange that maintains the detergent concentration above the critical micelle concentration. For CIU workflows, the DynaChip maintained the ground state transition which can be observed with off-line benchtop workflows while eliminating the need for benchtop buffer e

The DynaChip platform enables rapid online buffer exchange for MS analysis, reducing sample preparation time to under 15 minutes while preserving native structures. Its broad compatibility with diverse samples, buffers, and analytical techniques significantly enhances the accessibility and efficiency of complex biological analysis by MS across multiple applications.

Dynamic Spray Mass Spectrometry

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The desire to develop a universal ion source for mass spectrometric (MS) analysis of various compounds in a single experiment has remained but a dream. We hypothesized that a process that makes electrospray ionization (ESI) compatible with atmospheric pressure chemical ionization (APCI) will provide an opportunity to detect a broader range of analytes. In this presentation, we will describe a process in which nonthermal plasma can be fused into charged microdroplets to enable the ionization of both polar (including proteins) and nonpolar compounds. We have developed a dynamic high voltage power supply with DC voltage ramping capabilities. In this way, automatic voltage tunning is achieved, with each analyte in the mixture detected at a specific optimized voltage. The resultant composite mass spectrum includes various ion types such as [M+nH]n+, [M-H]+, [M-H]-, M•+, and adducts. Unlike dual hybrid commercial sources, the current dynamic spray process is achieved on a single emitter, eliminating the need to ascertain analyte properties before the analysis. Parameters of the voltage ramping process can be selected to terminate certain ionization mechanisms. The process can be accomplished using low flowrates in nESI or in traditional ESI at high flowrates for LC-MS. By altering the ramp period, a multitude of ionization mechanisms can be completed during the elution of a single chromatographic peak (up to 8 s peak width), enabling the effective ionization of coeluting species via different mechanisms. The power of the dynamic spray MS technology will be showcased by the analysis of various metabolites detected from untreated whole blood for malaria diagnosis.

Nonspecific Carbohydrate Adduction Increases with Increasing Collisional Cross-section of Globular Proteins during Native-ESI MS

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Presenting Author: Madeline Bannon

Native mass spectrometry can measure protein-ligand (PL) binding constants (Kd), which describe the strength of binding interactions. During ionization by the charged-residue model (CRM), unbound ligand and protein can interact, forming nonspecific PL complexes. Nonspecific binding (NSB) yields errors in calculated Kd. However, reference proteins, that have no specific interactions with the ligand, can be used to monitor NSB and correct Kd. We have observed increased NSB for lysozyme (pI=11.07, 14.2kDa) and α-lactalbumin (pI=5.09, 14.2kDa) compared to ubiquitin (pI=7.02, 8.56kDa). However, it is unclear whether increased NSB is related to protein charge or increasing surface area since α-lactalbumin and lysozyme are larger than ubiquitin. Here, we investigate the relationship between protein size and NSB using monomeric, dimeric, and tetrameric hemoglobin.

Denatured ubiquitin, cytochrome c, and myoglobin (Sigma-Aldrich, St. Louis, MO) were collisional cross section (CCS) calibrants. Holo-hemoglobin (P) (Sigma-Aldrich) was desalted into ammonium acetate with P6 columns (Bio-Rad, Mississauga, ON). Maltotriose (Cayman Chemical, Ann Arbor, MI) and 2,3'-sialyllactose (Elicityl, Crolles, France) were nonspecific ligands (L). P (5μM) and L were mixed at 1:20, 1:6, and 1:1 in separate samples. Samples were electrosprayed from glass capillaries (1-2μm) (Sutter Instrument Company, Novato, CA) on a Synapt G2-S HDMS (Waters Corporation, Milford, MA). Charge states and number of adducts were identified and peak areas were integrated. Fractional abundance was calculated by dividing the summed peak areas for all hemoglobin adducts by the summed areas for all hemoglobin peaks.

We hypothesize that increasing protein surface area results in increased formation of nonspecific adducts between ligands and globular proteins, such as hemoglobin. Here, collisional cross section (CCS) is used to describe gas-phase protein size. The experimental TWCCSN2He for monomeric hemoglobin are (1560±60) Å2 and (1680±60) Å2 for the 7+ and 8+ charge states, respectively. CCS increases with charge state due to Coulombic repulsion, as seen here. The fractional abundance of maltotriose (100μM) adduction to monomeric hemoglobin was 0.24±0.06 and 0.29±0.09 for the 7+ and 8+ charge states, respectively. TWCCSN2He values for dimeric hemoglobin were (2600±100) Å2, (2700±100) Å2, (2800±100) Å2, and (2900±100) Å2 for the 10+, 11+, 12+, and 13+ charge states, respectively. The resulting maltotriose fractional abundance was 0.42±0.08, 0.4±0.1, 0.4±0.1, and 0.42±0.09 for the dimeric 10+, 11+, 12+, and 13+ species, respectively. Finally, for tetrameric hemoglobin, the TWCCSN2He values were (4600±200) Å2 and (4600±200) Å2 for the 15+ and 16+ charge states. The 17+ and 18+ charge states each had two distinct populations: with TWCCSN2He of (4500±200) Å2 and (4800±200) Å2 for the 17+ and (4500±200) Å2 and (4900±200) Å2 for the 18+. Average fractional abundances of 0.5±0.2, 0.5±0.1, 0.5±0.1, and 0.5±0.1 were calculated for the 15+, 16+, 17+, and 18+ charge states, respectively. There was a significant increase in the average fractional abundance of NSB of maltotriose with higher order oligomers. We also investigated NSB of 2,3'-sialyllactose (L), a negatively charged trisaccharide. The same trend of increasing fractional abundance with increasing oligomeric state was observed. Averaged fractional abundances for all the charge states of the monomer, dimer, and tetramer were 0.28±0.07, 0.5±0.1, and 0.6±0.1, respectively. Overall, a positive correlation between protein TWCCSN2He and the fractional abundance for both maltotriose and 2,3'-sialyllactose was observed at all three ligand concentrations in +ESI.

This work shows that NSB increases with increasing protein CCS. Use of a reference protein to monitor and correct for NSB can ensure determination of accurate Kd values. However, this study highlights the need to select a reference protein with a similar size to the protein of interest.

Combining Glycan Engineering with Ion Mobility Selected Collision Induced Unfolding (IM-CIU) to Determine Glycoform Influence on Monoclonal Antibody Structural Polydispersity

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Monoclonal antibody (mAb) therapeutics dominate global biotherapeutic sales due to their large, dynamic, and structurally complex nature. Glycosylation, a key post-translational modification, contributes largely to this complexity. Glycan microheterogeneity can modulate activity and aggregation propensity, making glycoengineering a valuable tool for tuning the function of mAbs. Despite its importance, characterizing the full structural landscape of glycosylated mAbs remains a challenge, leaving gaps in our understanding of the sequence–structure–function relationship. Herein, we leverage a Waters Select Series Cyclic Ion Mobility (cIM) system to employ collision induced unfolding (CIU) and IM-CIU to explore mAb structural dynamics. By integrating enzymology and degradation studies, we explore how individual glycoforms influence mAb conformational ensembles, illuminating their structural role in both function and dysfunction.

TransGlycit© (Genovis) reactions were performed on Herceptin (trastuzumab, Mechem Express) to obtain individual glycoforms. Glycinator© (Genovis) was used to remove glycans and Fabricator© (Genovis) was used for middle-down digests. IM-CIU experiments were performed in triplicate on a cIM-MS system from Waters Corporation (Milford, MA). Gold capillaries were produced in-house for nano electrospray (nESI). To initiate IM-CIU, we quadrupole-select individual charge states for single-pass cIM-MS separations. Ions representing the leading or trailing portions of IM ATDs are stored in the cIM-MS pre-array and subjected to CIU for difference analyses. The selected ions were stored in the pre-array, activated to perform collision induced unfolding (CIU), and sent to the detector, while unselected ions were ejected. These data were evaluated using CIUSuite 3.

Native and deglycosylated Herceptin were indistinguishable by collision cross sections and FWHM, but CIU revealed a 14% destabilization in the high-energy transition (CIU50-3) upon deglycosylation, attributed to Fc domain unfolding. This CIU50-3 transition showed a bimodal feature (low CCS, A; high CCS, B), and the B form increased following deglycosylation. Remodeled glycoforms were analyzed by IM-MS and CIU. Though FWHM and CCS could not resolve structural differences, CIU-50 shifts at CIU50-3 highlighted glycoform-specific variation. A/B ratios correlated with glycoform identity and aggregation propensity in a pH-dependent study (pH 3–7). An A/B ratio of 0.9 correlated with increased aggregation (over 5%) with a sigmoidal fit (R^2=0.9353). G2S2F was most resistant to aggregation, while G2F and G0F were less so, aligning with prior observations. CIU-ECD of the bimodal feature revealed increased sequence coverage from the high CCS population (B form), especially in the CH2 domain where glycosylation occurs, suggesting potential glycan-driven structural divergence. IM-CIU enabled mapping of mAb polydispersity within the context of aggregation. Across Herceptin's IM ATD's leading and trailing portions showed different feature counts, indicating distinct coeluting structural populations. Native Herceptin at pH 7 lacked B form. Under pH stress, it appeared in the ATD's trailing half. In deglycosylated Herceptin, this feature was present even at pH 7 and intensified under stress. pH 7 G2S2F exhibited the B form in the trailing ATD half, while G0F did not. These data indicate glycoform-specific subpopulations may predispose the mAb to aggregation. By combining enzymology, degradation, and IM-CIU, we demonstrate glycoform-driven changes to the mAb structural ensemble. Our findings support the presence of multiple iso-CCS states and suggest mAb polydispersity is partly driven by glycosylation. This work highlights the potential of IM-CIU assays to enhance mAb therapeutic development.

Combining glycan engineering with IM-MS, CIU, and IM-CIU to parse apart the influence of glycoform on monoclonal antibody structural polydispersity.

IM-MS Unveils Global Protein Conformations in Response to Conditions that Promote and Reverse Liquid–liquid Phase Separation

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Presenting Author: Rebecca Beveridge

Liquid–liquid phase separation (LLPS) is a process by which biomolecules, particularly proteins and nucleic acids, condense into a dense phase that resembles liquid droplets. Dysregulation of LLPS is implicated in disease, yet the relationship between protein conformational changes and LLPS remains difficult to discern. This is due to the high flexibility and disordered nature of many proteins that phase separate under physiological conditions and their tendency to oligomerize. In published work, used ion mobility mass spectrometry (IM–MS) to investigate the conformational states of full-length ubiquilin-2 (UBQLN2) protein, LLPS of which is driven by high-salt concentration and reversed by noncovalent interactions with ubiquitin (Ub). We found that UBQLN2 exists as a mixture of monomers and dimers and that increasing salt concentration, which drives LLPS, causes the UBQLN2 dimers to undergo a subtle shift toward extended conformations. UBQLN2 binds to Ub in 2:1 and 2:2 UBQLN2/Ub complexes, which have compact geometries compared to free UBQLN2 dimers. Together, these results suggest that extended conformations of UBQLN2 are correlated with UBQLN2's ability to phase separate. In more recent work, we strove to understand the mechanism in which differentially linked tetra-ubiquitin chains, specifically those that are linked via lysine-48 (K48) or K63, affect the propensity of UBQLN-2 to phase separate. These results are surprising and interesting, as they do not reflect the expected behaviour that has been observed in the liquid phase. Overall, delineating protein conformations that are implicit in LLPS will greatly increase understanding of the phase separation process, both in normal cell physiology and disease states. References: Robb, C.G. et al. Journal of the American Chemical Society, 2023. 145(23): p. 12541-12549. Dao, T.P. et al. EMBO reports, 2022. 23(8): p. e55056.

Collision Induced Unfolding Detected by Hydrogen/Deuterium Exchange

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Mass spectrometry for structural biology is a rapidly evolving field, especially due to electrospray ionization and ion mobility mass spectrometry (IM-MS). Closely related protein isomers can be distinguished using collision induced unfolding (CIU) coupled with IM-MS. The different unfolding patterns relating to protein structure have been broadly applied to determine changes in protein stability under various conditions, modifications, and interactions. However, these experiments have been limited to mass spectrometers specifically equipped with low/intermediate pressure mobility cells. Here we demonstrate the use of gas phase hydrogen/deuterium exchange to detect structural changes resulting from CIU. As proteins undergo collisional unfolding, deuterium exchanges with newly accessible residues, resulting in observable m/z shifts.

Experiments were performed on a Synapt G2-Si Mass Spectrometer (Waters) equipped with a nanoelectrospray source. The instrument was modified to allow the introduction of ND3 into the transfer cell. Experiments were conducted in mobility mode with the initial trap collisional energy set to 5 V. A solution of 10 μM myoglobin in 200 mM ammonium acetate was sprayed in positive mode, and desired charge states were isolated using the quadrupole. After isolation and mobility separation, proteins were exposed to ND3 with a partial pressure of 8.3 μbar the transfer cell. The trap cell collisional energy was increased stepwise by 2.5 V and spectra were acquired.

Experiments using the model protein myoglobin 8+ have shown that at low collisional energies (CE) of 5 V to 15 V there is an increase of approximately 2 m/z, which corresponds to the exchange of approximately 16 deuterium. When the collisional energy is increased to 20 V the m/z distribution broadens but still appears to be a single distribution. By 22.5 V the distribution continues to broaden and includes an increase of nearly 5 m/z corresponding to the exchange of approximately 40 deuterium. At 25 V there are clearly two distributions overlapping with the second distribution, pertaining to the higher m/z, appearing to be favored based on a slightly higher intensity. After increasing the CE stepwise to 40 V collision induced dissociation takes place. As the instrument is equipped with ion mobility, it was used to verify the transition states changes in myoglobin 8+. Mobility data acquired at the same time shows the beginning of a major transition around 20 V CE and the completion of that transition by 30 V CE. This early experimental data demonstrates that the major transition state changes in myoglobin 8+ are verified with ion mobility as CIU took place in the trap cell prior to ion mobility.

This newly developed method enables the detection of structural changes CIU without requiring ion mobility, potentially expanding the adoption of CIU to a broader range of mass spectrometers and research labs.

UV Photofragments of Native-like Proteins Maintain a 'Memory' of the Precursor Protein Structure

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Determining the sequence and structure of a protein is crucial to understanding its cellular functions. Mass spectrometry offers the advantage of accurately identifying individual proteins within a heterogeneous biological sample. However, proposing a detailed three-dimensional protein structure directly from mass spectrometry-based data has not yet been accomplished. In this work, we show that UV photodissociation of native-like ubiquitin and myoglobin in a tandem-trapped ion mobility/tandem-mass spectrometer produces folded fragment ions. Taking advantage of the ability of Tandem-TIMS to determine the collision cross sections of both the precursor and fragment ions, we developed a machine learning-molecular dynamics method that proposes a protein structure using the cross sections of the folded photofragments as constraints.

All experiments were performed in an orthogonal tandem-TIMS/MS instrument coupled with a 213 nm UV laser produced from the fifth harmonic of a Nd:YAG laser (EKSPLA). The orthogonal tandem-TIMS/MS was constructed by modifying a commercial timsTOF Pro (in collaboration with Bruker Daltonics) with an additional trapped ion mobility spectrometry (TIMS) device and a linear ion trap operating at 2–3 bar between the two TIMS devices. 10 μM aqueous ubiquitin and myoglobin solution with 1v% acetic acid (Sigma) was infused into the electrospray source. RosettaFold was used to propose protein model strucures. Structural analysis of the experimental data was conducted using molecular dynamics-based structure relaxation approximation (SRA) method, estimating three-dimensional structures of the intact precursor proteins and the photofragment ions.

We selected the compact (native-like) and the extended (gas-phase) ubiquitin conformations of charge state 8+, respectively, and dissociated the backbone of the selected ubiquitin ions by collision-induced dissociation (CID) and UV photodissociation (UVPD). Fragments generated via CID from the native-like and gas-phase ubiquitin conformations exhibit negligible differences in the number of spectral features, their collision cross-sections, or their relative abundances. These findings can be rationalized by the slow heating nature of CID in which successive collisions with buffer gas neutrals produce a vibrationally hot electronic ground state, promoting isomerization into gas-phase conformations during the fragmentation process. On the other hand, we found that structures of many UVPD-generated fragments correlate with the precursor structure. UV photodissociation of the compact, native-like ubiquitin conformation produces fragment ions with folded conformations, whereas the extended gas-phase ubiquitin conformation produces extended, unfolded photofragment ions. The folded fragments produced from the native-like, folded ubiquitin conformation are ~20% smaller in cross section than those produced from the extended, gas-phase conformation. To test the metastability of the folded, compact fragment ions, we vibrationally activated the fragment ions by energetic collisions with buffer gas molecules prior to measuring their collision cross-sections in TIMS-2. We found that significant energetic activation is required to induce the unfolding of the compact UV fragment ions, implying that they are significantly stabilized by non-covalent contacts. We performed analogous experiments for myoglobin, a 17 kDa protein with a non-covalently bound heme group. Here, we observed multiple UV photofragments that maintain both a folded conformation and the non-covalent heme group. We then developed a computational machine learning-molecular dynamics approach in conjunction with RoseTTAFold to use the collision cross-sections of the native-like (intact) protein conformation and those of the folded fragments as constraints to propose the three-dimensional protein structure.

UV Photofragments of native-like proteins maintain a 'memory' of the precursor protein conformation

Investigating Conformational Heterogeneity of Various Caspase-9 Maturation States

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Uncontrolled cell-death can lead to neurodegeneration and cancers, underscoring the need for tight regulation. Caspase-9 (casp-9) is from a family of proteases involved in programmed cell death via apoptosis. Casp-9 has a pro-domain, called CARD, along with a large and a small subunit, that assemble to form a monomer (WTC9). There is currently no high-resolution structure of full-length holo casp-9, and many questions remain about domain interactions and dimerization. Ion mobility-mass spectrometry (IM-MS) data obtained under native solution conditions will provide insight into the casp-9 structure and conformation in various maturation states. We are using native IM-MS to explore formation of casp-9 dimers in the presence/absence of substrate by assessing the quantities of various cleavage states, number of charge state distributions, average charge state, and collision cross section (CCS).

IM-MS data obtained under native nanospray conditions (pH = 6.9; ionic strength = 100 mM ammonium acetate) was collected on a Waters Synapt G2. Drift times and CCS values were measured for various casp-9 constructs after expression in BL21 cells and purification. These constructs include the pro-domain (CARD), the pro-domain-deleted variant (ΔCARD), the full-length wild-type protein (WTC9), and WTC9 + z-VAD-fmk, which is a substrate mimic that causes dimerization by inactivating the enzyme. Samples with z-VAD-fmk were also subjected to activity checks prior to IM-MS to evaluate inhibition, as well as size exclusion chromatography- multiangle light scattering (SEC-MALS).

Caspase-9 (casp-9) exists in multiple conformational states, impacting its activity. We investigate the conformational landscape of casp-9 using native MS, focusing on the influence of the CARD domain and dimerization. Casp-9 consists of non-covalently bound small and large subunits, with the CARD domain covalently attached to the large subunit (CARD+Large). For all measurements, casp-9 activity in ammonium acetate was first confirmed. Native MS of WTC9 revealed three charge state distributions (CSDs), indicating conformational heterogeneity. Removal of the CARD domain (ΔCARD) resulted in a single CSD, suggesting conformational homogeneity. This construct was also able to facilitate dimerization at lower concentrations or substrate mimic ratios. Conversely, the CARD domain alone exhibited two CSDs, suggesting partial unfolding and indicating its contribution to the monomer's heterogeneity. Intriguingly, while the WTC9 monomer displayed three CSDs (compact, partially extended, and extended) under native nanospray conditions, only one CSD was observed for the unbound dimer. This suggests compaction and stabilization of the dimer through its additional interactions. Each of the three monomer conformations can exist in uncleaved, single-cleaved, and double-cleaved forms, resulting in nine total possible monomer states. The dimer, while exhibiting a single conformation, also displays these three cleavage states. Collision cross-section (CCS) measurements of the single-cleaved dimer (5600±40 Å²) were comparable to the partially extended monomer, suggesting that dimerization may induce a more compact conformation in the flexible CARD domain. Finally, we investigated dimer formation with and without z-VAD-fmk, a substrate mimic that inactivates casp-9 and initiates dimerization. Our goal was to identify the monomer states most susceptible to z-VAD-fmk-induced dimerization and quantify the resulting dimer states. In the presence of z-VAD-fmk, the double-cleaved dimer was the predominant form (56%), followed by the single-cleaved (20%), and uncleaved (24%) dimers. The impact of time on dimer formation in WTC9 with no inhibitor will also be investigated to similarly assess which of the nine observed states corresponds to dimer formation under these conditions.

Native MS revealed the loss conformational heterogeneity that occurs with caspase-9 dimerization, and the differences in dimer formation with/without substrate.

Probing Protein Interactions using Ultraviolet Photodissociation and Native Mass Spectrometry

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Presenting Author: Jenny Brodbelt

Native mass spectrometry has emerged as a versatile strategy for exploring protein structures, now well established for monitoring interactions between proteins and inhibitors or substrates.

All experiments were performing using Thermo Orbitrap mass spectrometers (HFX, UHMR, and Eclipse platforms), each modified to perform UVPD by addition of a 193 nm excimer laser. All data was processed using a combination of ProSight Native and MS-TAFI.

We have developed ultraviolet photodissociation (UVPD) to characterize protein complexes, binding sites, and monitor structural changes as a function of ligand binding or protein interactions. Here we report insights gained from these integrated methods to characterize SARS-CoV coronavirus proteins, including two proteases, Mpro and its interactions with covalent inhibitors and PLpro and its interactions with interferon-induced ubiquitin-like protein (ISG15) and both covalent and non-covalent inhibitors, and RNA-binding nucleocapsid proteins. We also highlight the use of UVPD to examine structure features of large macromolecular complexes, including the 20S proteasome, in conjunction with variable temperature ESI and charge reduction methods to probe disassembly of the complexes.

Deciphering binding sites and binding interactions using UVPD-MS

Intact Mass HDX-MS and IM-MS Reveal Differences in Protein Dynamics Resulting from Native MS Buffers

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Native mass spectrometry (nMS) is a technique that allows for the study of intact protein complexes by preserving noncovalent interactions between subunits. Two techniques that are often paired with nMS to infer protein complex dynamics and structure are hydrogen-deuterium exchange (HDX) and ion-mobility (IM). Here, we use intact mass HDX-MS to track mass shifts of protein complexes when exposed to a deuterated solution to obtain information on the solvent accessible surface area (SASA) under different buffer conditions. IM-MS was also used to monitor protein behavior in different buffer conditions by observing shifts in protein complex conformer abundances. Intact mass HDX-MS and IM-MS were used together to study the effect that various nMS buffers have on protein dynamics and structure.

HDX-MS experiments performed for streptavidin (SA) and transthyretin (TTR) were buffer exchanged into an H2O solution containing 200 mM ammonium acetate (AmAc), ethylenediamine diacetate (EDDA), or triethylammonium acetate (TEAA), pH 7.0. Protein samples were diluted 10-fold with 80% D2O solution with the same buffer conditions, and immediately electrosprayed into the mass spectrometer. Exchange was observed over a period of 96 hours. For IM-MS experiments, SA and TTR were buffer exchanged into H2O solutions that complemented the H2O solutions used for HDX-MS experiments. Spectra were collected for 3 minutes, and drift time was converted to CCS using Agilent tune mix for single field calibration and peaks were fitted using OriginLab Software.

HDX-MS experiments were performed in native conditions to observe how the intact protein dynamics differ for both TTR and SA in multiple buffers. In AmAc and TEAA, TTR underwent similar amounts of exchange, with final exchanges of 45.0% and 46.1% respectively. This was lower in EDDA, with a final exchange of 40.4%. A similar trend was observed for SA, where 44.2% exchange was observed in AmAc, 47.5% in TEAA, and 41.4% in EDDA. For both protein complexes, the least amount of exchange occurred in EDDA. It is possible that the three buffers alter the SASA of these protein complexes resulting in differences in HDX, or that the buffers themselves interact with different residues on the protein complex that inhibit HDX to different degrees. IM-MS data highlights additional differences that these buffers have on protein dynamics. When comparing the average collision cross section (CCSavg) of TTR and SA, there is a statistically significant difference in the AmAc, EDDA, and TEAA solutions. For both TTR and SA, AmAc has the largest CCSavg, followed by EDDA, then TEAA. When comparing the CCS profile of overlapping charge states between AmAc and EDDA (14+), along with those of EDDA and TEAA (11+ and 12+), two observations are made. The first is that different numbers and abundances of conformers for the overlapping charge states are observed, suggesting conformational diversity. For example, TTR has 3 conformers in the 14+ charge state in AmAc, but only 2 are observed for the same charge state in EDDA. The second observation is that the difference in CCS of TTR and SA in overlapping charge states is statistically significant. These data provided by IM-MS indicate that the different buffers affect the conformational landscape of proteins.

Data collected confirm that there exist differences in protein structure/dynamics among the nMS buffers AmAc, EDDA, and TEAA. This is seen in variations in both the HDX and CCS values for both TTR and SA, indicating differences in the SASA and conformational abundances of the proteins.

Tackling Biosimilarity Assessment using Top-Down and Middle-down Mass Spectrometry

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Presenting Author: Sara Carillo

With many biologic products going off-patent, the biopharmaceutical market is witnessing the exponential increase of biosimilar products being commercialized worldwide. To avail of a faster approval, deep structural characterization and understanding of which product quality attributes are critical to maintain drug quality, efficacy and safety is needed. An increasing effort needs to be pursued for accurate and sensitive analytical methods which can highlight similarities and differences between originator drug and biosimilar candidates. Mass spectrometry can play a unique role in establishing data rich methodologies for this characterization and comparability exercise. In this study, top-down and middle-down approaches were used to assess biosimilarity. The first case study will focus on trastuzumab, an anti-HER2 IgG1 monoclonal antibody used for breast cancer treatment. Strong cation exchange was used for native proteoform separation while native top-down mass spectrometry on an Orbitrap Ascend Biopharma Edition Tribid mass spectrometer was employed to localize the modifications in the originator and 4 different biosimilars. In the second case study, etanercept originator (an anti-TNF-α fusion protein) and 4 commercially available biosimilars were investigated with a multi-level approach on the Orbitrap Excedion Pro Biopharma Hybrid mass spectrometer. The workflow included native mass spectrometry, middle-up and middle-down of fusion protein subunits and bottom-up analysis for profiling and localization of several post-translational modifications including O-glycans.

High-resolution Ion Mobility-mass Spectrometry: Uncovering New Structural Details for Drug Compounds

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The severity of the ongoing drug crisis has been exacerbated by the relentless introduction of new substances into the recreational market. Ion mobility-mass spectrometry (IM-MS) has shown tremendous promise for rapid structural characterization of emerging drugs, but while higher-resolution techniques like cyclic IMS (cIMS) and structures for lossless ion manipulations (SLIM) provide improved separations over conventional methods, they can also reveal additional structural features which must be better understood. Conformational differences and protonation site isomers have been reported in the literature for fentanyl analogues and nitazenes, and therefore an accurate and robust structural characterization method is a critical need in the field.

A variety of drug compound classes including fentanyl analogues, xylazines, benzodiazepines, and nitazenes were surveyed in this study. Ion mobility-tandem mass spectrometry (IM-MS/MS) was performed using both Agilent 6560 IM-QTOF and MOBILion MOBIE SLIM platforms. Standards of individual drugs and isomer mixtures were prepared at 1 ug/mL in 50/50 water-methanol. For hydrogen deuterium exchange (HDX) experiments, standards were prepared in 100% D2O. Analysis was performed using flow injection analysis (FIA) with positive mode electrospray ionization (ESI). Following mobility separation, MS/MS was performed either at optimized collision energies or via energy-resolved MS/MS to study fragmentation pathways. All data analysis was performed using Agilent MassHunter IM-MS Browser 10.0.

Protonated fentanyl was first demonstrated to exhibit two peaks in the mobility dimension in a paper published by Butler and Baker in 2022 (JASMS, 33, 1904-1913), which they proposed resulted from structural conformers or protonation site isomers, “protomers”. Our group later showed that dozens of fentanyl analogues display similiar mobility pattern (Drug Testing and Analysis, 2024, 16, 369-379) and that several experimental parameters can affect that pattern (Journal of Mass Spectrometry, 2024, 59, e5070). Identification of those features was attributed to protomers based on work we recently published using mobility-aligned MS/MS, specifically annotating protonation site through different fragmentation patterns (International Journal of Mass Spectrometry, 2024, 496, 117185). Since then, our most recent work has focused on identifying the fragments, as we suspect there are actually multiple isobaric fragments for each pathway. For these studies, we’ve relied on a combination of computational modeling and experiments using hydrogen-deuterium exchange. Specifically in the latter studies, we’ve shown that many of these fragments are actually made up of a collection of isobars; deuterium labeling has shown that a deuterated precursor can dissociate to either [F]+ or [F+D]+, as determined by the mass shift in the fragmentation spectrum. Our computational modeling has demonstrated differences in intramolecular hydrogen bonding as well as the likelihood of a mobile proton during the activation stage; the latter phenomenon complicates the annotation of protomeric features, as initial protonation location does not preclude formation of fragments associated with other protonation sites. Nevertheless, the wealth of structural information garnered with only a single IM-MS/MS experiment has proven tremendously valuable for confident structure elucidation that will ultimately be applied to novel drug species and their biological metabolites.

Structure elucidation of novel fentanyl analogues and other drug substances can be attained using a combination of IM-MS/MS, HDX, and computational modeling

Photocrosslinking of Histones to Study Conformational Changes Induced by Post Translational Modifications

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Histones play a crucial role in gene regulation by controlling access to DNA. Rich in lysine residues, histones undergo lysine acetylation and methylation post translational modifications (PTMs) that regulate nucleosome conformation. The histone octamer consists of core histones including two H2A-H2B dimers that bind the H3-H4 tetramer. Because of the abundance of lysine residues and the PTMs that occur on them, nonselective photocrosslinkers may be better suited for the study of conformational changes of histones. To determine how these PTMs and their patterns modulate conformational space, various crosslinkers were used with top-down mass spectrometry. Cytochrome c was used as a model protein to develop the photocrosslinking workflow and isolated histones were used for method development.

For top-down photocrosslinking method development, cytochrome c was combined with the amine-to-nonspecific photocrosslinker sulfo-SDA at a 1:2 protein-to-photocrosslinker ratio in water, incubated at RT for 15-minutes, exposed to 365 nm light for 30-minutes, and quenched by removing the light source. Top-down MS with TOF and electron capture dissociation was used to determine the crosslinking sites and sequence fragmentation. The histone octamer protein was expressed in BL21(DE3)pLysS chemically competent E. coli cells with induction and was purified with FPLC using a His-Tag column. The histone sample was analyzed by gel electrophoresis and three bands were observed corresponding with the masses of the histones. Additionally, LC-MS was used to confirm the presence of each histone.

Previously, cytochrome c was successfully photocrosslinked with sulfo-SDA by the methods described here. The 7+, 8+, 9+ and 10+ crosslinked charge states were identified by mass spectrometry. Additionally, the 9+ charge state was successfully isolated by MS/MS with electron capture dissociation (ECD) and looped links between peptides were observed and verified by bottom-up MS. BS2G was used to crosslink the H4 histone in PBS and the crosslinked 12+ charge state was isolated by MS/MS and fragmented with ECD. It was found that the N-terminus was linked to lysine 16, and lysine 77 was crosslinked to lysine 91. Various methods for the expression and purification of histones have been performed to produce histones with a purity compatible with mass spectrometry. In addition to the histone octamer being expressed in pLysS cells, histones were also extracted from chromatin following an acid extraction method. Different purification methods were used including cation exchange and size exclusion chromatography and His Mag Sepharose nickel magnetic beads. From the expressed histone octamer, the H2A, H2B, H3, and H4 histones were identified via LC-MS. Top-down mass spectrometry allows for the analysis of PTMs within the histones. This photocrosslinking method will also be used to study modified histones including those containing the acetylated, methylated, and phosphorylated PTMs.

We are using crosslinking to reveal how relevant PTMs influence conformational space of histones, histone octamers, and nucleosomes.

***(Late Submission) A Metabolomics/Proteomics-Inspired Workflow to Probe Prebiotic Chemical Systems*.**

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Presenting Author: Alexander Deans-Rowe

The study of prebiotic chemical systems is hindered by the difficulty in characterizing the compounds that comprise them. Even relatively constrained systems produce hundreds of compounds that are infeasible for many methods to separate and characterize. High resolution MS, tandem MS and ancillary techniques have shown great promise in unraveling such chemical complexity. Knowledge of predicted product structures and fragmentation patterns allows in silico MS/MS libraries to be created as in proteomics or lipidomics. Molecular networking, heavily used in metabolomics to annotate unknowns structurally similar to knowns, can also contribute to the annotation pipeline. Here, we propose a workflow incorporating principles used in both metabolomics and proteomics to study a model prebiotic system capable of forming oligo-depsipeptides.

Mixtures containing ribose, glycolic acid and, in some experiments, glycine in LC-MS-grade water were placed in open Eppendorf tubes and allowed to dry at 45°C, with periodic rounds of rehydration every 24 hours. For the duration of the third drying round, some tubes were instead subjected to drying at 85°C. Aliquots were taken of the reaction mixture at cycles 0, 1, 3, 6, and 10 and frozen at -80°C until analysis. These samples were diluted 1:5 in acetonitrile and analyzed by LC-MS/MS using a Z-HILIC column at pH 9 and an Orbitrap Exploris 240 (Thermo Fischer Scientific) mass spectrometer operating at a mass resolution of 120,000 in both positive and negative ion mode. Data were collected with an AcquireX workflow.

The raw files generated by the instrument were converted with MSconvert to the .mzML format. These files were imported to MZmine for feature detection and alignment. Feature annotation was performed both in MZmine using the GNPS library, and directly through GNPS. Both annotation methods yielded only a few hits against pre-existing databases, which were judged to be starting material and likely contaminants or false positives. We attribute the scarcity of matches to a lack of overlap between compounds in the GNPS database and the compounds produced in our experiments. Despite this, MS1 data were used to identify features corresponding to oligo-depsipeptide tetramers. Semi-quantitative analysis of these features indicated that depsipeptides mostly formed during the high temperature step. Ester-rich depsipeptides were largely destroyed over subsequent wet-dry cycles, while amide-rich depsipeptides continued to accumulate.

To characterize the sequences of these depsipeptides, we will construct data-dependent artificial MS2 libraries as in (Forsythe, J. G. et al. Proc. Natl. Acad. Sci. (2017). 114(37): E7652-E7659) and use these libraries with Compound Discoverer and mzvault to detect series of anticipated products. Molecular networking will be used to identify any features from derivatives of these predicted products. We intend to use this technique to identify compounds in our prior work (Matange, K. et al. Nat. Chem. (2025)) that have resisted identification through other techniques, to clarify the role that amide bonds play in causing the trends we observed, and to identify compounds with significant differences between the normal and temperature-spike experiments we have conducted. Widening the analytical bottleneck of prebiotic chemistry will enable the creation and testing of more sophisticated hypotheses about the nature of evolving chemical systems and thus potentially shed light on the processes that led to life's emergence.

Use of a combined metabolomics and proteomics approach to study model prebiotic systems. Preliminary evidence that amide bonds can allow chemical systems to retain information about past system events via kinetic trapping.

Mass Spectrometry Reveals Effects of Sequence Complexity on Structure of α-Synuclein

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Presenting Author: Olivia Dioli

α-Synuclein is a disordered, presynaptic protein responsible for synaptic vesicle transport via membrane binding. α-Synuclein fibrillation is a pathogenic hallmark of Parkinson's Disease. Like many disordered proteins, α-synuclein contains low complexity domains (LCDs) in its sequence that are comprised of only a few repetitive amino acids. LCDs are thought to contribute to proper physiochemical functions of disordered proteins. Introducing missense mutations to α-synuclein's sequence that alter the protein's sequence complexity opens the door for probing the effects that sequence complexity has on proper disordered protein function. Herein, we present the ability of native mass spectrometry to probe the sequence dynamics of α-synuclein, laying the foundation for investigating the effects that sequence complexity mutations have on the structure of α-synuclein.

Recombinant α-synuclein was expressed in E.coli BL21 cells. Samples were prepared at 5-10 μM in 100 mM ammonium acetate, loaded into borosilicate emitters, and ionized by electrospray using 1.6 kV. Experiments were performed on a Thermo Scientific Q Exactive HF-X quadrupole-Orbitrap mass spectrometer modified with a Coherent Excistar excimer laser for 193 nm UVPD. Ion mobility experiments used an atmospheric pressure drift tube made of printed circuit board electrodes with a 10 cm desolvation region and 10 cm drift region. UVPD was performed using 1 laser pulse, 3 mJ per pulse. Spectra were collected at 240k resolution with 250 averages per replicate. Experiments were also undertaken using a variable temperature ESI (vT-ESI) source to modulate solution temperature during ESI.

Electrospray ionization of wild type (WT) α-synuclein produces a broad charge state distribution (CSD) from [M+6H]6+ to [M+18H]18+ owing to its disordered structure which affords many accessible protonation sites. Ion mobility measurements show a large range of collision cross sections (CCS), averaging 1553 Å2 for [M+6H]6+ to 2878 Å2 for [M+12H]12+, indicating that α-synuclein occupies a broad ensemble of conformations from compact to elongated. Similar charge state distributions were observed for three sequence complexity mutants: E83Q, A18T, and the G68P/V70I double mutant. In the presence of copper, WT apo α-synuclein, α-synuclein•Cu, and α-synuclein•2Cu complexes were detected. The E83Q and A18T mutants show increased intensity for the α-synuclein•2Cu species, with E83Q also yielding a third unidentified species. The double mutant shows slightly higher α-synuclein•2Cu intensity compared to the WT. UVPD, a fast high-energy activation method, is known to be sensitive to arrangements of non-covalent interactions as evidenced by suppressed fragmentation and/or loss of sequence coverage in regions stabilized by networks of non-covalent interactions. For the 7+ charge state of α-synuclein, there is a lack of fragmentation in residues G101-Y125, suggesting stabilization by noncovalent interactions. However, the 14+ charge state shows extensive fragmentation in the same region, suggesting fewer intramolecular interactions and more efficient release of fragment ions upon backbone cleavage. The absence of fragmentation is consistent across WT replicates and has been observed for each of the sequence complexity mutants, suggesting that protection is not due to random gas phase collapse. The thermal stability of α-synuclein-copper binding was probed using vT-ESI. The presence of α-synuclein•Cu complexes was tracked as the temperature increased from 20°C to 90°C in 10°C increments per minute. There was no evidence of dissociation of the α-synuclein•Cu or α-synuclein•2Cu complex. These data suggest these two copper binding sites are thermally stable.

Native MS can detect differences in copper binding affinities for the various sequence complexity mutants. UVPD identifies regions of noncovalent folding in the compact conformers of WT and mutant α-synuclein. vT-ESI analysis of α-synuclein•Cu complexes suggest highly stable copper binding sites.

Osmolyte-induced Structural Changes in Wild Type and Phosphorylated Alpha Synuclein

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Presenting Author: Ashlyn Dollar

Alpha synuclein (aSyn) is an intrinsically disordered protein (IDP) known to play a role in the pathology of multiple neurodegenerative diseases, particularly Parkinson's Disease and Lewy Body Dementia. In Lewy Body dementia, 95% of aSyn in brain aggregates is phosphorylated at S129, making this proteoform important to study. Osmolytes are small organic molecules found within cells that maintain osmotic pressure, water flow, and protein folding. Studying the effects of osmolytes on aSyn helps elucidate the aSyn conformationome within a cellular environment. We utilize mass spectrometry techniques including crosslinking, covalent labeling, and top-down fragmentation to explore how osmolytes such as trimethylamine N-oxide (TMAO) and N-acetyl aspartate (NAA) affect the aSyn ensemble of conformers.

Reactions were conducted with and without 1.8M TMAO with 50uM aSyn or p-aSyn. Crosslinking was performed in 1X PBS for 15min at RT. For aSyn, we utilized 150uM BS2G or BS3 crosslinkers. For phosphorylated aSyn (p-aSyn), we utilized 75uM crosslinker with TMAO and 150uM without TMAO. Covalent labeling was performed with 150uM DEPC for aSyn and 300uM DEPC for p-aSyn in 100mM MOPS pH 6.8 for 1min at 37C. Reaction mixtures were digested with trypsin and peptides were then analyzed by LC-MS/MS. For top- down analysis, 5uM aSyn or p-aSyn and 10mM NAA were incubated at RT for ~20min before electrospray on a Thermo Orbitrap Fusion. Multiple charge states with NAA bound and unbound were isolated and fragmented with EThcD.

Crosslinking data using BS2G and BS3 without cell crowder demonstrated that p-aSyn has a well-folded N-terminus. Crosslinking data revealed that aSyn demonstrates more long-range interactions within the TMAO solution than without. In contrast, p-aSyn crosslinking patterns remained largely unchanged in TMAO solutions versus controls. This suggests that p-aSyn has more stable, folded ensembles of conformations than aSyn due to it having less conformational change in the presence of the cell crowder. Preliminary studies have also shown that less crosslinker is needed to get same equivalence of 0-3 crosslinkers for p-aSyn compared to wild type aSyn due to p-aSyn having more folding, so crosslinking more quickly. Covalent labeling data without cell crowder using the DEPC label demonstrated that p-aSyn has a more hydrophobic microenvironment than aSyn as seen with S, T, and Y residues labeled on p-aSyn that were absent on the wild type aSyn. Our top-down analysis of the wild type aSyn in the presence of NAA demonstrated that NAA is loosely bound to aSyn as evidenced by NAA dissociation from aSyn during ETD and EThcD fragmentation. Fragments from EThcD suggest that binding occurs within the N-terminal region of the protein, but further experiments are needed to confirm this. pH of the aSyn/NAA has also been seen to play a significant role in the stability of the protein. aSyn/NAA pH 6.5 has shown to cause the protein to begin autoproteolysis, while this same mixture at pH 7.5 has shown a charge state distribution shift of aSyn more towards the higher, extended charge states but without proteolytic fragments.

In a mimicked cellular environment, wild type aSyn adopts a more compact conformation resembling p-aSyn, while p-aSyn structure remains largely unaffected by cellular crowding conditions. This suggests inherent structural stability of the phosphorylated form.

Proteomics at Scale - Empowering Biopharmaceutical Discovery & Translational Research

Ashok R. Dongre, Discovery & Development Sciences, Bristol Myers Squibb

Presenting Author: Ashok Dongre

Proteomics as a discipline has steadily matured over the past three decades. More recently, proteomics is enjoying a renaissance of sorts with wide adoption and embrace of various methodologies that include reagent-based profiling approaches (antibodies / aptamers-based profiling techniques) in addition to mass spectrometry which continues to be the "gold standard". Like all technologies, mass spectrometry-based proteomics has been through its own Gartner hype cycle over the past three decades. As LC-MS platforms and technologies have matured we are certainly on the plateau of productivity and continue to rapidly build on the successes. Even with these advances the discipline is still not quite as mature as genomics. There are still barriers (technical, training & others) that curtail the wide adoption across the biological and biopharma research continuum. However, the significant value proteomics investigations bring when performed with appropriate rigor is undeniable. The objective of this presentation is to share how transformative technological and methodological leaps in LC-MS over past 5 years coupled with robust deep learning data analytics pipelines at biopharmaceutical enterprise scale provide a vigorous framework to deliver "just-in-time" actionable insights to drug discovery & translational research. The technological advances are awe inspiring in and of themselves, however, coupling these advances to address complex questions and generate new hypotheses at the intersection of disease pathophysiology & biopharma research brings into focus the exponentially rising impact of mass spectrometry-based proteomics. Briefly, the presentation will try to illustrate through examples the impact and incredible versatility mass spectrometry-based proteomics delivers across the drug discovery process. Early on in drug discovery process coupling frontline high throughput screening technologies with proteomic profiling enables identification of compound hits for specific target modulation or molecular / cellular pathways and/or associated biological / pathophysiological phenotypes in a "post-reductionist" unbiased manner. When coupled with high content imaging and other -omics datasets, these high-dimensional, high content datasets create unique opportunities to understand pharmacology at a systems level. As the discovery program progresses through the drug discovery continuum, unbiased proteomics profiling is enabling chemotype SAR (structure activity relationship) campaigns, deeper understanding of pharmacology and compound mechanism of action (MoA) at systems level that allow simultaneous and early assessment of off-target liabilities. These capabilities can hasten the path to discovery by enabling better compound selection powered by wholistic understanding of mechanism of action, which also includes safety. Additionally, chemical proteomics methods when coupled to chemical biology techniques allow for therapeutic target deconvolution from phenotypic screening efforts. AI/ML can also better predict compounds that could progress along the drug discovery pipeline by evaluating relevance of their mechanism of action in the pre-clinical and clinical disease datasets. Translational studies allow for systematic assessment of pre-clinical models for markers of target engagement, compound MoA, pharmacodynamics (PD) and safety with an eye towards forward translation of these markers to clinical settings. Similarly, studies performed with clinical cohorts to elucidate novel markers can be reverse translated to achieve systematic assessment in a pre-clinical model systems including non-human primates. This dynamic and crucial translational research space of biopharmaceutical development benefits from the power of unbiased proteomics capabilities where the methods are enabling elucidation of novel markers of pharmacological response (PD biomarkers), disease, patient stratification, efficacy and even guiding human dose projection to enable early clinical trials. Borrowing from the famous quote by Winston Churchill "it is perhaps the end of the beginning" of what is possible with proteomics to enable biopharmaceutical discovery and translational research. The future is full of possibilities and opportunities to bring innovation that translates to breakthrough therapies.

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successes. Even with these advances the discipline is still not quite as mature as genomics. There are still barriers (technical, training & others) that curtail the wide adoption across the biological and biopharma research continuum. However, the significant value proteomics investigations bring when performed with appropriate rigor is undeniable.

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Native Taylor/Non-Taylor Dispersion – Mass Spectrometry (TNT-MS) Allows Rapid Protein Desalting and Ligand Screening using an Unmodified LC-MS Instrument

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Presenting Author: Jonathan Eisert

Native mass spectrometry provides valuable insights into protein structure and interactions but is incompatible with non-volatile buffers, including those traditionally used in molecular biology. Typically, this incompatibility results in a need for offline buffer exchange or online size-exclusion chromatography. We introduce an alternative that exploits capillary flow dynamics to separate molecules based on their hydrodynamic radii. By optimizing flow rates, smaller species (e.g., buffers, salts, small molecules) remain in a Taylor dispersion regime and travel slowly, while larger molecules (e.g., proteins, multi-protein and protein-ligand complexes) enter a non-Taylor regime and travel faster. As a result, the large molecules elute earlier, which enables online protein desalting and multiplexed ligand screening.

TNT-MS was implemented using an unmodified LC-MS system (Waters Synapt XS with Acquity M-Class UPLC) operated without a chromatographic column. Samples were injected via an autosampler into an open tubular PEEK capillary (90 cm x 254 µm internal diameter) and pumped toward the mass spectrometer at controlled flow rates (5-60 µL/min). Flow rates were optimized for each analyte to exploit the transition between Taylor and non-Taylor regimes based on its hydrodynamic radius. Model proteins (e.g., enolase, BSA, carbonic anhydrase) covered a mass range between 12-150 kDa and were injected in high-salt buffers (e.g., 20 mM HEPES, 200 mM NaCl, 1 mM TCEP, 5% glycerol). For ligand screening experiments, we used five pharmacologically relevant proteins and a library of 26 compounds.

Using TNT-MS, desalting of model proteins injected in buffers containing up to 200 mM NaCl was achieved in less than 2 minutes, with native-like protein signals typically being detected after only 30 seconds. Salts and buffer components were effectively separated, only reaching their elution maximum after approximately one minute. Besides desalting, native TNT-MS enabled multiplexed ligand screening with clinically relevant kinases (AAK1, LIMK1, STK17A), BIRC4, and the E3 ligase cereblon. Protein stock solutions (65 – 230 µM) were made in conventional HEPES buffers containing 150 – 200 mM NaCl. Other than dilution to 25 µM protein with aqueous ammonium acetate, no effort was made to reduce the concentration of buffer components prior to injection. Proteins and protein-ligand complexes entered a non-Taylor regime and eluted rapidly, while unbound ligands eluted later. This approach thus avoided potential protein ion suppression, which allowed multiplexed screening of all 26 co-incubated compounds in a single injection, including simultaneous detection of multiple hits. Besides observation of native-like protein-ligand complexes, controlled gas-phase dissociation was used to release ligands, which enabled their detection in the low-m/z range with high mass accuracy. Furthermore, the extracted ion chromatograms (EICs) of ligands released in the gas phase showed the same non-Taylor profile as the proteins, while non-binding compounds showed a narrow, Gaussian profile typical for species with small hydrodynamic radii. We developed a software for automated classification of small-molecule EICs into these two bins using Pearson correlation and dynamic time warping, significantly accelerating data analysis. Finally, we investigated the relationship between protein size and the critical flow rate for transition from Taylor to non-Taylor regimes. Careful choice of flow rate thus enabled limited, ‘columnless’ size-based separation. TNT-MS consumed sample amounts comparable to static nano-ESI, while offering significant gains in speed, automation, and throughput.

TNT-MS enables ultra-fast protein desalting and multiplexed ligand screening by exploiting size-based differences in the dynamics of molecules flowing through an open, tubular capillary. This approach is compatible with high-salt buffers, preserves labile interactions, and is easily implemented on standard LC-MS systems.

Native and Top-down Mass Spectrometry Reveals Remodeling of Brain-derived mGluRs in Major Depressive Disorder

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Presenting Author: Tarick El-Baba

Major depressive disorder (MDD) is a debilitating psychiatric condition. Individuals afflicted with severe MDD have patterns of reduced cellular activity in regions of the brain linked to reward processing (e.g., anticipation), learning, and emotion. Diminished cellular activity in the orbitofrontal cortex has well-established links to severe MDD. Metabotropic glutamate receptor 2 (mGluR2) is a dimeric G protein-coupled receptor responsible for inhibiting neurotransmission upon activation by glutamate, the most abundant neurotransmitter in the brain. We reasoned mGluR2 may have hyperactivity in the OFC of individuals with severe MDD. To explore this hypothesis, we developed a strategy to retrieve it from post-mortem brain for downstream native MS and top-down MS (nTDMS).

Recombinant proteins were expressed and purified from E. coli and HEK293 GNTI-/- cells. Post-mortem brain tissue dissected from the orbitofrontal cortex of n=9 control subjects and n=10 MDD subjects were obtained from the UK Brain Bank. The major inclusion criteria in the MDD group was suicide as the cause of death. A depressive syndrome consistent with human MDD was induced in mice using the chronic social defeat stress (CSDS) paradigm (Golden et al, Nat Protoc. 2011). VGluT1 and mGluR2 were immunoprecipitated from detergent solubilized brain tissue using immobilized nanobodies (Nbs). Native MS and nTDMS were carried out on an Orbitrap UHMR and modified Orbitrap Ascend, respectively. Fluorescence microscopy assays were carried our using a Leica Thunder microscope.

I will first describe the overall pipeline involving: (1) retrieval of brain-derived VGluT1 and mGluR2 from individual mouse brain tissue sections, (2) analysis of the protein complexes and interactions using native MS and PTCR, (3) determination of sequences by nTDMS, and (4) characterization of N-glycan architectures via glycoproteomic analysis. mGluR2 was predominantly dimeric, and had a range of N-glycan architectures. Native MS analyses found evidence for both monomeric and dimeric forms, with dimers representing the highest population (>90%). No significant differences in composition, lipid binding, or PTMs were observed for VGluT1 isolated from mice exhibiting pro-depressive behaviors relative to unstressed controls. We did observe a ~2-fold increase in the fraction of mGluR2 monomers relative to dimers in SUS mice. Thus, we conclude that CSDS leads to destabilization of mGluR2 dimers. VGluT1 from a depression human tissue cohort was again unremarkable with respect to differences in interaction, sequence, or PTMs between control and depressed groups. Alike the mice, in the depression group relative to controls, mGluR2 dimers were significantly destabilized. Accompanying dimer destabilization was a significant depletion in bound phosphatidylserine lipids. The role of phosphatidylserine in stabilizing mGluR2 dimers was validated using a battery of biophysical, biochemical, and cell-based assays. We therefore conclude that phosphatidylserine affords stabilization to mGluR2 dimers uniquely. We used nTDMS to explore the extent that mGluR2 homodimers differentially interact with other proteins between depression and control groups. Relative to fragment ion peaks for mGluR22, we observed a significant increase in intensity for fragment ions corresponding to mGluR33. This provides direct evidence for mGluR2/3 heterodimers directly in human brain. Interestingly, there was a slight statistically-significant increase in the fraction of mGluR3 peptide fragment ions relative to mGluR2 in depression relative to controls, indicating that the formation of mGluR23 complexes is associated with MDD.

Immunoprecipitation and native MS studies of SLCs and GPCRs from mouse and human brain uncovers remodelling of protein-lipid and protein-protein interactions in a stress-related psychiatric disorder.

Evaluating Changes to ADP Binding using HDX and Variable Temperature to Provide Insight into the Hydration of SR1 GroEL

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Presenting Author: Kacie Evans

Protein complexes can adopt numerous forms in a given solution, referred to as microstates, and changing the hydration of a molecule can alter the distribution of microstates. Deciphering how the distribution of microstates changes with hydration could provide relevant information on complex stability and binding mechanisms; however, conventional structure techniques lack the sensitivity or resolution to perform these analyses. Recent advancements in mass spectrometry (MS) provide the ability to analyze shifts in microstate distributions. Here, native MS, variable-temperature ESI (vT-ESI), and hydrogen-deuterium exchange (HDX) aid in determining the role of water through changes in binding affinities and thermodynamics. Our results provide evidence that modifying hydration alters the microstate distribution of a single-ring mutant of GroEL (SR1), which alters ADP binding.

SR1 was dissolved in a solution containing magnesium acetate (MgAc2), ADP, and either 200mM ammonium acetate (AmAc), ethylenediamine diacetate (EDDA), or triethylammonium acetate (TEAA). Corresponding deuterated buffers were made by diluting the 1M buffer stock in H2O, resulting in a final 80% D2O. Each buffer used was adjusted to pH 7 using ammonium hydroxide or ethylenediamine. Samples were injected into custom-pulled nano-ESI borosilicate capillaries then positioned inside a ceramic sleeve and inserted into a vT-ESI source controlled by a TEC module. Temperatures investigated were 4 – 40ºC. The raw mass spectra were collected on a Thermo Q Exactive UHMR orbitrap mass spectrometer and collected for 1 minute. Unidec was used to assign charge states, mass, and abundance of each individual species.

vT-ESI and ADP binding were performed in AmAc, EDDA, and TEAA since different buffer molecules interact with SR1 in varying degrees. AmAc, EDDA, and TEAA results in varying average charge state (Zavg), 42.8, 40.0 and 32.7, respectively, indicating differences in solvent accessible surface area. When vT-ESI was performed from 4-45 ºC in the different buffers, the Zavg value in AmAc increased, whereas the Zavg value for TEAA and EDDA decreased. SR1 also displayed differences in binding affinity for ADP in the different buffers. In AmAc the required ADP concentration for saturation was 50 µM, in EDDA the required ADP concentration for saturation was >200 µM, and in TEAA the required ADP concentration for saturation was 100 µM. Differences in Zavg and binding affinities between the different solutions provide evidence that the distribution of SR1 microstates shifts depending on the osmolyte present in solution. The role of hydration in shifting the distribution of SR1 microstates was evaluated using deuterated buffers and calculating correlating thermodynamics to compare enthalpy-entropy contributions (EEC). In deuterated solutions containing AmAc, EDDA, and TEAA, 50%, 42%, and 47% of the exchangeable hydrogen atoms were exchanged with deuterium after 96 hours, respectively. The effect of deuterated solutions on ADP binding affinities was then investigated for the SR1 complex. The data revealed that the binding affinity of SR1-ADP7 increased 2x in deuterated AmAc. The thermodynamic profile was calculated to investigate the increased binding affinity in deuterated AmAc, showing SR1-ADP0-3 are enthalpy-driven with diminished entropy contributions. Alterations in the EEC indicate that D2O alters the dynamics of SR1-ADP complex. Future directions include evaluating ADP binding in deuterated TEAA and EDDA to probe how hydration differs by buffer molecules. With this data, we hope to provide further evidence that hydration shifts the microstate distribution of SR1, which alters its behavior in solution.

Native MS and HDX indicate that shifts in the microstates distribution of SR1 alters the binding of ADP. Shifts in the binding affinities and EEC of the SR1-ADP complex when using D2O provide insight into the role of hydration.

A Mass Spectrometry Imaging Annotation Workflow Combining Cyclic Ion Mobility and Machine Learning Molecular Predictions

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Presenting Author: Facundo Fernandez

Confidently annotating metabolites helps rationalize their biological role and changes in their abundances in nontargeted metabolomics studies. However, collecting high quality MS2 fragmentation data on hundreds of differential metabolites is challenging, particularly during lengthy mass spectrometry imaging (MSI) experiments. MS2 coverage is often incomplete or uninformative, and many unknowns remain. Without proper annotation, spectral features do not offer meaningful biological insights, so a wealth of information remains untapped. Collision cross section (CCS) measurements have emerged as an additional aid for metabolite annotation. Here, we present a desorption electrospray ionization cyclic ion mobility mass spectrometry imaging (DESI cIM MSI) workflow leveraging CCS and fragment ion pattern machine learning prediction tools for increased confidence in metabolite and lipid annotation.

Imaging data were collected on a SELECT SERIES Cyclic IMS (Waters) mass spectrometer equipped with a DESI-XS ion source (Waters). Four human renal cell carcinoma tissue sections with matched normal tissues were imaged in positive ion mode using one pass through the cyclic mobility cell. CCS values were calculated using the default instrument approach. Ion mobility data at various separation times was also collected for one normal kidney section for calculating multi-pass CCS values using the Lin and Costello (doi.org/10.1021/acs.analchem.4c01758) method. To complement imaging experiments, LC-MS/MS data were collected on kidney extracts and uploaded to SIRIUS. SIRIUS candidates' CCS values were predicted using CCSP 2.0 (doi: 10.1021/acs.analchem.2c03491) and compared to high accuracy multi-pass CCS values, filtering out unlikely SIRIUS candidates.

Ion abundance data were extracted from the 4 tumor kidney samples and 4 matched normal tissues. Using a 1.5-fold change and 0.1 p-value threshold the initial feature list was narrowed down to 179 moderately differential features. Amongst these, 70 putative database matches were obtained in LIPID MAPS and HMDB. CCS values for these features were measured using both the default vendor approach and the perturbation corrected multi-pass approach, then compared to the Baker METLIN CCS database. The average %CCS error for the 13 lipids matching the Baker database was 0.7% and 0.4% using the default and multi-pass approaches, respectively. These results demonstrate that multi-pass CCS calculations yield higher accuracy CCS values, allowing to filter out closely related, but incorrect structures. LC-MS/MS data was collected on all moderately differential features and uploaded to SIRIUS. CCS was predicted for the top 20 SIRIUS candidates of each lipid and compared to multi-pass CCS values. Each candidate structure was examined to determine whether the lipid sum composition matched the MS1 database annotation. Most correct candidates were within 1% CCS error, whereas incorrect candidates with epoxide or hydroxyl groups had far greater errors and could be readily discarded. For example, for the PC(34:1) [M+K]+ ion, all candidates were incorrectly predicted with an average 5.3% error, relative to PC(34:2) [M+Na]+ where the correct candidates had an average 0.4% error. This workflow was used to annotate an unknown species at m/z 529.3989. Using a 1% CCS cutoff, 78/108 SIRIUS candidates could be discarded. Amongst the 8 database candidates, only four fell within the expected CCS error and only one was reported in the literature as being metabolized in the kidneys. The unknown species was annotated as rocuronium, a drug used in surgery that was administered to all patients.

Combined high accuracy CCS values derived from DESI-cIM-MSI and machine learning predicted CCS increase annotation confidence and identify unknowns.

Steroidal Hormones Fragmentation Mechanisms Revealed by Infrared Ion Spectroscopy

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Presenting Author: Laura Finazzi

Steroidal hormones play a crucial role as chemical messengers within organisms. Their profound influence on various physiological processes makes them a prime focus for analysis in healthcare and many other biochemical fields. While their metabolic pathways are extensively studied and numerous MS(/MSn) spectra are available (e.g., in the HMDB), the fragmentation pathways of steroidal hormones and their derivatives in tandem mass spectrometry are yet to be completely understood. The complex fragmentation mechanisms of steroids pose significant challenges in interpreting MSn spectra and elucidating fragment structures. This work aims to characterize MS/MS fragments using infrared ion spectroscopy (IRIS) and propose potential candidate structures.

Infrared spectra are obtained for various mass-selected MS/MS fragment ions of the protonated steroid molecules cortisol, progesterone, testosterone and estradiol. Ions are produced by electrospray ionization of ~10 uM solutions of the respective precursor molecule and fragment ions of interest are produced by collision-induced dissociation in a Bruker AmaZon ion trap mass spectrometer. Mass-selected ions are then exposed to the tunable IR radiation of the free-electron laser FELIX. An IR spectrum is reconstructed from a series of mass spectra by plotting the fractional dissociation as a function of laser frequency over the 800-2000 cm-1 range. Experimental IR spectra are compared against DFT-computed IR spectra to extract likely fragment ion structures.

Steroid hormones are derivatives of cholesterol and their aromatic rings nomenclature (A-D) is based on cholesterol core structure. These hormones exhibit distinct fragmentation behaviours, allowing for their classification into groups: corticosteroids, progestogens, androgens and estrogens. This work aims to elucidate the fragmentation pathways within each category by investigating a representative molecule: cortisol, progesterone, testosterone, and estradiol, respectively. IR spectra of protonated parents and MS/MS products are compared with theoretical calculations to identify likely fragment structures. Testosterone MS/MS fragments m/z 271, 123, 109 and 97 were investigated. The precursor ion is protonated on ring A carbonyl, leading to water loss and formation of m/z 271. The spectroscopically determined structures of m/z 123 and 109, resulting from ring B cleavage, corroborate previous literature findings. Our study also reproduces previous spectroscopic investigation of m/z 97, resulting from rearrangements of ring A, supporting the presence of two isomeric contributors. Cortisol MS/MS fragments m/z 327 and 121 were investigated. Protonation of cortisol is speculated to occur at ring A carbonyl and subsequent double water loss leads to the formation of the m/z 327 fragment, for which a novel structure is proposed. Based on our observations, a structure for m/z 121 is speculated to retain ring A structure, incorporating hydrocarbon substituents resulting from cleavage of ring B bonds . For estradiol, we investigated fragments at m/z 255, 173, 159, and 135. Protonation occurs at the hydroxyl group, resulting in water loss and the formation of m/z 255. Possible structures for m/z 173 and 135 are proposed, and m/z 159 structure is compared to previous literature findings, with m/z 173 potentially serving as a precursor for m/z 159 due to structural similarities. Finally, we characterized progesterone fragments at m/z 297 and 279, resulting from single and double water loss, respectively, and proposed candidate structures for each.

Accurate molecular structures are established for several steroid MS/MS fragments based on IR ion spectroscopy and DFT calculations.

In-depth N-Terminomics Profiling and Dynamics of N-Terminal Proteoforms in Human Cells

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Presenting Author: Longping Fu

N-terminomics is pivotal for dissecting protease specificity and deciphering the regulatory roles of N-degron pathway in protein turnover. Here, we developed a Galactosyl Aldehyde Azide (GAA) probe for the selective enrichment of proteoforms with diverse N-termini. Integrated with state-of-the-art mass spectrometry, our approach identified 18,777 unique N-terminal proteoforms from 3,160 proteins across HEK293T, Jurkat, and THP-1 cells. Additionally, we systematically investigated the dynamics of distinct N-terminal proteoforms alongside their corresponding proteins. Bioinformatic analyses revealed that fast-turnover proteoforms and stable ones exhibited distinct function and subcellular localizations. Furthermore, intrinsic structure, N-terminal sequence physicochemical characteristic, and E3 ligase recognition, could dramatically affect the proteoform degradation. Together, our findings provide new insights into the regulatory impact of N-terminus on proteoform function and dynamics.

We developed a chemical proteomic approach for efficient N-terminal proteoform profiling and systematic analysis of their dynamics. Using galactose oxidase (GAO), we synthesized a Galactosyl Aldehyde Azide (GAA) probe by converting the hydroxyl group on C6 of β-D-Galactopyranosyl azide into an aldehyde. This probe was integrated into a workflow that combines click chemistry with a cleavable linker for effective enrichment of N-terminal proteoforms. Furthermore, we systematically investigated N-terminal proteoforms turnover in HEK293T cells by integrating N-terminal proteoform profiling with pulse-chase labeling approach and multiplexed proteomics.

Using the N-terminal proteoform profiling workflow, we systematically investigated the proteoforms in three human cell lines: HEK293T, Jurkat, and THP-1. For HEK293T cells, duplicate experiments were performed, yielding approximately 9,000 unique N-terminal proteoforms per experiment, with over 8,300 proteoforms overlapping between replicates, demonstrating high reproducibility. In Jurkat cells, 10,015 N-terminal proteoforms were identified, while 8,747 ones were detected in THP-1 cells. Across all three cell lines, 2,880 proteoforms were commonly identified. Additionally, 4,434, 3,757, and 3,735 cell-specific N-terminal proteoforms were detected in HEK293T, Jurkat, and THP-1 cells, respectively. Functional enrichment analysis of the common proteoforms highlighted their involvement protein folding, as well as RNA processing and intracellular trafficking. Investigation of cell-specific proteoforms revealed distinct biological functions. In HEK293T cells, cell-specific function of N-terminal proteoforms were primarily associated with biomolecular condensates and cell cycle regulation, while in Jurkat cells, NADH-related processes were overrepresented. In contrast, unique proteoforms in THP-1 were predominantly linked to secretory pathway, including endomembrane system and cell surface. Integrated with protease cleavage data from TopFIND database, we identified events mediated by multiple proteases, underscoring their diverse roles in N-terminal proteoform generation. Bioinformatic analysis of N-terminal proteoform dynamics demonstrated that rapidly degrading proteoforms were related to cell cycle, whereas stable proteoforms were associated with translation. Additionally, structural analysis revealed that proteoforms with N-termini situated within ordered regions, such as helix and strand, exhibit markedly greater stability than those whose N-termini are in unstructured, high solvent accessibility regions. Furthermore, we compared the half-lives of proteoforms associated with different N-degron classes. Interestingly, proteoforms regulated by the GASTC/N-degron pathway and those with bulky, hydrophobic residues (FYLIW, type-II N-degron) displayed significantly longer half-lives than those controlled by type-I N-degrons, suggesting inherent differences in N terminal sequence properties and recognition efficiencies by E3 ligases underlie their distinct degradation kinetics.

We developed the GAA probe for unprecedented N-terminomics coverage in human cells, revealing both common and cell-specific functions of N-terminal proteoforms. Investigation of N-terminal proteoform dynamics identifies critical determinants of proteoform turnover, including intrinsic protein function, subcellular localization, structure context of N-terminus, N-terminal sequence physicochemical properties, and N-degron pathway regulation.

Novel Insights into Native Ion Mobility from Nucleic Acid Studies

Valérie Gabelica; School of Pharmaceutical Sciences, University of Geneva, Switzerland

Presenting Author: Valérie Gabelica

Most native ion mobility mass spectrometry (MS) studies focus on proteins or protein complexes. Our group has been studying nucleic acid structures by native MS for many years.1 However, we never assumed that the underlying mechanisms and data interpretation would be directly applicable to our systems. Our scientific approach always involved questioning the fundamentals, leading us to novel insights into the underpinnings of native MS and ion mobility MS. This lecture will trace some of these fundamental studies, including collision cross-section calibrations, modeling oligonucleotide rearrangements in the gas phase, and the effects of the electrospray charging mechanism on gas-phase structures. Additionally, we will discuss recent efforts to interpret charge-induced and collision-induced unfolding to deduce the strength of intramolecular interactions.
Largy, E.; Konig, A.; Ghosh, A.; Ghosh, D.; Benabou, S.; Rosu, F.; Gabelica, V. Mass Spectrometry of Nucleic Acid Noncovalent Complexes. Chem. Rev. 2022, 122 (8), 7720-7839.

Resolving the Binding Efficiency and Disassembly Behavior of BCL-2/xL-Specific PROTACs by Native MS

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The anti-apoptotic BCL-2 family proteins play a central role in cancer progression by regulating apoptosis, making them key targets in cancer therapy. Although dual inhibition of BCL-2 and BCL-xL promotes cell death, it frequently leads to adverse effects such as thrombocytopenia. Proteolysis-targeting chimeras (PROTACs) offer an alternative by inducing selective degradation of target proteins via E3 ligase recruitment. In this study, we investigate PROTAC-induced ternary complex formation, disassembly mechanisms, and complex stability using native mass spectrometry, gas-phase activation (HCD and UVPD), and variable-temperature ESI. These results provide structural and mechanistic insights that can guide the design of next-generation PROTACs with improved selectivity, degradation efficiency, and therapeutic potential.

BCL-2, BCL-xL, and VCB (a heterotrimeric complex of VHL, EloC, and EloB) were recombinantly expressed and purified in-house, followed by buffer exchange into 100 mM ammonium acetate using Bio-Spin P-6 size exclusion columns. Equimolar concentrations of BCL proteins and VCB were incubated with selected PROTAC candidates, including DT2216, 753b, and PZ6252. Samples were introduced via static nano-electrospray ionization using Au/Pd-coated borosilicate capillaries at a spray voltage of 0.9–1.1 kV. Native MS experiments were conducted in positive ion mode on a Thermo Scientific Q Exactive Plus UHMR instrument, equipped with a 193 nm excimer laser for ultraviolet photodissociation (UVPD) in the HCD cell. A variable- temperature ESI source was used to assess stability and thermal dissociation of ternary complexes in solution.

Ternary complex formation was evaluated for a panel of PROTACs, including 753b, DT2216, and PZ6252, BCL-xL in the presence of VCB (VHL–EloC–EloB). Native MS titration experiments revealed concentration-dependent formation of 1:1:1 ternary complexes. Among the tested compounds, 753b exhibited the highest binding affinity, consistent with its reported therapeutic potency, while PZ6252 displayed relatively weak interactions, particularly with BCL- xL. These findings highlight the utility of native MS in predicting target engagement and potential therapeutic efficacy. To investigate gas-phase stability and dissociation mechanisms, CID and UVPD experiments were performed. At low collision energies (≤70 V), CID promoted release of elongin B and elongin C, generating subcomplexes that retained VHL, the PROTAC, and the BCL protein—indicating weaker peripheral interactions between VCB components compare to core tertiary complex. As the collision energy increased, the PROTAC was dissociated, resulting in subcomplexes containing only the core protein–protein interactions. In contrast, UVPD showed distinct dissociation behavior: while low UVPD energy also released elongin B and C, higher-energy irradiation yielded subcomplexes that retained the PROTAC, VHL, and BCL protein together, suggesting strong and possibly cooperative PROTAC-mediated binding. Solution thermal stability was further evaluated using a variable-temperature ESI source. At 40 °C, ternary complexes remained intact. Upon increasing the temperature to 55 °C, elongin B and C were released; above 55 °C, complete disassembly of the ternary complex was observed. These trends reflect a progressive destabilization of the complex under thermal stress, with early release of peripheral subunits and eventual dissociation of the core. Together, gas-phase and solution analyses revealed complementary disassembly patterns: gas-phase activation resolved interaction hierarchies and structural robustness, while variable temperature ESI measurements reflected complex stability in near-physiological conditions. This integrated approach enables a more comprehensive understanding of PROTAC-induced ternary complex behavior, informing degrader design beyond static affinity measurements.

Native MS titration enables direct comparison of PROTAC binding specificity and affinity for BCL-2/xL targets. By using CID, UVPD, and variable temperature ESI, this integrated approach reveals complementary dissociation pathways and stability profiles, offering insight to guide the design of selective, high-affinity degraders that may offer improved therapeutic potential.

Facilitating Native Ion Mobility-Mass Spectrometry and Collision Induced Unfolding of Proteins in Nonvolatile Buffers via Online Tangential Flow Filtration

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Native ion mobility-mass spectrometry (IM-MS) and collision induced unfolding (CIU) have been established as powerful approaches for evaluating protein structure in the gas phase. Prior to these analyses, samples must be exchanged into an ammonium acetate solution using molecular weight cut off spin filters, gel filtration spin columns, or preparative-scale size exclusion chromatography (SEC). However, some buffer components are challenging to remove even by successive spin columns/filters, or comprehensive SEC clean-up. These challenges prevent analysis of samples prepared in complex buffers, such as monoclonal antibodies (mAbs) in their storage buffers, and limit experimental capability. A device enabling automated tangential flow filtration online with nano-electrospray ionization (nESI) source was recently described. We evaluated this technology for native IM-MS and CIU applications.

Infliximab, and standard proteins were purchased from Millipore Sigma. Samples were prepared in ammonium acetate or 5 mM sodium phosphate (pH = 7.5). Samples were buffer exchanged by injecting 0.001 ml of 0.1 mg/ml protein onto an automated tangential flow filtration device (DynaChip, Andson Biotech) which exchanged samples into 200 mM ammonium acetate, and transferred samples directly to the nESI source of a Waters Select Series CyclicIMS ion mobility-mass spectrometer. Samples were ionized via nESI using fused silica emitters (CoAnn Technologies). All CIU measurements were conducted in 2 or 4 minutes by ramping the Trap Collision Energy to 200 V. IM-MS, and CIU data was analyzed using Waters Masslynx, TWIMExtract, and CIUSuite 3.

The DynaChip tangential flow filtration device enables rapid online cleanup of protein samples, while maintaining the analytical advantages of nanoflow electrospray ionization (sensitivity, low sample consumption etc.). We first evaluated the ability of this device to clean up a range of standard proteins including ubiquitin, β-lactoglobulin, pyruvate kinase, glycerol dehydrogenase, β-galactosidase, and GroEL. These samples typically require at least one round of buffer exchange by spin column/filter to remove buffer salts from production. Samples exchanged by DynaChip at least matched the level of buffer exchange achieved by spin columns/filters, and in several cases significantly outperformed conventional offline buffer exchange methods. The higher degree of desalting resulted in unprecedented mass resolution and enabled the identification of several low abundance protein signals which were not previously observed. Satisfied with the ability of the DynaChip to exchange standard proteins, we opted to evaluate this device for rapid CIU measurements. A 0.001 ml injection of 0.1 mg/ml protein results in ~6 minutes of stable sample spray. We designed an automated method which conducted a 2-minute CIU measurement and a 4-minute CIU measurement in this timespan for every sample. We evaluated the CIU of the monoclonal biotherapeutic antibody Infliximab, which was either prepared fresh, or exposed to 5 days of stress by shaking at 220 RPM at 37 C. We conducted the stress for infliximab reconstituted in ammonium acetate or 5 mM sodium phosphate buffer, to evaluate the protective effects of buffering agent against stress. All samples were cleaned up online using the DynaChip device, enabling the consistent analysis of samples which were stressed in ammonium acetate or phosphate buffer. Our results demonstrated the high reproducibility of rapid CIU and show that phosphate buffer had a protective effect against the 5-day stress protocol.

This work demonstrates the utility of the DynaChip technology in exchanging protein samples from buffer components such as phosphate buffer. Additionally, to match the time scale of this sample clean-up we demonstrated the possibility and utility of rapid CIU measurements in 2 minutes with remarkably high reproducibility.

Comparing Native-protein Charging and Structures Associated with Different Modeling Techniques

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Presenting Author: Elyssia Gallagher

Native mass spectrometry (nMS) is valuable for characterizing proteins and complexes. When nMS is coupled with ion mobility (IM), protein structure can be characterized by measuring collisional cross sections (CCS). Recently, there have been efforts to correlate these measured CCS values with CCS predicted from crystal or NMR structures. However, when native proteins transition from solution to the gas-phase during electrospray ionization (ESI), they form protonated ions. A current limitation of the modeling software is identifying where the charges should be placed for gas-phase protein ions. Herein, we use a modified molecular dynamics (MD) code to perform dynamic (de)protonation of proteins in evaporating droplets. Additionally, we compare the charging and resulting structures to those determined by Structure Relaxation Approximation (SRA).

Previously, we reported the development of a code (simESI) that utilizes a stitching method to perform MD simulations and enable proton-transfer reactions. After 4 ps of MD simulation, we pause the simulation to perform energetics calculations to determine the feasibility of proton transfers between aqueous solvent (water and Grotthuss diffusion of H3O+ and OH-), ammonium acetate, and/or protein. This code accounts for changes in gas-phase basicity as proteins transition from solvated to gaseous ions. We analyze the dynamic charging in ubiquitin and NISTmAb. We compare the charged sites to those identified using an implementation of SRA, which charges amino acids based on their decreasing order of solvent accessibility. We also examine differences in CCS associated with differences in residue charging.

Following replicate simulations, both ubiquitin (8.6 kDa, n=30) and NISTmAb (~150 kDa, n=10) yielded average charge states that correlated well with experimental values. The average simulated and experimental charge states for ubiquitin were 5.90+ and (5.8±0.2)+, respectively. The simulated charge states for NISTmAb ranged from 25+ to 29+, while the experimental charge states have been observed between 23+ and 28+. Next, we compared the protonation patterns for both ubiquitin and NISTmAb using either our droplet simulations with dynamic charging (simESI) or SRA. In simESI, the proteins begin the simulations at a charge state representative of their solution-phase charge and undergo dynamic protonation and deprotonation to yield a final charge for the desolvated gas-phase ion. However, in SRA, a protein charge must be set based on a used-defined value. Therefore, we compared the charge states produced by simESI to the same charge states in SRA. When comparing the sites of protonation determined by simESI and SRA, there was a significant difference in which amino acids were protonated, with 29% and 51% difference in average proton occupancy for ubiquitin and NISTmAb, respectively. In general, protonation resulting from simESI was more evenly distributed across the protein structures compared to SRA, with many residues from SRA not being protonated in any of the conducted trials. This suggests that in simESI, protonation is not primarily determined by solvent accessible. Dynamics in the protein structure from incremental changes in charge, interactions with ammonium and acetate, and solvent loss may all play a role in these differences. These differences in charging resulted in differences in calculated CCS (using Collidoscope), with ions from SRA compacting 35% less than those generated from simESI.

These findings suggest that modeling droplet dynamics, including protonation and solvent loss, may be important in considering gas-phase protonation sites. This, in turn, impacts calculated CCS. A better understanding of protein charging during ESI is necessary to correlate structures measured by IM-MS to those associated with traditional structural techniques.

Interaction of Substrate and Inhibitors with Salmonella FraB Deglycase, a Drug Target

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Presenting Author: Yuan Gao

Salmonella enterica serovar Typhimurium is the second leading cause of death from foodborne illness in the United States. FraB, a deglycase, catalyzes the final step during the catabolism of fructose-asparagine. FraB is a promising drug target since its inhibition results in build-up of its substrate (6-phosphofructose-aspartate, 6-P-F-Asp) and Salmonella intoxication. However, the substrate/inhibitor binding sites are not well characterized, a limitation that hinders the understanding of the mechanism of FraB catalysis and inhibition. We leveraged native mass spectrometry (nMS) to characterize the stoichiometry of substrate and inhibitor binding, as well as top-down electron capture dissociation (ECD) to characterize the binding sites and potential conformational changes during catalysis. Results from these studies should help advance the design of FraB-based, anti-Salmonella therapeutics.

FraB was overexpressed in Escherichia coli and purified using affinity chromatography. MicroBio-spin P6 columns were used to buffer exchange FraB wild type (WT) and FraB E214A into pH-adjusted 200 mM ammonium acetate or 67 mM ethylenediaminediacetic acid. 6-P-F-Asp was diluted in water, while inhibitors were diluted in MeOH or DMSO. Samples were analyzed at 3 μM FraB monomer concentration ± substrate (150 μM to 600 μM) ± inhibitor (5 μM to 100 μM). Total DMSO was < 1% (v/v) in all experiments. nMS and ECD experiments were performed on a Thermo Q Exactive UHMR (ultra-high mass range) Orbitrap mass spectrometer modified with a custom ECD-SID device. Mass Spectra were deconvolved using UniDec and ECD data were processed using ProSight Lite.

We first used nMS to characterize the binding of substrate to FraB, a homodimer. Peaks corresponding to enzyme-substrate (ES) and enzyme-product (EP) were evident. Moreover, the stronger and weaker binding, respectively, of glucose-6-phosphate and L-Asp, products from FraB cleavage of 6-P-F-Asp, indicates that FraB must leverages the sugar moiety during substrate binding. Furthermore, we isolated the different complexes E2/E2S/E2P/E2SP/E2P2 for WT and E2/E2S2 for the inactive mutant E214A and performed native top-down ECD experiments. We observed fragmentation pattern differences from multiple regions for different complexes. For FraB E214A, we observed ECD cleavage sites for Ser32 and Glu85 only for E2, but not E2S2, suggesting that Ser32 and Glu85 are potential substrate binding sites, consistent with a previously published RosettaCM-based model. Moreover, we observed different ECD fragmentation patterns post-substrate binding (e.g., less cleavage from Thr13-Ala16 and Phe42-Glu44, more cleavage from Tyr33-Ala35 and Lys45-Asn62). These differences in fragmentation locations coincide with the dimer interface and possibly relates to potential conformational changes associated with substrate binding. For FraB-inhibitor complexes, we observed enzyme-inhibitor (EI) complexes without the presence of substrate and enzyme-inhibitor-substrate (EIS) complexes, enzyme-inhibitor-product (EIP) complexes and enzyme-inhibitor-substrate-product (EISP) complexes with the presence of substrate, suggesting the binding of these inhibitors to a functional FraB albeit not at the active site. We also compared the binding affinity of an uncompetitive inhibitor ± competitive inhibitor. Through titration, we determined that the uncompetitive inhibitor binds less when the competitive inhibitor is bound, likely due to the conformational changes induced by the competitive inhibitor. Finally, our identification of FraB ECD fragments bound to an uncompetitive inhibitor allows the localization of the inhibitor's binding sites and local conformational changes elicited by it. Our work on FraB illustrates the unprecedented utility of native MS in characterizing the different species present during catalysis and inhibition of a homodimeric enzyme.

•Identify different types of inhibitors bound concomitantly to an enzyme, thereby affording a roadmap for design of inhibitor cocktails. • Identify the inhibitor-binding sites and conformational changes by native, top-down ECD •Integrate native and top-down MS to characterize inhibition of Salmonella FraB, a drug target; broader implications for mechanistic enzymology studies.

Advancing Native Top-down Proteomics for Structural and Proteoform Analysis

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Presenting Author: Ying Ge

Native top-down proteomics (nTDP) is a powerful approach for characterizing intact proteins and complexes in their native states, enabling detailed insights into macromolecular structure, protein–ligand interactions, and proteoform heterogeneity. We have developed an advanced nTDP platform that integrates trapped ion mobility spectrometry (TIMS) with ultrahigh-resolution Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), allowing precise analysis of protein conformers, gas-phase isomers, and structural proteoforms. Using this platform, we achieved complete structural characterization of intact O-glycan proteoforms in the SARS-CoV-2 spike receptor-binding domain (S-RBD). We further introduced a “native nanoproteomics” workflow that enables direct enrichment and nTDP analysis of endogenous cardiac troponin complexes from human heart tissue, revealing structural dynamics with functional relevance. To streamline data analysis, we developed MASH Native, a unified software tool for processing native TDP datasets, offering an integrated solution for characterizing both protein complexes and proteoforms. To address limitations in native protein separation, we established online mixed-bed ion exchange chromatography under non-denaturing conditions, enabling preservation of noncovalent interactions and successful detection of high-mass proteins (>146 kDa), including native metal-binding and oligomeric complexes from human heart tissue. We are now expanding to multi-dimensional native liquid chromatography that combines orthogonal separation strategies for deeper proteome coverage and analysis of large native complexes directly from tissues. Leveraging ultrahigh-resolution FTICR-MS, we continue to uncover higher-order structures, ligand interactions, and proteoform diversity. Together, these innovations in instrumentation, separation, and informatics significantly advance native top-down proteomics, offering new tools to investigate protein structure–function relationships and disease mechanisms at the proteoform level.

Understanding Biomolecular Behavior with Mass Photometry

Austin Graves, Refeyn Inc.

Presenting Author: Austin Graves

Biophysical characterization describes a wide variety of scientific problems, whether it is solving a crystal structure, investigating the interaction between two proteins, or simply optimizing a protein purification process. There are many techniques available for each of these problems, but they can be very time and resource consuming or too specialized to be applicable to multiple questions.

Mass photometry allows for accurate mass measurements of single molecules in solution in only a few minutes. The masses of these molecules are obtained by measuring the amount of light scattered by the molecules as they bind to a glass surface. This technique enables its users to characterize both proteins and nucleic acids in a label-free environment, in their native state. The ability to measure the mass of single molecules in a bulk sample provides access to subpopulations, which makes it possible to determine complex stoichiometry and oligomeric state. Furthermore, the single molecule counting over a wide mass range that mass photometry provides enables the detection of low abundance species and the characterization of sample heterogeneity.

We believe that mass photometry can help accelerate scientific discovery by making sample characterization more straightforward and accessible, and enabling analysis of complexes, assembly, and interactions in new ways.

Nanoscale Molecular Cartography of the Cellular Membranes

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Presenting Author: Kallol Gupta

Macromolecular organization between proteins and lipids at the cellular membrane is fundamental to any membrane-associated cellular signaling events. Capturing these associations demands molecular resolution that can unambiguously determine both the identity of large protein complexes, as well as small bound lipids and ligands. Simultaneously, we need nanoscale spatial resolution to capture these assemblies directly from their endogenous membrane of action. Addressing these analytical challenges, we will present our ongoing work in the lab that combines native mass spectrometry with chemical biology, molecular imaging, and other orthogonal tools to render a quantitative molecular view of the protein-lipid organization in the membrane and how that drives downstream cellular siganling.

Structural Analysis of Divalent Cation Bound α-Synuclein by Cross-linking Ion Mobility-Mass Spectrometry.

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Presenting Author: Ankita Gurav

Alpha-synuclein (a-syn) is an intrinsically disordered protein implicated in Parkinson's disease. Its disordered structure poses a challenge for traditional methods of structural characterization. Ion-mobility mass spectrometry is a powerful analytical technique used for studying conformational ensembles of such disordered proteins. Chemical crosslinking provides insights into the conformational space of the protein with residue-level selectivity. The information thus obtained gives detailed information about the most prominent conformer families as well as conformations change during biochemical events. Since a-syn is predominantly found in the synapses, it is susceptible to metal ion exposure. Here, we study the effects of metal ion binding on the conformational distribution of a-syn.

Purified α-Synuclein was 2x buffer exchanged from ammonium acetate to 1x Phosphate buffered saline (PBS) which was diluted to 50μM and reacted with 50mM metal chloride for 30 mins at a protein:metal ratio of 1:1000. To this 125μM crosslinker (BS3 or BS2G) was added and allowed to react for 12 mins where protein: crosslinker ratio was 1:2.5. The reaction mixture thus obtained (~25μM) was 2x buffered back into 50mM ammonium acetate and electrosprayed into a Waters Synapt G2Si HDMS instrument. The crosslinks thus formed were m/z isolated fragmented by electron capture dissociation in CID mode. Analysis was performed using MassLynx and Viewer and fragment maps were prepared using ProSight Lite. Experiments were repeated with N-terminal acetylated α-Syn.

Since our instrument allows for selection of individual charge states of metal bound and crosslinked a-syn, the individually isolated charge states were subjected to ECD fragmentation enabling identification of crosslinked sites. The reactions were optimized to result in formation of primarily single crosslink product. We used divalent metal ions (Ca, Co, Ni and Cu) for the metal binding step wherein only copper was found to produce peaks with metal ion and crosslink. Other metals produced peaks were the protein was simply crosslinked without presence of the metal ion. Control experiments performed indicated that our copper was bound to the aspartic acid residue at second position to the N-terminus. Since the reactions were carried out in solution phase, the crosslink positions observed had minimal differences across the different charge states (8+, 9+, 10+, 11+) explored. The most common crosslink site across the different charge state for BS3 crosslinker appeared at the N-terminal to either the lysine at position 6 or 10. For the BS2G crosslinker this crosslink was at N-terminal to the lysine at 10 or 12. The crosslinks assignments also appeared to get more ambiguous at the C terminal end. In case of the copper bound data, it was observed that the fragment maps had little coverage indicative of the possibility that the crosslink was affecting the fragmentation. To validate these assignments, a Glu-C enzymatic digest was undertaken which further confirmed the assignments. Alongside these studies, we are also undertaking similar reactions with N-terminally acetylated a-syn to observe for changes in conformational landscape due to metal binding. The results subsequently achieved will allow for changes to be made to existing models by applying distant restraints.

Studies help understand effects of metal ion interactions on the conformational distribution of a-syn.

Precursor Ion Size Effects in Tandem Mass Spectrometry of Posttranslationally-Modified Peptides and Proteins

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Presenting Author: Kristina Hakansson

Labile posttranslational modifications (PTMs) are known to show improved retention in top-down vs. bottom-up tandem mass spectrometry (MS/MS), thus potentially improving their site determination. For example, top-down collision-activated dissociation (CAD)-MS/MS has been shown to proceed without loss of N-glycosylation1 and phosphorylation;2 however, we demonstrated that the type of N-glycan affects the degree of retention in both bottom-up and middle-down infrared multiphoton dissociation (IRMPD) MS/MS.3 Electron capture/transfer dissociation (ECD/ETD) are known to show much improved PTM retention compared with CAD but, for highly labile PTMs such as sulfation, significant PTM loss can still be observed.4 Here, we systematically examine the influence of precursor ion size, PTM type, and charge state on PTM retention in bottom-up, middle-down, and top-down CAD and ETD.

Bovine β-casein, coral tree lectin, bovine fetuin, and bovine fibrinogen (Millipore Sigma) were subjected to top-down (intact), middle-down (TCEP, cyanogen bromide, or Arg-C treatment) and "bottom-up" (trypsin digestion) analysis. Direct infusion electrospray ionization-CAD and LC/CAD were performed with a 7 T quadrupole-FT-ICR (SolariX; Bruker) mass spectrometer. For LC/CAD, an Agilent 1290 HPLC with an AdvanceBio RP-mAb SB-CB or Agilent Poroshell 120 EC-C18 column was used with an acetonitrile:water/0.1% formic acid solvent system. nanoLC-MS/MS experiments were performed using a Waters ACQUITY UPLC M-Class system with custom-fabricated Agilent Poroshell 300 SB-C8 trap and analytical columns. CID, HCD, and ETD experiments were conducted using an Orbitrap Eclipse™ Tribrid™ instrument (Thermo Fisher Scientific) coupled with a custom 21 T FT-ICR mass spectrometer.

Top-down LC-CAD of a ~29 kDa lectin with two N-glycans yielded a few ~11-13 kDa fragments with intact glycosylation. However, low sequence coverage (9-11%) was observed. For tryptic glycopeptides (~3 and 5 kDa), no glycan-containing fragments were observed, while larger, Arg-C-generated, glycopeptides (~6.5 and 8.5 kDa) showed some glycan-containing fragments from low precursor ion charge states. A ~17 kDa, CNBr-generated, glycopetide yielded more glycan-retaining fragments/higher sequence coverage for low charge states. In top-down CAD of β-casein (~24 kDa), containing five N-terminal phosphoserines, the 15+ charge state showed the highest number of b-type fragments containing all phosphates/the highest sequence coverage (17%). However, 1-2 PTM losses were also seen for 26% of these fragments. An ~11 kDa CNBr-generated peptide with all phosphates yielded 6 PTM-retaining fragments in LC-CAD. The fibrinogen beta chain (~53 kDa), which contains a sulfotyrosine near the N-terminus, showed partial sulfate retention in b-type ions; however, desulfated b-type fragments were more abundant. Partial sulfate retention was observed for both the beta and gamma chains in bottom-up ETD. By contrast, complete sulfate retention was seen in top-down beta chain ETD. Overall, our data show that labile PTM loss is reduced for the glycoprotein but significant PTM loss is seen for the phospho- and sulfoproteins in top-down CAD-MS/MS. For the lectin, middle-down analysis of the ~17 kDa glycopeptide yielded minimum glycan cleavage with higher sequence coverage than top-down analysis. By contrast, the ~11 kDa phosphopeptide showed more prominent PTM loss, suggesting that ~20 kDa at a low charge state is a sweet spot for optimum PTM retention/sequence coverage with the exception of sulfation, which requires alternative activation, e.g., ETD. [1] Reid et al. Anal. Chem.2002;74:577-583. [2] Meng et al. Nat. Biotechnol.2001;19:952-957 [3] Adamson and Hakansson. J. Proteome Res.2006;5:493-501 [4] Youssef et al. J. Proteome Res.2024;23:2386−2396

Middle-down CAD-MS/MS provides higher labile PTM retention and sequence coverage compared with both bottom-up and top-down CAD, except for sulfation which requires ETD.

Analysis of Heterogeneous Higher-order Transthyretin Oligomers using Direct Mass Technology and Ion Mobility-mass Spectrometry

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Transthyretin (TTR) is a 55 kDa tetrameric protein complex responsible for retinol and thyroxine transport as well as having a propensity to undergo amyloidosis. TTR amyloidosis is caused by the disassembly of the tetramer into monomers that become misfolded leading to formation of higher-order oligomers and fibers. Native mass spectrometry (nMS) is an established technique for studying TTR dynamics but cannot resolve higher order oligomers due to unresolvable charge and oligomeric states. This works provides insights into TTR amyloidosis by utilizing Direct Mass Technology (DMT) and Ion Mobility-Mass Spectrometry (IM-MS) to observe differences in TTR oligomerization and the misfolding of the monomer respectively based on solution conditions of pH and incubation temperature.

TTR was incubated in 100 mM ammonium acetate at a pH of 3.4, 4.4, or 5.4 at an incubation temperature of 4, 21 or 37°C for up to 3 days. For DMT measurements, an UHMR mass spectrometer (ThermoFisher Scientific, Bremen, GE) is used with the Direct Mass Technology software. Data collection was done with a resolution setting of 200,000 and at least 10,000 ions were collected per DMT spectrum and processed with STORIboard (Proteinaceous, Evanston, IL). IM-MS measurements were performed in triplicate with an Agilent 6560 mass spectrometer (Agilent, Santa Clara, CA). Data is collected initially after sample prep and 24 hours later at room temperature at different pH. Single-field collision cross section measurements were collected for IM-MS.

DMT analysis shows differences in oligomerization of wild type (WT) TTR based on changing solution conditions of pH and incubation temperature. At 21°C, pH 3.4 formed oligomers instantly, pH 4.4 formed oligomers after 12 hours of incubation, and pH 5.4 did not form oligomers due to being above the pI of TTR. Additionally, pH 4.4 experienced the greatest difference in rate of oligomerization when incubating at different temperatures. At 4°C oligomerization started after 6 hours of incubation and did not occur at 37°C. Oligomerization was studied at pH 4.4 for the mutants V30M, L55P, F87A, T119M, and V122I. F87A and L55P formed oligomers at all temperatures after 1 hour. V122I formed oligomers within 1 hour at temperatures lower than 37°C where oligomerization starts after 6 hours of incubation. V30M formed oligomers at 4°C after 3 hours of incubation, and after 48 hours for 21°C and 37°C. Lastly, T119M did not form oligomers at any temperature which is attributed to enhanced tetramer stability of this mutant. DMT reveals differences in TTR oligomerization brough on by changing solution conditions of temperature and pH. IM-MS of TTR reveals when looking at the monomer charge states the 8+ charge state has 3 conformations. Lower solution pH created a higher abundance of extended conformations. The extended conformations are attributed to a misfolded state due to them not existing at pH conditions above the pI of TTR. Preliminary analysis shows V122I as a stable monomer compared to WT and F87A due to a higher abundance of the extended conformations. An increase in the abundance of the extended conformations can be seen after 24 hours of incubation for the mutants tested. All mutants of TTR will be studied and compared to one another to relate to the start of TTR amyloidosis.

DMT resolves TTR oligomeric states that are currently not resolvable with other analytical techniques. Distinguishing differences in TTR oligomerization via DMT and monomer stability with IM-MS gives further insights into TTR amyloidosis and could be applied to other amyloid proteins.

Elucidating the Connection Between Acid Beta-Glucosidase Variant E326K and Parkinson's Disease

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Acid beta-glucosidase (GCase) is an enzyme associated with the lysosome and is responsible for the processing of glucosylceramide into glucose and ceramide. Homozygous mutations resultant in a loss of activity of GCase, and concomitant substrate accumulation, are causative for Gaucher disease (GD). GCase, in addition to its connection with GD, is the strongest genetic link to Parkinson disease (PD). However, the molecular mechanism underlying PD association is unclear, as many of the variants linked to PD retain some of their enzymatic activity. Of particular interest to the PD community is the GCase variant GCaseE326K. There is a strong genetic link of this variant to PD, but some control subjects harboring GCaseE326K do not develop PD. GCaseE326K has been shown to retain activity, and is not associated with GD, unless combined with other mutations, in which case loss of activity is enhanced. The E326K mutation is on the surface of GCase, removed from the active site, and should be tolerated. We hypothesize that the charge reversal mutation interferes with binding to GCase-activating protein Saposin C (SapC) and substrate trafficking to the GCase active site.

We have used a suite of biochemical and biophysical methods to investigate the GCase-SapC protein-protein interaction. Recombinant proteins were prepared in both E. coli and mammalian cell cultures and purified to homogeneity. The effect of SapC on enzyme (mutant, wild-type) activity was probed with fluorescence-based kinetics activity assays. Cell imaging was used to determine the impact of mutant GCase on protein abundance and distribution within cells. Microscale thermophoresis, as well as solution NMR, were used to estimate the strength of GCase-SapC interaction, and analytical ultracentrifugation was employed to determine reaction stoichiometry between the two proteins. Details of the protein-protein interaction interface were investigated with a combination of X-ray crystallography, solution NMR, and high-resolution cross-linking/mass spectrometry.

Our preliminary data suggest that the presence of the E326K mutation on GCase results in a weakened, aberrant interaction with SapC. While kinetics assays show little impact of SapC on either wild-type or mutant enzyme, expression of the mutant enzyme in cells results in a greater abundance of SapC present and a higher degree of co-localization with mutant GCase; in vitro assays do not fully recapitulate cellular context for this protein-protein interaction. Biophysical experiments point to a one-to-one interaction stoichiometry and weakened binding in the presence of the E326K mutation. Cross-linking/mass spectrometry data result in an interaction interface map for GCase and SapC.

Results of our experiments have informed a model of the GCase-SapC protein-protein interaction that is consistent with our hypothesis that the E326K mutation on the surface of GCase results in an aberrant interaction with its activator protein SapC. This manifests in the increased presence of SapC in cells expressing mutant GCase, and a greater extent of co-localization with the enzyme, perhaps to compensate for weaker or compromised interactions.

Vibrational Amplitude of Capillary Vibrating Sharp-Edge Spray Ionization (cVSSI) Emitters Influences Ion Intensity of Nucleic Acid and Protein Systems

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Presenting Author: Thomas Hughart

Biopolymer structure analysis is increasingly pertinent to drug discovery. Because of their flexibility, species such as intrinsically disordered proteins form co-existing conformational ensembles. Such conformations undergo transitions that affect ligand binding, which is an important consideration in drug design. Currently, experimental studies of conformer structural changes are somewhat limited. Recently, a novel ionization technique, capillary vibrating sharp-edge spray ionization (cVSSI), has been developed to expand experimental tools for the study of flexible species. This method allows for gentler ionization and the preservation of native structure. However, much work yet remains in the optimization of cVSSI for native MS.. Here, the influence of emitter tip mechanical amplitude on ion intensity and native structure preservation for DNA and protein systems is investigated.

Device Fabrication: A pulled glass capillary emitter tip was epoxied to a piezoelectric transducer. The devices were tested for nebulization before use. cVSSI Workflow: The cVSSI device is connected to a Waveform Generator and Amplifier and undergoes RF actuation. Sample is infused from a sample syringe into PTFE tubing, which has been slip-fitted over the pulled capillary emitter tip. Sample preparation: 50 µM 10-23 DNAzyme solution and a 20 µM Bovine Serum Albumin (BSA) solution were prepared. Mass Spectrometric Analysis: A Q-Exactive Orbitrap Mass Spectrometer was used for the analysis of the two analytes. Mass spectral data were collected at varying amplitude settings: 8.0-15.9 Vpp; 1.15 V increments.

Mass spectra collected for 10-23 DNAzyme in negative ion mode produced a bimodal charge state distribution (CSD). To determine the effect of emitter tip vibration amplitude on the preservation of native structure, the ion intensities of the lower charge states in the bimodal distribution were monitored as the piezoelectric device RF voltage was incremented. Overall, the intensities of the globular DNA ions increased from ~8.5 Vpp to ~10.3 Vpp. From ~10.3 Vpp to ~13.8 Vpp, the ion intensities remained essentially unchanged. After 13.8 V, the ion intensity for all charge states drops off. Separate experiments showed that the amplitude of the cVSSI emitter tip influences albumin CSDs. Increasing the amplitude beyond ~11.4 Vpp caused a shift in the CSDs. A higher abundance of the native and multimeric charge states was observed when applying ~11.4 to ~12.6 Vpp. This was accompanied by a lower number of higher charge states, suggesting greater preservation of albumin native structure. Beyond ~12.6 Vpp, the intensities of all ions decrease.

For the first time, experiments show that the amplitude of cVSSI emitter tips influences the ion intensity and native CSDs of DNA and protein systems.

Mass Spectrometry Imaging and Proteomic Analysis of Liposomes in 3D Cell Cultures

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Presenting Author: Amanda Hummon

Three-dimensional cell cultures are attractive models for biological research. They combine the flexibility of cell culture with some of the spatial and molecular complexity of tissue. For example, colon cancer cell lines form spheroids, in vitro mimics of poorly vascularized tumors. The spheroids are composed of a central necrotic core, a middle quiescent layer and an outer proliferative layer of cells, similar to a rapidly growing colon tumor. Our laboratory has characterized the distribution of endogenous proteins via MALDI imaging mass spectrometry in colon spheroids and determined that the molecular gradients correlate with the pathophysiological changes in the structure. We have also developed an approach to employ 3D cell cultures to evaluate the penetration of compounds into cellular masses. We are employing microfluidic devices to enable dynamic dosing, thus investigating the pharmacokinetics and pharmacodynamics of chemotherapy regimes in these attractive model systems. In particular, we have evaluated liposomal drugs with this approach, examining the downstream protein changes in addition to mapping the therapeutic and associated metabolites by imaging mass spectrometry.

Progesterone Metabolism and Breast Cancer Risk using Simultaneous Quantitation and Discovery (SQUAD) Liquid Chromatography Mass Spectrometry

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Presenting Author: Katherine Kenney

Breast cancer is the most prevalent cancer globally, accounting for 12.5% of new annual cases worldwide. Women with BRCA1/2 mutations face a significantly increased risk, with up to 80% higher likelihood of developing breast cancer. Emerging evidence suggests that the steroid hormone progesterone may be a critical intrinsic factor for breast cancer risk in BRCA1/2 carriers. Using metabolomic analysis, the association between relevant metabolomic alterations and progesterone levels can be examined among donor groups, including BRCA1/2-positive women who developed breast cancer, BRCA1/2-negative women who developed breast cancer, and healthy controls. The goal of this study is to identify metabolite and lipid changes linked to progesterone levels to determine whether these alterations are associated with breast cancer risk and cancer development in BRCA1/2 carriers.

Plasma samples from 450 women were obtained from the Komen Tissue Bank at Indiana University. These plasma samples were extracted with IPA before analyzing with reverse phase liquid chromatography mass spectrometry (RP-LC-MS). An innovative workflow, titled, simultaneous quantitation and discovery (SQUAD) LC-MS approach, was used to collect both targeted and non-targeted metabolomics data in a single experiment. SQUAD is an innovative acquisition scheme that combines targeted and non-targeted scans on a mass spectrometry platform. Targeted analysis focused on progesterone-related steroids, including beta-estradiol, estrone, cortisone, hydrocortisone, corticosterone, testosterone, androstenedione, and 17alpha-hydroxyprogesterone, producing data that can be compared across both batches and over time. These target analytes were selected to ensure wide coverage of the various enzymatic steps along the steroid biosynthetic pathway. The collection of non-targeted data allowed for wider lipidomic analysis, enabling the generation of subsequent hypotheses associated with greater effects of progesterone and breast cancer based on BRCA1/2 status.

Both targeted and non-targeted SQUAD LC-MS experiments yielded informative preliminary data, highlighting the potential differences among steroid pathways. The targeted data analysis focused on species of interest that contribute to the steroid biosynthetic pathway, including progesterone, cortisone, beta-estradiol, hydrocortisone, corticosterone, estrone, testosterone, androstenedione, and 17alpha-hydroxyprogesterone. All steroids were analyzed in positive ion mode except for beta-estradiol, which was analyzed in negative ion mode. However, most of these analytes fell below the corresponding limits of detection or did not show a significant change. Additionally, MS/MS was collected for both the targeted and non-targeted data, allowing for the identification of several steroids of interest via the corresponding internal standards. This MS/MS data also aided in unknown metabolite annotation for those with significant fold changes. MS2 data was compared against an in-house mzVault database along with accurate mass measurements. Annotation of known and unknown metabolites presents both an opportunity to better understand breast cancer biology but also a significant challenge. The SQUAD LC-MS approach ensures that both qualitative and quantitative differences in the steroid and metabolome profiles are thoroughly investigated, providing deeper insights into breast cancer biology.

SQUAD LC-MS detection and quantification of specific steroids and their metabolites related to breast cancer risk and development in BRCA-mutation carriers.

Deciphering the GlycoCode through Native Mass Spectrometry

John S. Klassen

Presenting Author: John Klassen

Native mass spectrometry (nMS)—electrospray ionization (ESI)-MS performed under conditions that preserve non-covalent interactions—has become an indispensable tool in glycomics. It offers a sensitive, label-free means of quantifying the stoichiometry and affinity of glycan interactions with glycan-binding proteins (GBPs). When coupled with the catch-and-release (CaR) technique, nMS enables high-throughput screening of both defined and natural glycan libraries, facilitating ligand discovery and providing detailed insights into GBP specificity. Integration with model membranes such as nanodiscs further expands nMS capabilities to detect glycolipid ligands and quantify their interactions with GBPs. This talk will highlight recent methodological advances, including concentration-independent (COIN)-nMS for screening natural glycan libraries, slow-mixing mode (SLOMO)-nMS for quantifying glycoprotein–GBP interactions, and membrane anchor-assisted (MEAN)-nMS for discovering glycolipid ligands on intact cells.

Analysis of Conformations and Microstates that Form Protein Free Energy Landscapes: A Mass Spectrometry Perspective

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Presenting Author: Carter Lantz

Proteins and protein complexes can adopt numerous structures in solution and the distribution of structures form a free energy landscape (FEL). Structures with large differences in collision cross section (CCS) (i.e. conformations) can be measured with ion-mobility mass spectrometry (IM-MS). Structures with similar CCS values (i.e. microstates) cannot be measured by IM-MS, so shifts in microstate distributions must be inferred using average charge state (Zavg) and ligand binding data from native mass spectrometry (nMS). In this work, we monitor changes in CCS to probe protein conformational shifts and changes in Zavg and ligand binding to probe protein microstate distribution shifts, and the results provide insight into how differences in water molecule structure (i.e. hydration) alter protein dynamics and stabilities.

Transthyretin (TTR) was expressed in house and buffer exchanged into H2O or D2O solutions containing 20mM ammonium acetate (AmAc). IM-MS was performed by electrospraying intact TTR solutions on an Agilent 6560 Mobility Q-TOF in Hadamard mode (Santa Clara, CA) or a Q-exactive EMR (San Jose, CA) using custom nanoelectrospray needles. Single ring GroEL (SR1) experiments were performed by buffer exchanging the protein in AmAc, EDDA, or TEAA with various concentrations of ATP. Mobility and ligand binding experiments for SR1 were performed on a Thermo Q-executive UHMR (San Jose, CA). Variable temperature electrospray ionization (vT-ESI) experiments were performed using a device described previously (McCabe, Anal. Chem., 2021).

IM-MS can be used to monitor protein conformational entropy (i.e., the distribution of conformers in solution) and probe alteration in conformational entropy due to shifts in hydration. Wild type (wt) TTR shows increases in abundance for compact conformers in deuterium oxide (D2O) relative to water (H2O). We interpret the compaction of TTR tetramers in D2O as evidence that D2O shifts hydration of TTR tetramers, which alters their conformational entropy. Furthermore, our results show that differences in buffer conditions can alter single ring GroEL (SR1) conformational entropy. When SR1 is inserted into ammonium acetate (AmAc), CCS measurements for observed charge states range from 150-155nm2; however, when SR1 is inserted into EDDA, CCS measurements for observed charge states range from 160-165nm2. We interpret the extension of SR1 in EDDA as evidence that EDDA interacts differently with SR1 relative to AmAc, which rearranges H2O molecules and shifts SR1 conformational entropy. Analysis of Zavg values and ligand binding can be used to monitor subtle changes in protein structure (i.e. microstates) that alter protein dynamics. Shifts in protein microstates can not be observed with IM-MS because changes in structure are small; however, they do shift the structure enough to change side chain protonation and ligand binding, which can be measured with nMS. Here, we vary temperature with vT-ESI directly in the nanospray tip to shift protein hydration, which alters microstate distributions. vT-ESI analysis of wtTTR in H2O and D2O shows that Zavg values increase at cold and hot temperatures indicating TTR tetramers can undergo cold and hot restructuring. In addition, variation of temperature with vT-ESI modulates ATP binding to SR1, which further indicates that microstate distributions can be altered by differences in hydration. Overall, monitoring shifts in conformations and microstates provide evidence that hydration should not be ignored when considering protein dynamics.

IM-MS and nMS can be used to probe protein complex conformations and microstates, which provides insight into how shifts in protein hydration alter their dynamics and stabilities.

A Protein-centric Mass Spectrometry Approach for Species Identification Within Harmful Algal Blooms

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Native mass spectrometry is a well-established tool in monitoring single protein/protein complexes that have been over-expressed recombinantly and purified. However, recent exciting developments within the field mean that we can now rapidly extract structural information on complex mixtures with minimal sample preparation. In this work, we demonstrate how native mass spectrometry can be used to analyse proteins extracted directly from lake water revealing critical information about the species they contain and their resulting environmental threat.

Lake water from 6 different lakes was collected. The lake cellular content pelleted and the material lysed using sonication. The cellular lysate was buffer exchanged through a 30kDa MWCO into 100 mM ammonium acetate pH 6.8 and its contents directly infused into either an Orbitrap Eclipse or Orbitrap Ascend Tribrid mass spectrometer (Thermo Fisher Scientific). MS2 and MS3 were performed to verify protein complexes observed.

We aimed to use native mass spectrometry analysis directly from lake water to assess harmful cyanobacterial blooms formation. Harmful cyanobacterial blooms present severe environmental threats, impacting water quality, aquatic ecosystems, and human health. However, only certain species are toxic. Here, we show how native mass spectrometry can readily detect colourful light harvesting complexes from within cyanobacteria directly from lake water. Furthermore, these complexes detected enable us to discriminate between different species that are actively growing within the cyanobacterial bloom. Moreover, our data highlights how highly resolved native mass spectrometry can excel above light microscopy in the early detection of harmful cyanobacterial blooms.

Native mass spectrometry directly from lake water discriminates between toxin and non-toxin producing algal blooms.

Unraveling Multispecific Antibody–antigen Interactions using Tandem-trapped Ion Mobility Spectrometry (Tandem-TIMS)

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Multispecific antibodies recognize two or more epitopes located on the same or distinct targets and are the latest breakthrough in therapeutic interventions for cancers, autoimmune diseases, and infectious diseases. Enhancing the stability and improving the binding affinity for multiple targets without cross-reactivity remains a significant challenge in the rational design and engineering of multispecific antibodies. This work explores the molecular interactions between multispecific antibodies and target binders using tandem-trapped ion mobility spectrometry (Tandem-TIMS). Utilizing the ability of Tandem-TIMS for conformational specific collision-induced unfolding and collision-induced dissociation, we characterize the impact of distinct protein conformations on the stability of various multispecific antibodies and the ligand binding affinities.

We employ orthogonally aligned Tandem-TIMS/tandem-MS instrument, constructed based on commercial timsTOFPro (in collaboration with Bruker Daltonics). Ion apertures were incorporated between the TIMS cells in Tandem-TIMS, allowing mobility selection and ion activation. Mobility-resolved collisional activation measurements were performed by mobility separation in TIMS-1 cell, followed by isolation and activation of ions with specific mobility in the interface and mobility separation in TIMS-2. Solutions of various multispecific antibodies in the apo and holo forms (Johnson & Johnson, designed for research purposes) in 0.5 μM concentration were infused into the electrospray source. Structural analysis of the experimental data was conducted using molecular dynamics-based structure relaxation approximation (SRA) method, providing detailed three-dimensional structures of unliganded and liganded proteins.

Mobility-resolved CIU (mr-CIU) was utilized to mobility-select a specific conformation from the full ensemble and probe the relative stability of the wild-type and mutants of mono, bi, and trispecific antibodies toward unfolding in a solvent-free environment of Tandem-TIMS. Overall, the AAS mutant displays slightly higher stability towards the gas phase unfolding than the wild-type, which correlates with solution melting temperatures. We evaluated the ability of Tandem-TIMS to characterize antibody-antigen complex formation using the humanized IgGk NIST monoclonal antibody (NIST mAb) and RSVF ligand. The data demonstrate specific binding of one and two RSVF ligands to the Fab regions of NIST mAb. We explore these approaches to study conformational-dependent binding affinity in multispecific antibodies. We use titration and collisional activation measurements to examine the relative binding affinities of distinct ligands non-covalently bound to binding arms of a mono, bi, and trispecific antibody (provided by Johnson & Johnson, designed for research purposes). We perform mobility-resolved collisional-induced unfolding (mr-CIU) and molecular dynamics-based SRA calculations to elucidate the three-dimensional structures of unbound and antigen-bound multispecific antibodies.

Tandem-trapped ion mobility spectrometry characterizes conformation-dependent antigen bindings in multispecific antibodies.

High-throughput Molecular Glue Screening via Native Mass Spectrometry and Cryo-EM Analysis

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Targeted protein degradation targeting conventionally undruggable proteins is a transformative approach in drug discovery. Molecular glues (MGs) achieve this by enhancing weak intrinsic interactions between targets and E3 ligase, enabling ubiquitin-proteasome-mediated degradation. While online affinity selection mass spectrometry (MS) struggles to identify weak binders due to on-column dissociation and lack of ternary complex interrogation, native MS facilitates the direct identification of E3-MG-target complexes. However, manual sample preparation and direct infusion limit its throughput. This study demonstrates high-throughput MG screening using nMS for WEE1 binding to CRBN-DDB1. It enables multiplex screening and analysis of over 2,500 compounds per day. Gas-phase ligand release and fragmentation support identification of unknown binders, and cryo-EM analysis further characterizes ligand-bound complexes, advancing MG discovery and validation.

Compound library, CRBN-DDB1 and WEE1 were provided by Dr. Eric Fischer. Thermo Fisher Scientific Vanquish Flex LC equipped with a UV detector, fraction collector, dual-injection autosampler, and dual Flex pumps was used. Proteins were online buffer exchanged into ammonium acetate (AmAc) using a 3-cm prototype SEC column and collected into a 96-well plate at the rate of 0.7 min/run. Each well of the plate was preloaded with ≥4 ligands. Protein-ligand mixtures were incubated and flow-injected into Thermo Fisher Orbitrap Ascend using Thermo Scientific EASY-Spray source for MS analysis. MS1 identified ternary complexes, MS2 determined bound ligands, and MS3 elucidated unknown ligands. Data processing was done using High-Throughput Screening feature in Prosight Native (Proteinaceous).

We initially compressed 96 compounds into 24 mixtures (4 compounds/well) and employed a 3-cm prototype SEC column for rapid online buffer exchange. LC-MS screening of all 96 compounds via native MS (nMS) took less than an hour, enabling throughput exceeding 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. However, identifying individual binders within mixtures was challenged by compound multiplexing, native adduct interference, and non-specific interactions. To resolve this ambiguity, 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 to assist structural elucidation. This workflow leverages MS1 to detect intact ternary complexes, MS2 to identify bound ligands, and MS3 to characterize unknowns, thereby increasing both throughput and specificity in MG screening. To eliminate compound competition and ensure sufficient molar excess of each ligand, we also screened compounds individually. This approach confirmed 16 of 96 compounds as potential molecular glues, exhibiting varying degrees of binding strengths. This nMS-based workflow enhances screening throughput, resolves compound ambiguity, and facilitates discovery of novel MGs. Selected hits were further characterized using cryo-electron microscopy (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 mechanistic insights to guide drug design and optimization.

This work introduces a high-throughput native MS workflow combining online buffer exchange, polarity switching, and multi-stage MS to identify and characterize molecular glues. It enables rapid, multiplexed screening with structural elucidation of unknown binders and is validated by cryo-EM, offering a powerful platform for MG discovery and optimization.

Flow-induced Dispersion Analysis – Mass Spectrometry (FIDA-MS) for Structural Characterization of Native Proteins from Conventional Molecular Biology Buffers

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Presenting Author: Edvaldo Maciel

Flow-induced dispersion analysis (FIDA) describes the size-dependent longitudinal dispersion of molecules under laminar flow conditions, based on the interplay between radial diffusion and axial convection. As the extent of this dispersion correlates inversely with the analyte's diffusion coefficient, larger molecules exhibit significant band broadening, while smaller molecules remain in a narrower band. This concept was recently applied for online buffer-exchange of intact proteins. We show that FIDA coupled with native MS not only allows protein desalting, but also provides insights into molecular size in solution and binding events. We used FIDA-MS to determine hydrodynamic radii (Rh) of native proteins and establish correlations with collision cross-section (CCS) values from ion mobility measurements, bridging the gap between solution- and gas-phase structural parameters.

FIDA-MS measurements were performed with a Synapt XS ion mobility–mass spectrometer (Waters), coupled either to a FIDA-1 instrument (Fida Biosystems ApS, DK), or with a Waters Acquity UPLC operated without a column. Samples were injected without desalting via an autosampler into either a 100 cm fused-silica capillary (FIDA-1 coupling) or into a 91 cm PEEK capillary (Acquity coupling), both running under isocratic conditions with aqueous ammonium acetate. Hydrodynamic radii (Rh) from peptides and proteins were calculated after Gaussian fitting of their extracted ion chromatograms (EICs). These values were compared with theoretical values based on PDB files as well as Rh values measured with the built-in fluorescence detector of the FIDA-1 system.

Flow-induced dispersion analysis coupled with native MS was successfully applied in the Rh determination of analytes over a significant molecular weight range (300 Da up to 133 kDa) with both setups described above. Rapid and effective desalting due to the band broadening effect described earlier was observed. Consequently, ion suppression only occurred in the central region of the elution window due to concentration of small molecules and salt clusters there, even when injecting samples in high-salt molecular biology buffers (e.g., 20 mM HEPES, 150 mM NaCl, 5% glycerol). The advantage of FIDA-MS over fluorescence-based detection is the mass-selective generation of many EICs from the same dataset, allowing for simultaneous Rh determination of multiple native proteins (including FKBP12, carbonic anhydrase, and enolase). Separately, the Rh of angiotensin II and glutathione were determined under the same conditions. The calculated Rh values from FIDA-MS showed a strong linear correlation ($R2 > 0.99$) to values obtained using the fluorescence detector of the FIDA-1 system. BSA provided a striking example of avoiding the ensemble averaging inherent to fluorescence-based detection, as separate EICs were generated for the monomer and dimer, which enabled Rh calculation of both oligomeric states in the same experiment. These values were validated against Rh control values from crystal structures. The stability of BSA monomers and dimers in solution has been demonstrated before, as they can be separated with size-exclusion chromatography. Therefore, native FIDA-MS provides insights about oligomers dynamics in solution: A similar Rh value for different oligomeric states can indicate a measurement artefact (e.g., dissociation during ESI) or rapid interconversion in solution, while distinct values indicate relatively slow interconversion. Moreover, the measured Rh values also showed a strong quadratic correlation ($R2 = 0.991$) with CCS values from ion mobility experiments, consistent with previous findings.

FIDA-MS enables multiplexed Rh determination of native proteins from standard buffers in less than 10 minutes and correlates well with orthogonal solution- and gas-phase measurements. This method provides insights on stability and dynamics of oligomeric states of proteins and is easily implementable with an unmodified LC-MS instrument.

Investigating the Effects of Cu/Zn Loss on Superoxide Dismutase 1 Structure using Ion Mobility and Electron Capture Dissociation

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Superoxide dismutase 1 (SOD1) is a homodimeric Cu,Zn metalloenzyme. Mutations in SOD1 cause familial amyotrophic lateral sclerosis (fALS), in part by disrupting its metal-binding properties. Loss of zinc from SOD1 generates a toxic proteoform that is harmful to motor neurons. Using native mass spectrometry, we previously demonstrated that a single metal form of SOD1 is enriched in fALS mouse models and found that therapeutically decreasing this pool results in an extended lifespan. Biophysical characterization of the two different metal proteoforms indicated significant structural differences. We hypothesized that ion mobility could resolve these structural differences and allow clear assignment and measurement of Cu- and Zn-bound SOD1. This would help clarify the role of zinc-deficient SOD1 in ALS pathology.

An Agilent 6560 ion mobility Q-TOF mass spectrometer was retrofitted with an Agilent ExD cell to enable ECD fragmentation. Recombinant human SOD1 and fALS mutant constructs were expressed, and standards of Cu-SOD1, Zn-SOD1, and Cu,Zn-SOD1 were prepared in-house. Samples were resuspended in 20 mM ammonium acetate at 100 µM and introduced via direct infusion using a syringe pump. Ionization was performed using an Agilent dual AJS ESI source under native conditions, with sheath gas at room temperature and reduced fragmentor voltage, gas temperature, and capillary voltage. Data were acquired using IM-MS. ECD was used to provide top-down characterization of the enzyme. Data analysis was performed using Agilent IM-MS Browser 10.0 and ExDViewer 4.6.

To investigate the effects of metal status on SOD1, we compared the monomer (7+) and dimer (11+) using IM to compare structural differences to compare single-metal to Cu,Zn-bound SOD1. We did not observe a significant differences between either single-metal SOD1 to the Cu,Zn-bound SOD1. However, ALS associated mutants, G93A and G37R, showed distinct structural changes with more conformers detected compared to controls. In both monomer (7+) and dimer (11+) states, the Cu,Zn-mutants appeared more compact and stable by showing fewer conformers than either metal-deficient form. Zn-only forms showed approximately double and Cu-only forms showed approximately triple the number of conformers as compared to Cu/Zn-bound SOD1. ECD fragmentation resolved metal-bound proteoforms and localized metal binding through detection of metal-retaining fragments. This top-down native approach provided structural and site-specific insights that IM alone could not. Overall, while metal loss increased structural heterogeneity, only the disease mutants introduced new conformers, supporting their role in structural disruption.

This study introduces a novel IM-MS and ECD approach to characterize SOD1 metalation. While IM-MS alone cannot resolve single-metal SOD1 proteoforms, ECD enables their distinction. In contrast, ALS-linked mutants show structural changes detectable by IM-MS, offering insights into how metal loss and mutation affect SOD1 structure.

Evaluating Stress-induced Stability Changes in Antibody Monomers and Dimers via Collision-induced Unfolding

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Presenting Author: Rowan Matney

Antibodies are a predominant class of biotherapeutics due to their high specificity, efficacy, and well-established manufacturing processes. However, biotherapeutic molecules are complex, requiring precise quality control over composition and higher order structure. Antibodies, like all proteins, experience changes in their stability and oligomerize when exposed to external stress. The presence of both oligomers and structurally-altered monomers in therapeutic formulations can reduce efficacy and trigger unwanted immunogenic responses. In this work, we characterize monomers and dimers exposed to stressors including heat, freeze-thaw cycling, and acidic/basic conditions, using native ion mobility-mass spectrometry (IM-MS) and collision-induced unfolding (CIU). We hypothesize that different forms of stress will result in stability changes of antibody monomers and dimers.

Immunoglobulin G1κ (IgG1κ) was purchased from Sigma Aldrich, aliquoted, and flash frozen. Prior to analysis, samples were thawed on ice and buffer exchanged into 100 mM ammonium acetate using Bio-Rad MicroBiospin 6 centrifugal columns. Stress was induced by incubating samples at 37°C for 4 days, 3 rounds of freeze-thaw cycling in liquid N2, or buffer exchange into 100mM ammonium acetate at pH 10 or pH 3, followed by 90 minute incubation. Samples were analyzed at a concentration of 1 mg/mL and ionized via gold-coated borosilicate emitters produced in-house. Native IM-MS measurements were conducted on a Waters SELECT SERIES Cyclic ion mobility-mass spectrometer. Data analysis was performed using Waters MassLynx, Driftscope, TwimExtract, and CIUSuite 3.

Native IM-MS can be used to characterize the structures of biological macromolecules in the gas phase. Natively structured protein ions undergo gas-phase unfolding upon activation by collision-induced dissociation (CID), which can be detected by the changes in ion mobility. This technique, collision-induced unfolding (CIU), has been previously used to successfully characterize antibodies. We used CIU to examine the relative stability of both monomer and dimer IgG1κ before and after exposure to stressors such as heat, freeze-thaw cycling, and changes in pH. CIU data shows that monomer IgG1κ is stabilized by exposure to both acidic and basic conditions but is relatively unchanged by both heat and freeze-thaw cycling. We used surface-induced dissociation (SID), an alternative gas-phase activation method which is ideal for studying protein complex stability. Our SID measurements show that dimer IgG1κ is stabilized at its interfaces by all forms of stress; preliminary results of the CIU of IgG1κ dimers show that some stress conditions, notably acidic conditions, lead to an increase in overall structural stability as well. However, the degree of this stabilization seems more significant than that shown by SID. We aim to use these shifts in stability as a characterization method for antibodies exposed to stress, so this work will ultimately employ supervised machine learning-based classification to differentiate biotherapeutic antibodies. Previous research has demonstrated machine learning classification models based on CIU (and surface-induced unfolding or SIU) to accurately distinguish between antibodies based on their subclass. This work will build on that tool with the goal of producing a clinically- and industrially-relevant tool for the quality control of antibodies.

Stress-induced IgG oligomerization is a well-documented concern. IgG monomer stability has previously been studied through collision-induced unfolding. This work additionally examines differential changes to monomer and dimer stability after exposure to stressors.

Ion/Ion Reactions in Native Mass Spectrometry: Methods and Instrumentation

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Presenting Author: Scott McLuckey

Since its inception, practitioners in the native mass spectrometry (MS) community have sought to extend the role of mass spectrometry in biophysics and structural biology. The ions generated under native conditions, however, pose technological challenges for mass measurement and structural characterization. For this reason, significant effort has been, and continues to be, placed on the development of novel technologies to facilitate native MS. In the spirit of expanding the native MS tool-box, we have been exploring the use of charge manipulation strategies via gas-phase ion/ion reactions to facilitate mass measurement and structural characterization. This work has involved both the development of instrumentation to enable ion/ion reactions and the high m/z ions and the application of various ion/ion chemistries and methods to ions generated under native conditions. This presentation will summarize modifications and performance of a commercial quadrupole/time-of-flight for ion/ion reactions and routine mass analysis extending beyond m/z 200,000 as well as the development of a tandem 2D/3D ion trap for high m/z operation using, inter alia, digital waveforms for mass analysis. Ion/ion reaction methods include ion parking, a tandem MS approach that facilitates the analysis of complex mixtures and heterogeneous analytes, single proton transfer reactions, and multiply-charged ion attachment reactions.

The Power and Pitfalls of AlphaFold for Structure Prediction Beyond Rigid Globular Proteins

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Presenting Author: Andrew McShan

Artificial intelligence driven advances in protein structure prediction in recent years have begged the question: has the protein structure prediction problem been solved? Here, with a focus on non-globular proteins, we highlight the many strengths and potential weaknesses of DeepMind's AlphaFold (AF) in the context of its biological and therapeutic applications.

We summarize the subtleties associated with evaluation of AF model quality and reliability using predicted local distance difference test (pLDDT) and predicted aligned error (PAE) values.

We highlight various classes of proteins and biomolecular complexes that AF can be applied to, and the caveats involved. Concrete examples of how AF models can be integrated with experimental data in the form of SAXS, solution NMR, cryo-EM, X-ray diffraction, and mass spectrometry are discussed. Finally, we highlight the need to move beyond structure prediction of rigid, static structural snapshots towards conformational ensembles and alternate biologically relevant states.

The overarching theme is that careful consideration is due when using AF-generated models to generate testable hypotheses and structural models, rather than treating predicted models as de facto ground truth structures.

G-quadruplex – Ligand Interaction Biophysics Explored by Native Mass Spectrometry and Trapped Ion Mobility Spectrometry

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Guanine-rich nucleic acid sequences are able to form G-quadruplexes (G4), both in vivo and in vitro. Due to their involvement in many important biological processes, such as gene expression and telomere maintenance, G-quadruplexes became a prominent target for anticancer therapy by small G4-binding molecules, or G4 ligands. Mass spectrometry combined with electrospray ionization source (ESI-MS) provides a great tool to relatively quickly asses the in-solution binding properties of G4 ligands, as was determined by previous studies. Coupling ESI-MS with the trapped ion mobility (TIMS) and the in-house made variable-temperature nano-electrospray source (vt-nESI) allows to obtain a detailed biophysical characterization of G4-ligand interactions.

The ESI-TIMS-MS measurements were performed on TIMS-TOF SCP upgraded with the 4th generation TIMS cartridge (XR TIMS; Bruker Daltonics, USA), a capacitance manometer (CERAVAC CTR 100), Leybold, Germany) and 2 pressure regulators (Speedivalve, Edwards Vacuum, UK). The pressures before and after TIMS cartridge, as well as TIMS voltages were optimized for better TIMS resolution, and declustering of nonspecific ammonium adducts while preserving the G-quadruplex specific ones. For standard ESI source, 2 µl/min injection flow rate was used; for nESI, metal-coated borosilicate capillaries were used (Thermo Fischer Scientific, USA). The vt-nESI device was described previously. The effective concentrations of the folded DNA structures and G4 ligands were determined by temperature-controlled UV spectroscopy (UV mc², SAFAS, Monaco).

Several novel G4 ligands (PyDH2, PhenDH2 and PhenDH9) were tested on 8 different DNA targets representing various G4 topologies: 1) (TG4T)4, parallel and tetramolecular; 2) (G4T4G4)2, antiparallel and bimolecular; 3) 23TAG, telomeric hybrid intramolecular; 4) 24TTG, telomeric hybrid intramolecular; 5) 5YEY, telomeric antiparallel intramolecular; 6) Pu24, c-MYC promoter parallel intramolecular; 7) (CGCGAATTCGCG)2, guanine-rich duplex control; 8) 24nonG4, guanine-rich single-strand control. As a ligand control, well-established PhenDC3 was screened on all DNA sequences as well. In native conditions (room temperature), all the tested compounds showed very high affinity to parallel folds (both intra and intermolecular), as well as telomeric variants (i.e., 23TTG, 24TTG and 5YEY). The remarkable affinity commands a very precise determination of DNA and ligand concentrations, and the ability to perform native MS from dilute solutions. For the telomeric hybrid topologies, the displacement of one of the two ammonium cations coordinated between 3 G4-tetrads, suggesting that the new ligands behave like previously studied PhenDC3. For 24nonG4 single strand control, unusual ammonium distributions were observed in the spectra for 1:1 and 1:2 (DNA : ligand) complexes; this allows to postulate the existence of ligand-induced two or even three G4-tetrad assemblies, as was observed for PhenDC3 earlier. All the analyzed systems were also characterized by TIMS, which enables the detection of changes in collision cross-sections of DNA systems upon the ligand binding. The melting experiments conducted by using the vt-nESI source allowed to monitor the temperature-dependent G4 unfolding, as well as to estimate thermodynamic parameters of ligand binding, including enthalpies and entropies associated with ligand binding and conformational changes associated with cation ejection. Future work will be devoted to the influence of ligands on protein binding to G-quadruplexes.

The similar type of experiments conducted in previous years were now performed using TIMS-TOF SCP upgraded with the latest XR cartridge. This allowed to add the high-resolution ion mobility dimension to the G4 ligand screening and therefore obtain structural data on G4-ligand complexes.

Capillary Zone Electrophoresis Based Native Proteomics of E. coli Cell Lysate

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Traditional denatured top-down proteomics provides valuable information on sequence identity and post-translational modifications but provides limited structural information about higher-order protein complexes. We demonstrate native proteomics, where we can characterize the quaternary structure of protein complexes from cell lysate. Native capillary zone electrophoresis (CZE) is a separation technique that preserves the structures of proteins and protein complexes while using nanoliter volumes of sample. Combined with native mass spectrometry, where the protein structure is preserved in the gas phase, it is possible to characterize protein complex structure directly from complex samples. We also implement gas-phase activation such as with surface induced dissociation (SID), which fragments protein complexes along their weakest interface, yielding information about the assembly of higher order protein complexes.

Proteins and protein complexes were extracted from E. coli cell lysate grown in rich media. Protein extracts were fractionated using size-exclusion chromatography prior to separation by CZE. CZE was performed on a CESI 8000 system (SCIEX) using LPA coated capillaries with an electrokinetically pumped sheath flow nanospray source (EMASS-II, CMP Scientific) then analyzed using an Q Exactive Ultra High Mass Range Orbitrap Mass spectrometer (Thermo) fitted with a custom-built SID device. Samples were additionally run on a timsTOF Pro mass spectrometer (Bruker) to leverage ion mobility separation. Traditional bottom-up proteomic analysis was later performed to create a library of proteins present in each fraction.

We have analyzed multiple E. coli lysate fractions generated by size exclusion chromatography using CZE-based native proteomics. We have collected masses of intact protein complexes using MS1, followed by activation by SID and higher energy collision induced dissociation (HCD). We were able to determine the monomer mass and stoichiometry using HCD and SID, while SID yields additional information regarding complex connectivity. We have determined the putative identities of more than 10 protein complexes including a 200 kDa Tryptophanase that appears as a tetramer under MS1 conditions and is revealed to be a dimer-of-dimer geometry after activation by SID. We have also collected bottom-up data to generate a library of proteins present in each fraction. Fractions were further analyzed using trapped ion mobility to give an additional dimension of separation. Ion mobility separation has allowed us to assign additional protein identities including an enzyme involved in glucose metabolism.

Capillary Zone Electrophoresis based native proteomics of fractionated cell lysate with surface induced dissociation to identify protein complexes and higher order structures.

Light Triggered Time-resolved Ion Mobility Mass Spectrometry Allows to Investigate Changes in Conformation and Oligomerisation in Cryptochromes

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Presenting Author: Nina Morgner

Cryptochromes (CRYs) are a compelling class of light-sensitive proteins that play an essential role in biological signal transduction. Their ability to undergo structural changes upon light stimulation enables diverse physiological processes, including circadian rhythm regulation, photomorphogenesis and flowering or developmental timing. This study explores the photoactivation dynamics of cryptochromes, with a particular focus on their oligomerization behavior and functional modulation. To enable time-resolved light-triggered measurements, we adapted both a Synapt G2S mass spectrometer and a home-built LILBID mass spectrometer, to allow continuous or short pulse illumination of samples using a high-power LED prior to analysis. With these set-ups we can assess mass and ion mobility spectra over time, allowing us to explore the CRYs response to blue light activation. Comparative analyses of CraCRY (Chlamydomonas reinhardtii) and PdL-CRY (Platynereis dumerilii) and AtCRY (Arabidopsis thaliana) reveal diverse evolutionary adaptations in cryptochrome functionality across species. Using time-resolved mass spectrometry (MS), ion mobility (IM) analysis, and UV/Vis spectroscopy, we characterized both conformational transitions and oligomerization state shifts under different light conditions. These findings provide insights into species-specific strategies for converting light energy into biochemical signals. Our CraCRY analysis allowed us to track light-induced conformational changes, enabling the determination of rate constants for activation and dark-state recovery. Comparative studies of CraCRY wild-type and mutants identified key molecular interactions, including a salt bridge, that regulate the repositioning of the C-terminal extension (CTE) upon blue light stimulation [1]. Other CRY species have the ability to oligomerise, as we show for PdLCry or AtCRY, for which we characterized the oligomerization regulation process, demonstrating species specific transitions into different oligomeric states depending on light intensity. Our investigation of AtCRY included the role of Blue light Inhibitors of Cryptochrome (BICs), elucidating their mechanism in modulating oligomerization. Overall, this research provides a deeper understanding of light-induced structural transitions in cryptochrome signal transduction, expanding knowledge on biological light-sensing mechanisms. [1] Rene Zangl; Sejla Soravia; Martin Saft; Jan Gerrit Löffler; Jonathan Schulte; Christian Joshua Rosner; Jens Bredenbeck; Lars-Oliver Essen; Nina Morgner; Time-resolved ion mobility mass spectrometry to solve conformational changes in a cryptochrome; Journal of the American Chemical Society (2024), 146, 21; https://doi.org/10.1021/jacs.3c13818

Advancing Health Disparities Research Through Protein Footprinting on PBMCs

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Presenting Author: Jalah Morris

Health disparities research requires a better understanding of protein structure and biology. Since structural biology in human patient samples is difficult to do, the Jones lab has a unique opportunity to greatly impact the advancement of health disparities research with the utilization of in-cell fast photochemical oxidation of proteins (IC-FPOP) coupled with mass spectrometry. This technique has allowed the identification of protein-protein interaction sites and regions of conformational change through hydroxyl radical modifications on solvent accessible sites. IC-FPOP has revolutionized the way we study in vivo systems by modifying thousands of proteins in a single experiment, enabling proteome-wide structural biology. IC-FPOP has been employed to study human patient samples through peripheral blood mononuclear cells (PBMCs).

PBMCs were cultured in fibronectin coated 24-well plates. For IC-FPOP, 500,000 PBMCs were plated per well and allowed to adhere for one day prior to experiments. For IC-FPOP, H2O2 was introduced to each sample after removal of media. Photolysis was achieved via a 248 nm KrF excimer; specifically, one laser pulse at 50 Hz and 26 kV was applied. Immediately after the pulse H2O2 was quenched. Cells underwent lysis, reduction, alkylation, and trypsin digestion. Control samples were not laser irradiated. Peptides were analyzed on the Orbitrap Astral Mass Spectrometer and searched against a Homo Sapien database using Proteome Discoverer 3.1.

PBMCs are a new cell type to our lab; therefore, optimizing cell culture, IC-FPOP, and LC-MS/MS conditions is important for our method development. Pilot studies showed PBMCs are best cultured in 24-well plates because it allows for best cell adherence and cell recovery. IC-FPOP experiments require adherent cells due to the removal of cell culture media prior to laser irradiation. Coating 24-well plates in fibronectin resulted in ~95% total cell adherence. The use of 24-well plates instead of previous work that utilized 6-well plates, results in less total cells necessary per technical replicate to coat an entire well. With lower total cell count, the optimal hydrogen peroxide concentration to achieve quantifiable modifications was explored. 100mM hydrogen peroxide resulted in the most total numbers of quantifiable IC-FPOP data. This research will require hundreds to thousands of patient samples to show biological significance of the IC-FPOP data. The previous setup, which was suitable for a 6-well plate, included a platform incubator with a movable XY stage (PIXY) which allowed the user to position the stage, add and remove reagents using peristaltic pumps, and mirror optics for laser beam guidance. The expansion of PIXY to labeling cells in 24-well plates would increase the throughput of IC-FPOP and provide the ability to study large patient sample cohorts. One key factor to this expansion is ensuring single-well irradiation. To irradiate an entire well in a 24-well plate in a single shot, multiple configurations that incorporated either an iris or cylindrical lens and different sized mirrors were evaluated to optimize the number of quantifiable data from a single 24-well plate experiment. IC-FPOP pilot studies on PBMCs from a healthy Black male and White male showed 642 and 183 quantifiable modified peptides respectively with 69 in common.

This research probes the current gap in health disparities by identifying structural differences in key proteins that cause disease from racially diverse patient samples. We demonstrate a high throughput method for IC-FPOP coupled with mass spectrometry on PBMCs that modified hundreds of proteins.

Integrating Cyclic Ion Mobility Separations, Tandem Mass Spectrometry, and Collision Cross Section Measurements for Carbohydrate Sequencing

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Carbohydrates, and carbohydrate-containing molecules, are biologically important, but notoriously difficult to characterize largely owing to their high degrees of isomeric heterogeneity. Thus, new analytical methodologies are needed to improve the confidence of their characterization and work toward de novo sequencing. Ion mobility spectrometry-mass spectrometry has emerged as an orthogonal and complementary analytical technique to liquid chromatography-tandem mass spectrometry in omics-based analyses but has had limited application toward carbohydrate-based molecules. Here, the use of cyclic ion mobility spectrometry-mass spectrometry (cIMS-MS) in conjunction with multiple tandem mass spectrometry-based strategies and collision cross section measurements is presented as a new method toward the de novo sequencing of carbohydrate-based molecules, such as human milk oligosaccharides and glycolipids.

Photo-crosslinking for Identifying Residue-level Interactions Involving Proteins with Intrinsic Disorder

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Presenting Author: Lucas Narisawa

Intrinsically disordered regions are prevalent throughout the human proteome but are untenable to many structural biology techniques due to their many transient or plastic interactions. Small heat shock proteins (sHSPs) are holdases for disease-associated aggregation-prone proteins and contain a disordered N-terminal region (NTR) key for their protein-protein interactions. To probe the NTR interactions of sHSPs, we integrate a photoreactive crosslinking amino acid, benzoyl-L-phenylalanine (BPA) into specific NTR sites of sHSPs. Through crosslinking and tandem mass spectrometry with a subsequent bioinformatic pipeline, we have identified ensembles of interactions between sHSPs and aggregation-prone tubulin proteins. We propose that our identifications will be useful in a wide range of protein systems containing less-ordered regions or transient interactions.

sHSP variants that contain non-canonical amino acid BPA are expressed recombinantly in E. coli. Photoinduced crosslinking reactions are performed in 96 well plates with purified BPA-containing variants, combined with aliquots of purified target proteins. Following crosslinking, SDS-PAGE is performed on the mixture, and bands associated with crosslinked product are excised, destained, reduced, alkylated and digested in-gel via trypsinolysis or dual-enzyme trypsin-GluC digestion overnight at 37 °C. Solutions are then cleaned for nanoLC-MS analysis via C18 spin columns. Analysis is performed on an Orbitrap Fusion Lumos Tribrid using data-dependent acquisition. Raw data is fed into search and validation tools in the Trans-Proteomic Pipeline (TPP) and our open-source, Python code that is described elsewhere (DOI: 10.1021/acs.jproteome.4c00194).

Results include crosslink sites from sites 9 and 61 in the NTR of HSPB5 across the sequence of tubulin proteins at a 1% FDR. All identified crosslink sites are novel to our knowledge. Various crosslinking sites are to the interior of current structural models of soluble tubulin dimers (PDB 1TUB, 3RYF). These residues must become solvent exposed for BPA to crosslink, indicating that either through structural heterogeneity or misfolding, which occurs readily in vitro, these residues become exposed for HSPB5 interaction. Additional crosslinking sites agree more with expectations based on crystal structures; the distal residue 9 on HSPB5 interacts with the dimer interface in the αβ-tubulin heterodimer. Dimer to monomer dissociation is a known occurrence, though for microtubule assembly competency, dimers are needed. Interactions in the dimer interface occur in close spatial proximity to both subunits, but are separated in sequence, elucidating that the distal sub-region of the NTR accesses a small solvent accessible pocket in the dimer interface, though the purpose of this interaction is unclear. Given the role of HSPB5 as a holdase, these interactions may exist either to stabilize the soluble dimer, or as recognition sites to facilitate binding of the alpha-crystallin domain of HSPB5 oligomers, which has a more pronounced impact in preventing tubulin aggregation.

Novel residue-level interactions originating from disordered regions of HSPB5 to Tubulin isoforms.

Investigation into the Dynamic Structure of Heat Shock Proteins using Electrospray Ion Beam Deposition and Cryo-electron Microscopy

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Presenting Author: Noor Naseeb

Small heat shock proteins (sHSP) are an intriguing family of polydisperse proteins found across all kingdoms of life. Typically ranging between around 10-40kDa in size, these monomers commonly assemble into oligomeric, cage-like structures that act as ATP-independent chaperones to prevent protein aggregation or misfolding during instances of cellular stress such as a change in temperature, metal toxicity, or oxidative stress. sHSP have been linked to multiple different neurological diseases, like Alzheimer's, Parkinson's and Huntington's disease, emphasizing the importance of studying them. However, sHSP dynamic nature in solution, often shifting between varying oligomeric states, makes structure determination particularly challenging.

Conventional approaches like X-ray crystallography (XRC), nuclear magnetic resonance (NMR), and cryogenic electron microscopy (cryo-EM) struggle to study dynamic, heterogeneous proteins. New approaches such as electrospray ion-beam deposition (ESIBD), which directly couples native mass spectrometry (MS) with cryo-EM, allow direct visualization of chemically selected protein complexes in near-native states at high resolution. In ESIBD, protein assemblies are transferred into the gas phase via native electrospray ionization, mass-filtered to isolate the molecule of interest from aggregates and fragments, and deposited onto cryogenically cooled TEM grids. The grids are then coated with amorphous ice and transferred from the vacuum deposition chamber into liquid nitrogen for imaging and processing in the cryo-EM via single-particle analysis (SPA).

HSP16.5 was chosen to be the first system of study as it is one of two previously determined oligomeric structures of any HSP. The protein is a monodisperse chaperone that oligomerizes into a 24-mer with octahedral symmetry, forming a complex with a mass of 395 kDa. The protein was then sprayed via ESI and mass filtered between 7500-9000 m/z to produce a chemically selective ion beam of HSP16.5 24-mer. The mass-selected ion-beam was then deposited onto a cryogenically cooled, carbon-coated TEM grid and was imaged in the cryo-EM. 2D classifications of multiple different orientations of the protein revealed detailed secondary structure features. Ab-initio's created, generated with and without symmetry constraints, demonstrated that the oligomeric structure resembled both the crystallographic PDB structure of HSP16.5 lacking the N-terminal region (NTR) and the AlphaFold-predicted structure including the NTR. Although the resolution was insufficient to fully resolve specific secondary structures or the NTR, future efforts with improved sample preparation are expected to enhance resolution.

HSP16.5 can be isolated by native mass spectrometry and deposited intact onto cryo-EM grids while preserving its oligomeric structure. This provides an important proof-of-concept for applying ESIBD-cryoEM to more dynamic and heterogeneous small heat shock proteins, enabling structural studies of challenging assemblies that are difficult to capture by conventional methods.

Super Resolution Mass Spectrometry Imaging, Multimodal Integration, & Metabolic Barcoding

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Presenting Author: Efe Ozturk

Mass spectrometry imaging (MSI) is a cornerstone of spatial metabolomics but suffers from limited spatial resolution, constraining its application to single-cell biology. To address this limitation, we introduce a Guided Super-Resolution (GSR) framework that integrates high-resolution structural and molecular imaging modalities—including hematoxylin and eosin (H&E) staining, immunofluorescence (IF), and Imaging Mass Cytometry (IMC)—to reconstruct high-resolution MSI data. GSR bridges the resolution gap, enabling biochemical mapping at cellular and subcellular levels. This enhancement unlocks new possibilities in tissue phenotyping, disease microenvironment profiling, and spatially resolved metabolomics. We further introduce a graph-based metabolic barcoding framework to assign interpretable metabolite signatures to individual cells, revealing phenotype-specific biochemistry and microenvironmental structure within complex tissues such as Alzheimer's mouse brain and human lymphoma.

We developed a dual-encoder neural network architecture that fuses low-resolution MSI and high-resolution guidance images to generate super-resolved outputs. Multimodal datasets—including MALDI-IHC with IF/H&E (mouse brain), MALDI with IMC (human lymphoma), and MALDI-DESI with H&E (mouse brain)—were aligned using rigid and non-rigid transformations. MSI channels were normalized, and patches from overlapping image regions were extracted to train the GSR model. For metabolic barcoding, we constructed spatial graphs of single cells using IMC-based segmentations, assigned node features using super-resolved MSI intensities, and trained a Graph Convolutional Network (GCN) to classify cell phenotypes. Integrated Gradients were used to derive metabolite-level feature attributions, creating interpretable metabolic barcodes for cell types and spatial neighborhoods.

We evaluated our GSR framework across three multimodal datasets to demonstrate its generalizability and biological utility. First, we applied GSR to a tri-modal Alzheimer's disease mouse brain dataset combining dual-labeled antibody fluorescence, MALDI-IHC MSI (50 μm), and post-MALDI H&E histology. Fluorescence microscopy provided high-resolution phenotypic and structural guidance for enhancing the MALDI images. GSR outputs showed improved cellular boundary definition and spatial continuity, especially around amyloid-β plaques. Single-cell segmentation based on fluorescence revealed distinct plaque-associated microenvironments, including enrichment of microglia and astrocytes in peri-plaque zones. Metabolic barcodes derived from super-resolved MSI channels highlighted localized glial activation and lipid remodeling, consistent with known Alzheimer's pathology. Second, we used a publicly available human colorectal cancer dataset featuring co-registered IMC and MALDI-MSI on the same FFPE sections. IMC provided high-resolution phenotypic annotations, which guided GSR reconstruction of low-resolution MSI. The GSR-enhanced outputs showed improved delineation of tumor–immune boundaries. Using the IMC segmentations, we constructed spatial graphs where each node represented a cell and edges captured spatial proximity. Node features included super-resolved metabolite intensities. A Graph Convolutional Network (GCN) accurately classified cell phenotypes, and Integrated Gradients revealed key metabolites predictive of cancer, immune, and stromal cell types. These formed the basis of cell-type-specific metabolic barcodes. Third, we applied GSR to a dual-modality MSI mouse brain dataset, combining MALDI and DESI imaging on spatially matched sections with high-resolution H&E histology. Using H&E as structural guidance, our model effectively enhanced both MALDI and DESI images, preserving anatomical boundaries and improving spatial coherence across modalities. These results demonstrate the robustness and cross-platform generalizability of our GSR framework, highlighting its utility for super-resolution metabolomics in both lipid-rich and small-molecule-focused MSI platforms.

We present the first integrated framework combining guided super-resolution of MSI with graph-based single-cell metabolic barcoding. This enables cellular-resolution metabolic profiling in diverse tissues using multimodal imaging, revealing spatially organized biochemical phenotypes in Alzheimer's disease and human cancer.

Advances in Hardware Design and Function of the New timsOmni MS Platform

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Presenting Author: Dimitrios Papanastasiou

Innovations in mass spectrometry (MS) instrumentation continue to emerge, driven primarily by the need to identify and characterize proteins and other classes of compounds 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. This unique functionality is significantly augmented by the integration of trapped ion mobility spectrometry.

A variety of ion dissociation schemes can be accessed through a software interface designed for ease of use, while customization of the different MSn modes supports the objectives of various experiments. Electron-based fragmentation, photodissociation and collision induced dissociation methods are applied interchangeably to characterize proteins, oligonucleotides and small molecules. Protein samples are sprayed under native and denaturing conditions using an offline electrospray ionization (ESI) source for native MS and a low-microflow ESI source for online measurements. Top-down and middle-down data are processed in OmniScapeTM configured with an improved de novo sequencing algorithm for proteoform identification. A new DDA method supported by an ultra-fast charge state deconvolution algorithm for top down proteomics is evaluated.

A series of hardware developments are reported including (a) a linear stacked-ring RF ion guide providing efficient desolvation at ~5 mbar, upstream of the trapped ion mobility analyzer (tims), (b) an ion mobility gate for selecting ions separated in the tims device at ~2 mbar, (c) a new design of segment Q5 in the OmnitrapTM platform 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 orthogonal acceleration region of a time-of-flight (TOF) mass analyzer. The range of m/z ratios recorded in single TOF spectra is shown to extend from 10,000 Th, while the mass resolving power is enhanced using a new peak reconstruction algorithm. In addition, an offline ESI source is developed and applied for analysis of intact proteins and protein complexes. Deep sequencing of non-reduced 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 optimized 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 on digested antibody mixtures to optimize instrument performance for top-down proteomics. Dynamic control of the accumulation period of selected charge states is shown to improve sequence coverage for all the protein systems and subunits examined. A new DDA method combining electron-based fragmentation and tims separation is currently being developed and its utility for the analysis of glycopeptides will be demonstrated experimentally.

A versatile MS platform for multidimensional tandem mass spectrometry with ion mobility separation.

The Functional Dynamics of Membrane Proteins in Physiological Conditions using HDX-MS

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Here, we develop the hydrogen deuterium exchange-mass spectrometry (HDX-MS) technology to interrogate the molecular mechanisms underpinning membrane protein function. We showcase applicability of the approach to the study of transmembrane transporters and G-protein coupled receptors, important for human health and disease.

Structure and Solution Dynamics of Yeast RAD52 Homolog Mgm101: Snapshots with DNA Reveal a Full Single-strand Annealing Pathway

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Presenting Author: Zihao Qi

Single-strand annealing (SSA) repairs DNA breaks by annealing complementary ssDNA at sites with direct repeats. Human RAD52 (RAD52) is an anti-cancer target, and understanding RAD52-mediated SSA mechanisms can ultimately facilitate cancer treatment. Prokaryotic annealases form stable helical nucleoprotein filaments on paired DNA that grow linearly with DNA length, with the two DNA strands bound in the same groove on a single oligomer (cis). Here, to unravel SSA mechanisms, we investigated the solution assembly and structures in different DNA-bound states of an RAD52-family annealase from yeast mitochondria, Mgm101. Although a competing theory for RAD52 proposes that two ring-ssDNA complexes stack to catalyze SSA (trans), our data collectively support the cis mechanism for eukaryotic annealases, as seen for prokaryotes.

S. cerevisiae Mgm101 was expressed and purified as an MBP fusion by amylose-affinity, cleavage with Precision protease, followed by anion exchange and heparin chromatography. HPLC-purified ssDNAs were purchased from Integrated DNA Technologies (IDT), Inc. Mgm101 and ssDNAs were buffer exchanged into 200 mM ammonium acetate. Native MS experiments were carried out on a Thermo Scientific Q Exactive ultra-high mass range (UHMR) Orbitrap Mass Spectrometer modified with a 4-cm surface induced dissociation (SID) device. 20% triethylammonium acetate by volume was added for SID experiments. Mass photometry (MP) measurements were conducted on a Refeyn TwoMP. Cryo-EM images were collected on a Thermo Krios G3i Cryo-TEM with a 300 kV x-FEG source. 2D class averages and 3D reconstruction were performed through CryoSPARC (v4.6.2).

Mgm101 forms monomer, 18-mer, and predominantly 19-mer in solution, as experimentally measured by nMS and MP. As concentration decreases, the abundance of monomer increases to become dominant. SID for Mgm101 19-mer indicates a ring-shaped topology that matches the 2D class averages from cryo-EM single particle analysis (SPA). Native MS analysis of Mgm101 bound to 75-mer and 83-mer ssDNA and duplex intermediate shows that 18 and 19 subunits are associated with a respective ssDNA or duplex intermediate, suggesting that a stable complex is formed during annealing for Mgm101. For a longer ssDNA, poly-dT150, 38 Mgm101 subunits bound to one ssDNA were observed, suggesting that long ssDNA promotes ring-stacking formation. SID fragmentation of the 19-mer bound poly-dT44 shows a high abundance of 11-mer bound to poly-dT44 fragments, and SID fragmentation of the 19-mer bound to two poly-dT44 splits the 19-mer, with 9-10 Mgm101 bound to one poly-dT44 being the dominant species, suggesting a four-nucleotide per monomer binding stoichiometry. High-resolution (< 3Å) 3D structures of Mgm101 bound to ssDNA and duplex intermediate were obtained by cryo-EM SPA. The ssDNA-bound structure shows an extended ssDNA where the basic residues at the bottom of the groove interact with the phosphate backbone of the ssDNA, and the bases are pointing outward and poised for homology recognition. Each protomer binds to four nucleotides, as seen for RAD52. In the duplex intermediate-bound structure, the outer strand is held in the complex almost exclusively via normal Watson-Crick base pairs with the inner strand. These structures align well with the nMS and nMS-SID results, suggesting that they reflect the active species in solution. The structures also reveal a novel positively charged hairpin not present in RAD52 that could bind to pre-formed double-stranded DNA (dsDNA). Preliminary results from nMS and fluorescence polarization suggest that Mgm101 can bind pre-formed dsDNA weakly with an oligomeric assembly of 18 and 19, as for ssDNA.

•Native MS of a novel SSA protein alone and in complex with ssDNA, duplex intermediate, and pre-formed dsDNA reveals 18 and 19-mer in all states. •SID to probe the DNA localization. •Cryo-EM captures the structures of Mgm101 in complex with ssDNA and the duplex intermediate of annealing.

Atomic Resolution Molecular Imaging by Scanning Probe and Electron Microscopy Based on Soft-landing Electrospray Ion Beam Deposition

Stephan Rauschenbach

Presenting Author: Stephan Rauschenbach

Electrospray ion beam deposition (ESIBD), the deposition of intact molecular ions created by electrospray ionisation onto solid surfaces in vacuum after mass-selection, has been introduced in our lab as a tool for the handling of large and complex, usually non-volatile molecules.(1) Initially, the high-resolution single-molecule imaging by scanning probe microscopy (SPM) has been the major application for samples fabricated by ESIBD. It proved successful in the in–ves–tigation of structure, conformation, and properties of proteins, peptides, sac–charides, and synthetic mo–le–cules.(2,3) Here we present out recent efforts expanding the scope of ESIBD towards the investigation of protein structure using cryoEM by using native electrospray ionisation.(4,5)

For ESIBD we are using a modified Oribitrap UHMR instrument. Larger apertures and more pumping speed in the source region allow for high intensity ion currents. Beyond the HCD cell a two vacuum stages containing ion optics were added to transfer the ion beam to a deposition stage in ultrahigh vacuum. Here the molecular beams can be deposited onto a sample of thin freestanding carbon film whereby ion current (i.e. coverage), deposition energy (soft landing) as well as temperature (down to 40K). In addition, we can grow thin ice films from the gas phase to embed the proteins after deposition. After that the samples are transferred into liquid nitrogen and further to a cryoEM for imaging.

We optimised conditions for native deposition to promote imaging of individual proteins at a res-o-lu-tion sufficient for the con-struc-tion of atomic models from cryoEM data.(5) Several parameters are essential to achieve this: Beyond gentle deposition, the native proteins need to be transmitted gently in the mass spectrometer. Any activation, wanted or unwanted will lead to a loss in resolution when imaging. After depostion, the proteins need to be embedded in a thin layer of vitreous ice. Only after that atomic resolution details appear in the 2D classes. The structures ob--tained from cryoEM after embedding the landed proteins in ice grown from the gas phase shows a fold and subunit arrangement which is remarkably similar to the solution structure. Generally, the resolution of the cryoEM 3D reconstruction is lower in regions of the protein with more solvent exposure. Depending on the mechanical properties of the protein we observe conformational changes, typically a small compression or a shifting of a domain or subunit. For beta-Galactosidase for instance we find the closing of cavities and crevices' due to self-interaction in absence of water, a change readily reversed in MD simulations to find the native solution structure. GroEL retains it's cavity whereas it is compressed along the axis. Compact proteins like RuBisCo do not show any structural change at all. For the imaging of membrane proteins, in addition to the embedding in ice, the encapsulation of the protein in surfactants is needed for the imaging at high resolution. **References:** (1) Rauschenbach, **Annu Rev Anal Chem** 9 (2016) 16.1-16.26. (2) Wu, **Nature** 582 (2020) 375–378. (3) Anggara, **Science** 382 (2023) 219-223. (4) Esser, **PNAS Nexus** 1 (2022) pgac153. (5) Esser, **Science Advances** 10 (2024) eadl4628.

We achieved the imaging of proteins at atomic resolution by cryoEM after the deposition from molecular ion beams from native ESI. Structural changes occur due to dehydration. We minimise structural changes by fully controlling the ion beam and deposition.

Characterization of Protein Structure using Cleavable Crosslinking and Top-down LC-MS/MS

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Presenting Author: Erika Renbarger

Crosslinking enables protein structure analysis by providing information about structure, dynamics, and protein-protein interactions. The field of crosslinking was expanded by the development of cleavable crosslinking, which simplifies MS/MS spectra from crosslinked peptides. Since crosslinking reduces sequence coverage fron top-down proteomics, we examined cleavable crosslinkers for top-down approaches. Combining cleavable crosslinking with top-down enables increased sequence coverage for more confident fragment identification. This allows correct identification of crosslinks and proteoforms, and the relationship between proteoforms and higher order structure.

Transthyretin (TTR) from human plasma was crosslinked using disuccinimidyl dibutryic urea (DSBU) in 5 molar excess with 1X PBS (pH 7). This reaction was quenched by removal of excess reagent via buffer exchange with spin desalting columns. Samples were introduced via a Waters ACQUITY UPLC M-Class LC with a 5 cm C4 column and short gradient to a Thermo Scientific Orbitrap Fusion MS. The proteins were kept intact and sprayed under native conditions. ETD was used to fragment the protein backbone, and supplemental HCD was used to cleave the crosslinker and increase ETD sequence coverage.

Native MS analysis of the crosslinked proteins revealed the addition of up to 2 crosslinkers. The b, c, y, and z fragment ions from EThcD were analyzed and compared to assign the location(s) of the crosslinker within each protein. For TTR, the addition of collision energy not only cleaved the crosslinker but dissociated the tetrameric protein into monomers/trimers and dimers. The asymmetric cleavage of the crosslinker resulted in three new peaks that were able to be identified, one for each half of the crosslinker following cleavage and one for the intact crosslinker. The cleavage of the crosslinker allowed for enhanced sequence coverage of the protein. Reversed-phase LC of the intact reaction mixture separated non-crosslinked and crosslinked proteins, resulting in an enhancement of signal-to-noise for the products of interest. Utilizing ETD in combination with supplemental HCD allowed for the cleavage of the crosslinker to occur. Combining the results from the fragmentation of the intact crosslinker and the cleaved crosslinker allowed for further confirmation of the crosslinking sites and the comparison of the sites between two proteoforms of transthyretin. The two naturally occurring proteoforms compared of transthyretin, one cysteinylated and one with a point mutation at residue 10 from cysteine to glycine, showed unique fragmentation patterns with both the intact and cleaved crosslinker.

Application of cleavable crosslinking in a top-down workflow enables the characterization of the relationship between proteoforms and higher order structure.

Quantitation of Heterogenous Protein Samples Utilizing Orbitrap Charge Detection Mass Spectrometry

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Presenting Author: Robert Rider

Charge detection mass spectrometry (CDMS) concurrently measures the m/z and z of ions, allowing for a "direct" measurement of mass. Orbitrap based CDMS (O-CDMS) has increased CDMS utility, yet almost all samples are analyzed qualitatively. Transthyretin (TTR) is an amyloidogenic 56kDa thyroxine transport protein-complex that also happens to undergo subunit exchange (SUE) to form hybrid tetramers (i.e. tetramers containing wild type and mutant subunits). Analyzing TTR SUE and ligand binding reactions can be difficult because many signals have the same m/z values. Here we demonstrate that O-CDMS can be utilized to quantitatively monitor SUE kinetics and thyroxine binding of wild type (WT) and C-terminal (CT) TTR, providing insight into how protein-tags alter TTR stabilities and dynamics

WT and CT TTR homotetramers were expressed as described previously (Shirzadeh, Anal Chem., 2019), and buffer exchanged into 200mM ammonium acetate using 6kDa Bio-Spin columns. SUE samples contained equimolar amounts of WT and CT-TTR and were left in 4oC and monitored over 100 hours. Ligand binding experiments contained equimolar amounts of WT and CT-TTR, and 4:1 thyroxine to protein. All solutions were loaded into custom nanospray capillaries and electrosprayed using a nano-ESI source on a UHMR (ThermoFisher- San Jose, CA). Data processing of O-CDMS data was done using STORIboard. O-CDMS data was collected in triplicate and quantitation was done using peak fitting in OriginLab Software.

TTR SUE is often used to probe tetramer stabilities and gain insight into TTR disassembly, which is related to TTR amyloidosis. In this work we examine WT-TTR and CT-TTR homotetramers to gain information on the relative tetramer stabilities and the effect(s) the CT-tag induces on TTR using O-CDMS. Native mass spectra reveal the homotetramers overlap extensively at each charge state and cannot be confidently resolved, yet the added charge dimension provided by O-CDMS allows for accurate mass and abundance value assignment to each proteoform. After 6 hours of incubation, the hybrid-tetramer (containing WT and CT sub-units) are more abundant and the homotetramers decrease in abundance. Kinetic analysis of the homotetramer dissociation rates indicate the WT-TTR tetramer is ~3 times stable, providing evidence the CT tag is deleterious to the tetramers solution stability. The transport of thyroxine (T4) is pertinent to TTR's function and increases the stability of the protein complex. Mixing both homotetramers in solution with T4 present, we observe ligand bound states but cannot accurately distinguish the homotetramers in the native mass spectra because they overlap in m/z. Again, O-CDMS is able to confidently resolve each homotetramer and produce a subsequent mass plot that shows both homotetramers with 0,1 and 2 T4 bound states present. Quantitation of the ligand binding shows high reproducibility for ligand binding abundance to each homotetramer with statistical significance between each adjacent ligand binding event. Additionally, we observe that the abundance of T4 bound signals is greater for CT-TTR relative to WT-TTR , indicating that tag may be facilitating T4 binding. Both experiments align with previous literature that suggest the N- and C-termini loop regions can modulate TTR ligand affinity, specificity and tetramer stability. The data presented provides strong evidence that O-CDMS can produce meaningful quantitative data of heterogenous protein samples including proteoforms and ligands.

Orbitrap-based charge detection mass spectrometry (O-CDMS) enables quantitative analysis of protein complex dynamics, such as transthyretin subunit exchange and ligand binding, by resolving overlapping proteoforms and providing insights into protein stability and ligand affinity. These experiments are some of the first to complete O-CDMS quantitation.

Ion Mobility-mass Spectrometry and Collision-induced Unfolding Distinguishes Between Tryptophan and Methionine Oxidation and Reveals its Impact on mAbs' Structure and Stability

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Monoclonal antibodies (mAbs) are attractive therapeutic modalities due to their favorable properties. However, mAbs are complex molecules, susceptible to various chemical degradation pathways. Among these, oxidation can lead to alterations in mAb structure and stability. Such changes are commonly monitored by thermal unfolding in solution, but such techniques are time-consuming, sample intensive, an intolerant of mixtures. Ion mobility-mass spectrometry (IM-MS) are collision induced unfolding (CIU), are data-rich techniques that can be used to rapidly evaluate differences in mAb higher-order structures (HOSs). Here, we explored the ability of IM-MS and CIU to distinguish HOS changes in tryptophan and methionine oxidized mabs. Our preliminary data reveals that CIU can reliably and rapidly differentiate samples having undergone either methionine or tryptophan oxidation.

Data was collected on a Synapt G2 Q-IM-ToF-MS instrument (Waters). Humanized IgG1k Human IgG4k, 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), tert-Butyl hydroperoxide (tBHP) and hydrogen peroxide were purchased from Sigma Aldrich. Tryptophan oxidation was achieved by incubating mab with 5mM AAPH, 5mg/mL L-methionine at 37 °C for 3 days. Methionine oxidation was achieved by incubating mab with 0.25 % (v/v) tBHP or H2O2, 5mg/mL L-tryptophan at room temperature for 1-30 h. Samples were buffer exchanged into 200mM ammonium acetate and diluted to 1mg/mL for MS analysis. In CIU experiments, acceleration potentials were increased from 5 V to 200 V in intervals of 5 V. Location of oxidations were identified by peptide mapping analysis on a LC- Orbitrap Fusion Lumos MS system (Thermo Scientific).

We observe 3 CIU features in the average fingerprint (24+) for both the native and oxidized mabs. A quantitative comparison reveals that, for IgG1 and IgG4, methionine oxidation with tBHP resulted in an 8.4 % and a 4.5% decrease CIU50-1, while methionine oxidation with H2O2 resulted in a 5.2% and a 4.2% decrease in CIU50-1 respectively. Interestingly, tryptophan oxidation resulted in a 3% (IgG4) and 14% (IgG1) increase in CIU50-1. This increase in gas-phase stability is enhanced in the dimer population, where a 7-9% (IgG4) and a 17% (IgG1) increase in CIU50-1 was observed. Although the first transition of AAPH oxidized dimer IgG1 resulted in greater magnitude of difference than IgG4, the average fingerprint (33+) of IgG4 for native dimer contains 4 features, while the AAPH-oxidized IgG4 contains 3 features. A comparison of native-like collision cross sections (CCS) reveals no significant differences when comparing monomers (24-26+) of tryptophan and methionine oxidation to control mAb. When comparing dimers (33-37+), a 2-3% increase in size is observed for both tryptophan and methionine oxidation. This difference in dimer CCS suggests a topological shift mediated by chemical oxidation. A root-mean-squared deviation (RMSD) analysis of dimer CIU fingerprints resulted in a 17-18% difference between native and methionine-oxidized mAb, while the replicate RMSD analyses resulted in significantly less variation (<7%). Similarly, a RMSD comparison between native and tryptophan-oxidized mAb resulted in 13-17% difference, greater than the background RMSD obtained for technical replicates (<5%). Taken together, our data reveals that CIU is able to detect stability changes produced by oxidative modifications. Additionally, we can differentiate between tryptophan and methionine oxidation in mabs, given their distinct gas-phase unfolding from feature 1 to feature 2. Here, we will report on our methods to detect changes in the populations of antibodies as a function of small chemical modifications.

First report demonstrating that CIU can distinguish different oxidized mAb isoforms by their distinct gas-phase unfolding pathways.

Structural Characterization of Proteins Involved in Neurodegenerative Disease Using Ion Mobility and Electron Capture Dissociation

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Presenting Author: Blaine Roberts

Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) are heterogeneous neurodegenerative disorders marked by pathological protein aggregates and diverse molecular etiologies. While hallmark lesions such as amyloid plaques and Lewy bodies are well characterized, the underlying biochemical modifications driving protein dysfunction remain less defined. Using advanced mass spectrometry platforms including ion mobility (IM), electron capture dissociation (ECD), and ultraviolet photodissociation (UVPD) we identified post-translational modifications (PTMs) that contribute to disease mechanisms in AD, PD and ALS. These structural MS approaches are particularly well suited for resolving subtle and labile modifications, enabling site-specific detection of isomerization, metal binding, and oxidative cross-links. We discovered that isoaspartic acid-modified Aβ (isoD-Aβ) is the predominant form present in AD brain, distinguishable from other neurodegenerative conditions via chromatography, IM, and ECD fragmentation. In ALS models, ECD enabled top-down direct determination of the metal-binding state and isomerization of aspartic acid of superoxide dismutase 1 (SOD1). These findings demonstrate that isomerization, and metal dyshomeostasis are dominant features in proteins involved in neurodegeneration. The discovery and characterization of these PTMs have broad implications for biomarker and therapeutic development in age-related neurodegenerative diseases

Probing Tau Conformations and Early Aggregates using Native Mass Spectrometry

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Presenting Author: Isabella Rossetti

Aggregation of Tau protein in the brain is heavily correlated with neurodegenerative disease, but the molecular mechanism by which Tau begins to aggregate remains elusive. Traditional methods of structural characterization of globular proteins tend to fall short in providing structural information about intrinsically disordered proteins (IDPs) because of their dynamic nature. Native electrospray ionization mass spectrometry (nESI-MS), however, has proven to be a promising approach for probing the conformational heterogeneity of intrinsically disordered proteins such as Tau. A goal of our work is to obtain “coarse” information about the conformational profile of Tau under native conditions and observe changes in this profile as the protein begins to aggregate.

Our experiments utilized the Repeat Domain of Tau (Tau-RD), as it contains the important region for microtubule binding, and various core constructs of Tau-RD that are relevant to specific neurodegenerative diseases (e.g. chronic traumatic encephalopathy, Pick’s disease, Alzheimer's disease, and corticobasal degeneration). Tau constructs were expressed in BL21 E. coli cells. Each core construct was analyzed using nESI-MS on a Waters Synapt G2 to reveal the conformational heterogeneity of the proteins under native conditions. Aggregation of Tau-RD and core constructs was done using an established protocol, and the samples were analyzed by nESI-MS with complementary analyses done via Thioflavin T fluorescence assays.

Three distinct charge-state distributions are observed during nESI-MS of Tau-RD, representing three distinct conformations of the protein under native conditions. The highest charge-state distribution suggests that Tau-RD can take on a fully extended conformation, the lowest charge-state distribution indicates a more compact structure, and a moderate charge-state distribution suggests a partially extended conformation. It is possible that the low charge-state distribution corresponds to some Tau-RD molecules with an intact disulfide bond due to its two cysteines, however, reacting the protein with iodoacetamide to alkylate free thiol groups prior to nESI-MS indicates that all three conformations have both disulfide bonded and non-disulfide bonded structures. Moreover, reduction of the disulfide bonds with tris(2-carboxyethyl) phosphine hydrochloride (TCEP), still yields all three conformations during nESI-MS analyses. The results confirm that the conformations can exist in both disulfide bonded and non-disulfide bonded states. Further analysis with ion mobility mass spectrometry (IM-MS) confirms the existence of subpopulations within the distributions. Native MS analysis of other Tau-RD constructs in which specific repeat domains are removed can result in a different number of conformations. For example, the removal of the R2 domain results in two charge state distributions, indicating that specific domains are essential to the observed conformational profile. Additional experiments have also involved the mutation of disease-relevant residues, resulting in some cases in a fourth charge state distribution, suggesting another extended conformation can be formed. These preliminary results suggest that nESI-MS can be used to study conformational heterogeneity in intrinsically disordered Tau. Current work is focused on understanding which conformations of Tau and its structural elements are important for initiating aggregation. These findings will be presented.

Native mass spectrometry is used to probe the conformational heterogeneity and aggregation of intrinsically disordered Tau.

Investigating MSC Immune Suppressive Function with Quantitative Phase Microscopy and Single-cell Spatial Lipidomics

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Presenting Author: Kejie Rui

Mesenchymal stromal cells (MSCs) exhibit significant immunomodulatory potential, making them promising candidates for cell-based therapies in autoimmune and inflammatory diseases. However, due in part to the intra-culture functional heterogeneity of MSC and challenges in developing reliable and easy to deploy potency assays, the clinical translation of MSC-based therapies has been hampered. Current characterization methods for MSCs often rely on invasive or endpoint assays, which are unsuitable for real-time monitoring during manufacturing and frequently do not address single cell heterogeneity. To address this challenge, this study aims to develop a non-invasive methodology to predict single cell MSC immunosuppressive properties. As imaging cell morphology has been found to be associated with MSC immunosuppressive capacity, in this study we employed label-free quantitative phase imaging via differential phase contrast (DPC) microscopy to non-invasively extract features from live MSCs during biomanufacturing. We then correlated morphology directly with MSC function using live imaging of co-cultured T-cells, and explored the mechanism of action with single cell metabolomics using advanced mass spectrometry imaging.

Methods: Single-cell morphological profiles from 6 MSC donors were collected using non-destructive quantitative phase microscopy and used to predict single cell MSC immunosuppressive capacity as measured by indoleamine-2,3-dioxygenase (IDO) activity. Single cell indoleamine-2,3-dioxygenase (IDO) protein levels of interferon-gamma (IFN-γ) primed MSCs were then cross correlated with morphology-based IDO activity predictions. With additional co-culturing with PBMCs, MSC function (e.g. suppression on PBMC proliferation) was validated through DPC time-lapse imaging on live cells. We then investigated single cell lipid metabolite profiles of high functioning MSCs using matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI).

MSC immunosuppressive capacity was accurately predicted based on single-cell morphological profiles. In IFN-γ licensed MSCs, we found functionally distinct subpopulations based on measured IDO protein levels and identified their distinct morphological features. When co-cultured with T-cells, we identified functional subpopulations of MSCs with high levels of T-cell engagement with associated T-cell death after 16h. These functional MSC subpopulations had similar morphologies to the IFN-γ stimulated cells. In the same cells, we explored spatially-defined lipid abundances, and identified high abundances of specific immune-related lipid moieties, especially in the phosphatidylcholine family. By using arachidonyl trifluoromethyl ketone (ATK), we disrupted a critical pathway for phosphatidylcholine (PC) production and signaling. Following the inhibition, we observed a reduction of IDO activity as well as shifting in cell morphological features of MSCs. These findings support the role for PCs in MSC morphology and immune function.

This study highlights the potential of integrating label-free optical imaging and single cell spatial lipidomics to provide a mechanistic understanding of MSC function during biomanufacturing processes. Our approach allows for the prediction of MSC immunosuppressive capacity during cell expansion without disrupting the cells using quantitative DPC imaging, and insights into single cell functional metabolic pathways could be leveraged to improve MSC immune activity and reduce heterogeneity. Future work could move towards enhancing MSC lipid pathways and creating quantitative imaging that is compatible with larger scale biomanufacturing platforms for live evaluation of cell quality and functional activity.

Influences of Buffers and Water on Protein Structures, Stabilities, and Dynamics: A Variable Temperature-native ESI MS Perspective

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Presenting Author: David Russell

Native MS aims to study intact biomolecules in their native folded states. Since many factors (viz. cofactors, osmolytes, and ligands) influence protein structures, stabilities, and dynamics, the so-called “native state” is a nebulas concept. While temperature and water (hydration) influences protein “native state,” they also impact protein-ligand interactions by influencing protein conformation, binding affinities, and the folding energy landscape (FEL), viz. changes of enthalpy (ΔH) and entropy (TΔS) (enthalpy-entropy compensation) often with only minor changes in free energy (ΔG). The FEL harbors rich conformational entropy (distribution of conformation states) in which kinetic and/or thermodynamic traps reveal the presence of both native and “non-native” states (microstates) that may remain “hidden” for experimental approaches that report ensemble-averaged responses, e.g., ITC, XRD, and cryo-EM. We employ both nESI experimental approaches and molecular dynamics simulations to compare influences of cold and hot water for native and non-native states, viz. structure, stabilities, dynamics and thermodynamics for ligand binding. Water plays crucial roles in determining protein structure and stability, and mediating folding processes that form the ensemble of microstates. These studies capture ensembles of microstates states that proved further evidences for allostery without conformational change, e.g. dynamic allostery.

Molecular Dynamic Simulations of Denatured Proteins in Electrosprayed Droplets with H3O+ and Dynamic Protonation

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Presenting Author: Stewart Ryberg

Denatured proteins are analyzed by electrospray ionization-mass spectrometry (ESI-MS) to identify and sequence proteins based on molecular weight and fragmentation patterns. Unfortunately, protein ionization from electrosprayed droplets is difficult to observe experimentally. Prior work, using molecular dynamics (MD) simulations, examined the ionization mechanisms of denatured proteins using sodium ions as charge carriers and static, pre-set protein charges to model positive-ion-mode ESI. In these simulations, denatured proteins volatilized via ejection from the droplet, indicative of the chain ejection model (CEM). However, these conditions are not representative of experimental ESI, where proteins are dynamically charged via proton transfer. Here, we model ionization of denatured proteins using a novel MD workflow, H3O+-containing droplets, and dynamic protonation to perform more realistic ESI simulations.

Denatured proteins were produced in MD by heating ubiquitin (PDB:1D3Z) from 300 K to 1000 K over 200 ns, then equilibrating in water (200 ns). Denatured ubiquitin was then seeded into droplets (~3.4 nm or 5.5 nm) containing H3O+ with total droplet charge at 90% of the Rayleigh limit. The MD simulation proceeded via a trajectory stitching method, in which the MD simulation was paused every 4 ps to perform energetics calculations that were used to determine the favorability of proton transfers between chargeable amino acids, water, and H3O+. If favorable, protons were transferred before the MD simulation was re-initiated. This model included Grotthuss diffusion for H3O+ with up to five proton transfers per timestep between water molecules.

All simulations of denatured ubiquitin from ~3.4nm droplets showed the protein ejecting from the droplet in a linearized form, similar to CEM. However, in every trial, water was retained around a portion of the protein. In n=8 of 10 trials, one of the termini retained water. In the remaining two trials, the middle of the protein was ejected while the termini retained solvent, or both termini were ejected while the center of the protein retained solvent. Thus, ubiquitin appears to ionize by a partial-CEM mechanism. These results are different from previous simulations using Na+ as charge carriers, since we observed solvent being retained at some portion of the protein rather than the protein being fully ejected from the droplet. To increase the time for the protein to fully eject from the droplets, we ran simulations using larger droplets (5.5nm radii). In these droplets, solvent was retained either around one terminus of the protein (n=9 of 10 trials) or around the middle of the protein (n=1 of 10). Ejection of the linearized protein started when the droplets were similar in size, with 1000 ± 300 and 1100 ± 300 water molecules remaining from the 3.4nm and 5.5nm droplets, respectively, suggesting that the initial droplet size is not a factor in the protein maintaining solvent.

Using our MD protocol, we observed ionization of denatured ubiquitin by a partial CEM mechanism. Because ionization mechanisms impact ionization efficiency, this work may provide new insights for improving the ionization efficiency of denatured proteins analyzed by ESI-MS, further enhancing identification and sequencing.

AstroAgents: A Multi-agent AI for Hypothesis Generation from Mass Spectrometry Data

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Presenting Author: Daniel Saeedi

With the increasing availability of mass spectrometry data from solar system missions, there's an urgent need for methods to analyze such data within the context of astrobiology literature and generate hypotheses about life's emergence on Earth. Hypothesis generation from mass spectrometry data faces challenges including environmental contaminants, spectral peak complexity, and difficulties cross-matching with prior studies. To address these challenges, we introduce AstroAgents, a large language model-based, multi-agent AI system. AstroAgents consists of eight collaborative agents: a data analyst, a planner, three domain scientists, an accumulator, a literature reviewer, and a critic. The system processes mass spectrometry data alongside research papers to explore potential hypotheses. An astrobiology expert evaluated over a hundred generated hypotheses, finding 36% plausible, with 66% of those being novel. AstroAgents represents a promising approach for interpreting complex astrobiological data.

AstroAgents begins with user input of research papers and mass spectrometry data. The data analyst agent examines the data to identify patterns, anomalies, and potential contamination. The planner agent delegates specific data segments to three scientist agents for in-depth exploration, each focusing on different molecular groups. Each scientist agent generates hypotheses within their domain in structured JSON format, containing hypothesis statements and supporting evidence. The accumulator agent consolidates and deduplicates hypotheses from all scientists. The literature review agent uses Semantic Scholar to find relevant research for each hypothesis, extracting key insights. The critic agent evaluates hypotheses based on consistency with data, scientific rigor, theoretical basis, and integration with literature, providing structured feedback for iterative refinement.

We analyzed mass spectrometry data from eight meteorite and ten terrestrial samples using two-dimensional gas chromatography coupled with high-resolution mass spectrometry (GCxGC-HRTOF-MS). This analysis produced a detailed profile of 48 compounds with their peak information, including retention times, mass-to-charge ratios, and sample occurrence patterns.

To assess AstroAgents, an astrobiology expert evaluated the novelty and plausibility of more than a hundred hypotheses generated from data obtained from eight meteorites and ten soil samples. Of these hypotheses, 36% were identified as plausible, and among those, 66% were novel.

Unlocking Nature's Chemical Diversity: Genome Mining Strategies for Peptide Halogenases and Their Catalytic Potential

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Presenting Author: Nirmal Saha

While halogenation is one of the most versatile C–H functionalization strategy, regiospecific halogenation of peptides and proteins is outside the purview of traditional chemical catalysis. Enzymes that participate in the biosynthesis of ribosomally synthesized and post-translationally modified peptides and proteins can bridge this gap and offer a biocatalytic route for residue-specific incorporation of halogen handles onto amino acid side chains. Protocols described herein provide a guided approach for the discovery of peptide halogenases MppI in the context of natural product biosynthetic gene clusters (mpp BGC), and the preliminary reconstitution of their activity using a bacterial heterologous host E. coli. Also described are mass spectrometry-based analytical procedures and data analysis workflows that allow for deconvolution of halide specificity and preliminary insights into peptidic natural product biosynthetic schemes.

The paper describes a genome mining methodology to discover peptide halogenases capable of regioselective bromination. We used a flavin-dependent halogenase (SrpI) as a query sequence to mine sequence databases, organizing results into a sequence similarity network. They specifically searched for biosynthetic gene clusters containing both SrpI-like halogenase genes and lanthionine synthetase (LanM) encoding genes, focusing on a marine cyanobacterium to find brominases with minimal chlorination activity. We have co-expressed the precursor peptide gene (mppE) with LanM (mppM) and halogenase (mppI) genes in E. coli, resulting in lanthionine ring formation and site-selective tryptophan bromination. Mutagenesis studies and enzymatic degradation confirmed regiospecific indole-5-bromination of Trp80. The utility of this aryl bromide handle was demonstrated through palladium-assisted Suzuki–Miyaura cross-coupling reactions.

The preliminary data centers on a novel genome mining strategy to discover halogenases capable of producing brominated lanthipeptides. We used the flavin-dependent halogenase SrpI as a query sequence to mine sequence databases for similar enzymes. The hits were organized into a sequence similarity network (SSN) to visualize relationships between potential halogenases. We searched for biosynthetic gene clusters (BGCs) containing both SrpI-like halogenase genes and lanthionine synthetase (LanM) encoding genes to target macrocyclic halogenated peptides specifically. LanMs are crucial enzymes that catalyze macrocyclizing thioether bond formation through ATP-dependent dehydration of serine/threonine side chains and subsequent addition of cysteine thiols. This strategic approach identified four candidate BGCs containing an SrpI-like halogenase encoded near a LanM gene. The team selected a marine cyanobacterium, Moorena producens-derived RiPP BGC (designated mpp BGC) for further experimental evaluation, as marine cyanobacterial halogenases typically function as obligate brominases with minimal chlorination activity. The mpp BGC contains multiple genes: mppI (halogenase), mppM (LanM), mppE (RiPP precursor peptide), mppA and mppB (arginases), mppC (prenyltransferase), mppD (terpene synthase), mppG (oxygenase), and mppP (peptidase). The MppE precursor peptide follows typical RiPP architecture with a Nif11-like 77-amino acid leader sequence attached to a 7-amino acid ACWRWSG core peptide. In similar studies, we characterized SrpI, a novel iterative flavin-dependent halogenase that chlorinates specific tryptophan residues in a lasso peptide precursor. SrpI shows remarkable substrate flexibility, capable of halogenating internal tryptophan residues in linear peptides and at N- and C-terminal positions. Other genome mining approaches include using halogenase as a probe combined with halogen isotope patterns to identify new halogenated metabolites from fungal sources. Additional work has focused on characterizing the reactivity and regioselectivity determinants of halogenases through extensive sequence analysis of conserved motifs in the FAD-binding domain.

This study's key innovation is a genome mining strategy that identifies and characterizes MppI, a halogenase enabling site-selective tryptophan bromination in lanthipeptides. Co-expression of MppI, MppM, and MppE in E. coli produced brominated lanthipeptides, showcasing in vivo bromination (LCMS-characterization). The brominated residue was leveraged for downstream diversification via Suzuki-Miyaura cross-coupling.

Redesigning the Crosslinking Reaction for High-fidelity Cellular Interactomics: Almost There

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Presenting Author: David Schriemer

Crosslinking mass spectrometry should be the ideal method for mapping protein-protein interactions in situ; however conventional crosslinking methods distort the structural proteome and suffer from poor yield. We can overcome these problems by prestabilizing the cell prior to crosslinking, which preserves cellular ultrastructure and offers full control over crosslinker yield via a two-stage crosslinking protocol. We will describe the concept and illustrate that as much as 30% of the total lysine content of the cell can be crosslinked, presenting new analytical challenges that must be met before ultra-deep interactome sampling will truly be possible.

Novel Multi-modal Mass Spectrometry Imaging and Electron Microscopy Instrument to Improve Structural and Chemical Single-cell Visualization

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Presenting Author: John Sentmanat

While recent advances in mass spectrometry imaging (MSI) technologies have yielded unprecedented abilities to biochemically image spatially distinct samples, performance at single-cell resolutions is still lacking. Obtaining structural and chemical information spatially at the single-cell level has been highlighted as a crucial element in understanding biological function as heterogeneities and intercellular interactions can be elucidated. BeamMap, a novel MSI technique, combines electron microscopy (EM) to topologically image and vacuum electrospray-enabled mass spectrometry (vESI-MS) to chemically image bio-samples with sub-cellular resolutions. The instrument offers a 10-fold enhancement in spatial and chemical resolutions that will dramatically improve the characterization of highly complex biomolecule populations useful for the detection, diagnosis, and treatment of disease.

Bio-samples are imaged by scanning EM in environmental vacuum mode (~0.1-5 Torr) to reveal topological features (<100 nm). Chemical imaging is enabled by scanning the surface with a focused vESI-generated beam of droplets (<500 nm) that impinges a sample substrate, desorbs, softly-ionizes, and extracts surface molecules for MS analysis. Supersonic gas-jets assist in extracting analyte-laden secondary droplets from the EM, which are transported to the MS through SPion®, a flexible electrodynamic ion guide. Analyte desolvation through SPion® enables the detection of biomolecules for in situ mass analysis. The topological and chemical datasets are combined to create a high-resolution map of sub-cellular biomolecule populations.

vESI enabled the production of sub-micrometer diameter droplet “beams” that experienced minimal plume spread due to the fast acceleration of droplets in vacuum. The stability of the droplet beam is highly dependent upon the vESI tip diameter, tip-to-substrate distance, EM pressure, and spray voltage. Monitoring the vESI source with EM imaging allows for the measurement of beam size, substrate impact location, and impact spot size. Manipulation and focusing of the vESI beam can be achieved by confined gas jets expanding into the vacuum environment. The supersonic expansion of the jet produces gas velocities greater than Mach 2.5, allowing for a 4-fold increase in vESI droplet velocity when compared to atmospheric pressure as the result of gas-induced drag. Combining vESI and supersonic gas jets permits greater precision when sampling surfaces, assists with the extraction and transport of secondary droplets and promotes analyte desolvation needed for MS analysis. MS detection of desorbed analytes produced in the EM environment is accomplished through combined hydrodynamic transport and electrodynamic focusing of ions. vESI droplets are produced in the EM and a sheath-gas assisted flow helps transport droplets to sample surface for analyte desorption. Secondary droplets leaving the surface enter the inlet of the SPion® electrodynamic ion guide for extraction to the MS. Using a slight hydrodynamic pressure gradient between the EM and MS environments and an RF-potential applied to SPion®, analytes can be transported and desolvated over a 1 m distance and detected by a quadrupole time-of-flight (qTOF) MS system.

In-situ topological E-beam and chemical MS imaging instrument to visualize bio-samples at single-cell resolution

Fast and Robust Phosphoproteomics Sample Prep with AttractSPE® Disks C18 Tips for High Phosphopeptide Recovery and Identification

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Presenting Author: Mana Shafaei

Phosphorylation is one of the most prevalent and important post-translational modifications proteins can undergo. Over 50% of the human proteome is phosphorylated and understanding the dynamic phosphorylation across the proteome can understand the progression of many diseases including cancers. As the stoichiometry of phosphorylation sites is generally very low, enrichment steps, followed by SPE clean-up before LC-MS/MS analysis are required to enhance identification and quantification of each site. However, recovery of phosphopeptides can be greatly affected by the choice of clean-up method, resulting in severe losses.

Different SPE C18 options, including SPE Tips and cartridges, were compared for the desalting and purification of phosphopeptides, after automated enrichment using magnetic beads (Ti/Zr-IMAC). Effects of sample acidification prior to SPE clean-up on phosphopeptides detection were also assessed. To do so, the enrichment elution was acidified with different percentages (2, 3, 4 and 5%) of phosphoric acid or trifluoracetic acid (TFA).

Among all SPE options tested, AttractSPE® Disks Tips C18 provided the highest recovery of phosphopeptides (up to 2.4 times more identifications), with high reproducibility (RSD < 10%). Moreover, these SPE Tips captured hydrophilic peptides more efficiently, with smaller phosphopeptides also generally retained compared to other brands, thus leading to higher phosphopeptide identification. Quenching the enrichment elution with 3% phosphoric acid had the best results, with 8% more phosphosites identifications compared to 5% TFA. In addition, lower acid concentration interestingly provided more singly phosphorylated peptides, while higher acid concentration recovered more hydrophilic peptides. This trend was observed for both acids tested but was more pronounced for TFA. AttractSPE® Disks Tips C18 are shown to be the best choice for phosphopeptide purification, offering simplicity of use by centrifugation, high sample recovery, and robustness. These SPE Tips are easily scalable with their availability in different sizes and binding capacities to perfectly adapt to different sample amounts, and can be provided as 96 and 384 SPE well plates for high throughput processing and fully automated workflows.

Enhanced phosphopeptides identification following efficient and fast purification step on innovative C18 SPE Tips.

Radical Footprinting in Mammalian Whole Blood of a Murine Diabetes Model

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Presenting Author: Joshua Sharp

Hydroxyl Radical Protein Footprinting is a powerful tool to probe protein higher-order structure. It is mostly performed in vitro, but recent advances have enabled its use in live cells, nematodes, and 3D cultures. However, application in living mammalian tissues has not been accomplished. Here, we present the first successful use of radical protein footprinting in mammalian whole blood from wild-type (WT) and a type 2 diabetes mouse model. Using persulfate photoactivated with the FOX Photolysis System, we achieved effective protein labeling without significant dis-ruption to blood cell morphology. An optimized quenching protocol eliminated background la-beling. We report oxidative modifications in 11 selected proteins, revealing disease-associated conformational changes in multiple proteins.

EDTA-stabilized whole blood and serum were collected respectively from BKS.Cg Dock7m +/+ Leprdb/J diabetic 2 mice and control mice. RPF was performed using FOX Protein Footprinting System (GenNext Technologies.) with a flash voltage of 900 V at 2 Hz. Immediately prior to RPF, sodium persulfate or sodium chloride at final concentration of 200 mM was added to samples. Immediately after labeling, whole blood samples were collected in a quench solution containing 200 mM imidazole, 200 mM N,N'-Dimethylthiourea (DMTU) and 70 mM methionine amide. After RPF, samples were precipitated, lysed and digested with Profiti Micro S-trap. Eluted peptides were analyzed on an Orbitrap fusion hydribrid mass spectrometer, with data analysis by FoxWare.

Radical protein footprinting (RPF) was demonstrated in murine whole blood using photoactivation of sodium persulfate with a Fox Photolysis System. Analysis of blood showed that addition of up to 200 mM sodium persulfate resulted in no measurable gas generation and no gross changes in blood characteristics. Blood cell analysis by microscopy showed that addition of up to 200 mM sodium persulfate showed no observable changes in blood cell morphology save for a mild hypertonicity, comparable to addition of an equal concentration of sodium chloride. In vitro experiments using adenine dosimetry demonstrated that the Fox Photolysis System could effectively generate high concentrations of sulfate radicals at typical operating voltages (600-900 V) in the presence of as little as 50 mM persulfate. An improved quench solution consisting of 200 mM DMTU, 70 mM methionine amide and 200 mM imidazole was developed for sulfate radical RPF that eliminated background oxidation for all peptides measured. Using these conditions, we performed RPF on EDTA-stabilized murine whole blood taken from either wild-type or an age-matched Cg Dock7m +/+ Leprdb/J type 2 diabetes mouse model (n=4 for each group). Analysis of the top eleven proteins by sequence coverage showed a significant increase in oxidation for extracellular proteins for RPF samples compared to 0 V controls, and a much lower increase for intracellular proteins suggesting differential labeling, possibly due to limited cell permeation of the persulfate under the rapid mixing conditions used here. Multiple proteins in the top 11 were found to have disease-associated differences in protein topography. Among them are complement c3, which has been previously reported to be both a biomarker and a potential contributor to the pathophysiology of T2D due to the inhibitory effects of its activation products (including complement C3a) on insulin signaling and beta-cell function.

First reported use of radical protein footprinting in mammalian tissue; first use of RPF in a pre-clinical animal model of disease

Higher Order Structure Characterization of ADCs using Hydrogen-deuterium Exchange Mass Spectrometry (HDX-MS)

Yuqi Shi, Thermo Fisher Scientific; Cong Wang, Thermo Fisher Scientific; Peter Krueger, Thermo Fisher Scientific; Thaís de Faria, Thermo Fisher Scientific; Rosa Viner, Thermo Fisher Scientific; Thomas Moehring, Thermo Fisher Scientific

Presenting Author: Yuqi Shi

Antibody-Drug Conjugates (ADCs) are an innovative class of therapeutics that merge the high specificity of mAbs with the potent cytotoxic capabilities of small-molecule drugs. ADCs offer a more precise and effective treatment modality. Despite their therapeutic potential, ADCs present unique challenges in development, particularly in understanding their complex structural and functional attributes. Any structural perturbations during conjugation, formulation, or storage could impact the ADC's ability to bind its target antigen and maintain its therapeutic profile. To address these challenges, HDX-MS has emerged as a powerful tool for studying the HOS of ADCs. In this study, we showed the ability to use HDX-MS to probe the HOS change on the antibody upon drug binding at both peptide and residue levels.

Unconjugated and conjugated forms of Rituximab were analyzed by HDX-MS at peptide and residue levels using a fully automated HDX workflow with a Vanquish Binary Pump N™ and a modified Thermo Scientific™ Orbitrap™ Hybrid mass spectrometer. For peptide-level analysis, samples were incubated in D2O at multiple time points in triplicates, quenched, digested with an NEP II protease column (AffiPro), and separated on a 300 µm × 5 cm column with a 6-minute gradient. Full MS spectra were used to identify regions of deuterium incorporation. For residue-level analysis, MS2 spectra pinpointed incorporation at single amino acids in peptides showing significant changes. Both experiments were conducted under low-flow (capillary flow <10 ul/min) LC conditions.

Peptide mapping experiments were conducted using unconjugated Rituximab across a dilution series. At the highest injection amount (1.5 µg on-column), 570 peptides were identified for the heavy chain and 234 for the light chain, achieving 100% sequence coverage for both. At the lowest injection amount (300 ng on-column), 445 peptides were identified for the heavy chain (98% coverage) and 186 for the light chain (99% coverage). Based on these results, 300 ng was selected for labeling experiments, and the identified peptides served as the peptide library. Peptide-level HDX-MS experiments were performed using both MS1 full scan and MS2 DIA modes, with data analyzed using HDExaminer (Trajan) and AutoHX in MS Studio (Trajan). The drug-binding site was identified at the CH2 domain of the antibody, and allosteric changes were detected across the antibody, particularly at antigen-binding sites within the CH1 domain, to evaluate structural alterations that could affect antigen recognition. Both the binding site and allosteric changes were mapped onto a homology model for structural interpretation. For residue-level analysis, peptides covering the binding region in the CH2 domain were selected for targeted MS2 analysis with nonergodic fragmentation to determine deuterium incorporation at the residue level. The binding site was pinpointed to a Cystine residue, consistent with prior sample information provided for the study.

Investigate antibody conformation changes upon drug binding using HDX-MS

Structural Characterization of "Difficult" Proteins by Cross-linking Mass Spectrometry using MS-cleavable Cross-linkers

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Presenting Author: Andrea Sinz

Chemical cross-linking in combination with a mass spectrometric analysis of the created cross-linked products has proven highly valuable to derive 3D-structural information of proteins and protein assemblies. In my talk, I will present different examples of cross-linking mass spectrometry (XL-MS) using MS-cleavable reagents. Due to the formation of characteristic marker ions in the fragment ion mass spectra, MS-cleavable cross-linkers drastically diminish false-positive identification rates of cross-links. I will introduce disuccinimidyl disuccinic imide (DSSI) as a prototype of MS-cleavable cross-linkers that contains a phosphonate group for immobilized metal ion affinity chromatography enrichment of cross-linked products. I will present the application of MS-cleavable cross-linkers for "difficult" protein systems, such as (i) intrinsically disordered proteins (IDPs) and (ii) G-protein-coupled receptors (GPCRs). (i) α-Synuclein is an IDP that undergoes liquid-liquid phase separation, fibrillation, and forms insoluble, intracellular Lewy bodies in neurons, which are the hallmark of Parkinson's Disease. The molecular mechanisms underlying the early stages of phase separation are still elusive. To obtain structural insights into α-synuclein upon phase separation, an innovative approach relying on 15N-labeling of the protein, termed COMPASS (COMPetitive PAiring StatisticS), was developed. XL-MS data revealed a shift of α-synuclein from a "hairpin-like" structure towards more "elongated" conformational states during the initial stages of α-synuclein phase separation. (i)

Neuropeptide Y receptors 1 and 2 are GPCRs that are involved in food intake and memory. A photo-reactive, diazirine-containing neuropeptide Y analogon allowed mapping the interaction sites with its receptors by photo-XL-MS. Parallel Accumulation-Serial Fragmentation (PASEF) on a timsTOF mass spectrometer facilitated the detection and analysis of cross-linked products. The cross-links were directly obtained in cells and provided distance constraints for deriving structural models of neuropeptide Y-receptor interactions. Molecular dynamics simulations and mutagenesis studies confirmed the XL-MS results. Conclusively, the application of MS-cleavable cross-linkers proved highly beneficial for gaining insights into 3D-structures of proteins that cannot easily be studied by the classical methods of protein structure analysis. As XL-MS can directly be performed in the cellular environment, in vivo protein 3D-structures and protein-protein interaction networks are reflected.

CZE-MS is Reproducible and Robust Enough for Large-scale Native and Denaturing Top-down Proteomics

Liangliang Sun, Chemistry, Michigan State University

Presenting Author: Liangliang Sun

Capillary zone electrophoresis-mass spectrometry (CZE-MS) has been recognized as a useful tool for intact proteins (“proteoforms”) and protein complexes for decades. However, it is still not widely adopted due to its lack of robustness and reproducibility. During the last nearly 10 years, our research group has advanced the field of CZE-MS-based top-down proteomics (native and denaturing) substantially regarding reproducibility, robustness, applicability, and proteome coverage.

In our most recent work, we investigated the long-term reproducibility of CZE-MS for denaturing and native top-down proteomics to pave the way for its broad adoption.

We performed CZE-MS-based denaturing top-down proteomics of E. coli and HeLa cell lysates for about 100 runs using one linear-polyacrylamide-coated separation capillary during an over 100-hour period of MS time. The system produced reproducible proteoform identification and quantification across the runs. We further carried out native top-down proteomics of an E. coli cell lysate for over 20 runs with the reproducible measurement of many protein complexes in a mass range of 30-400 kDa. Further improvement of CZE separation results in the detection of hundreds of protein complexes up to 600 kDa from the E. coli cell lysate. We developed an efficient multi-step strategy for CZE-MS/MS-based native proteomics and enabled the confident identification of multiple protein complexes up to 300 kDa from the E. coli and HeLa cell lysates. Finally, a quantitative native proteomics study was performed and discovered significant protein-complex level changes between log-phase and stationary-phase E. coli cells.

In summary, we believe that CZE-MS is reproducible and robust enough for large-scale native and denaturing top-down proteomics.

Offline Tandem MSn Workflows on the timsOmni Platform for Deep Sequencing of Intact Proteins and mAbs

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The broader use of top-down mass spectrometry (TDMS) is hindered by signal dilution into multiple dissociation pathways and charge states, resulting in low signal-to-noise mass spectra and information loss. Multidimensional multiple-stage activation processes in RF ion traps show great promise for generating complementary information to enhance sequence coverage, however, their effectiveness in the context of TDMS is dependent upon whether the design is operable under heavy space charge loads. Offline MSn modes implemented on the new timsOmni platform are described, accommodating a flux of >5M charges/ms enabled by high-capacity linear ion traps and high-speed time-of-flight acquisition. Exceptional sequence coverage is demonstrated for proteins sprayed offline and through extended averaging across multiple scans to enhance the fidelity of fragment-ion isotopic-envelopes.

For native MS, proteins and intact mAbs were buffer exchanged into 150mM ammonium acetate (Sigma Aldrich) and diluted to a working concentration of 5μM. For denatured analysis, proteins were diluted to 5μM in water:acetonitrile in 50:50 ratio (v/v) with 0.1% formic acid. Electrospray ionization for native MS was performed using coated, open type nanospray tips (Cellomics) biased at 1.3kV, while direct infusion for denatured proteins was performed at ~300nL/min with the CaptiveSpray ionization source (Bruker). Protein charge states at m/z<4500 Th were mass selected in the quadrupole mass filter while isolation of ions in the higher m/z range was performed in segment Q2 of the Omnitrap TM platform. The sequence confirmation workflow available in OmniScape TM software was used for data analysis.

Collision induced dissociation (CID) is performed in an ion funnel and upstream of a quadrupole mass filter. Analyte ions or first-generation CID fragments are selected using the quadrupole mass filter and accumulated either in segment Q2 of the Omnitrap platform for an additional CID step, in segment Q5 supporting all the different flavors of electron-based fragmentation, or in segment Q8 for photodissociation and accumulation. CID can also be performed in the collision cell while the different activation regions can be mixed and matched providing a diverse set of instrument operating modes tailored for comprehensive analysis of mAbs.Such optimal MSn workflows for mAbs depend on spraying conditions (native or denaturing) and on the region of the sequence targeted in an experiment. MS2 ECD is shown to provide complete sequence coverage of CDR3 for the light and heavy chains via the formation of a complete series of c- and a-type fragments, while reduction of the first intrachain disulfide bond is observed only for the light chain. Similar results are produced by EID, exhibiting higher spectral complexity.MS2 CID of denatured mAbs produces abundant b-type fragments which are mass selected and further subjected to MS3 ExD providing less congested spectra and high confidence annotations. MS2 CID on the intact denatured mAb is shown to release the light chain with the interchain disulfide bond cleaved between the beta-carbons and sulfur atoms and between the two sulfurs. MS2 ECD and MS2 EID followed by broadband excitation CID of the charge reduced intact mAbs provide complete sequence coverage of CDR3 and framework regions on both the light and heavy chains.The steps comprising the MS3/MS4 experiments so far performed exclusively in the Omnitrap platform (MSn in-time) are now distributed across different components of the timsOmni platform (MSn in-space), increasing the speed of the analysis considerably.

Advancing offline MSn workflows for deep sequencing of proteins and antibodies in the new timsOmni platform.

Flying Viruses – Mass Spectrometry Meets X-rays

Presenting Author: Charlotte Uetrecht

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.

These capsids 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.

Signal from gas phase SAXS of protein complex ions; dynamics of viral protein complexes studied by native MS.

The Design and Characterisation of a New Charge Detection Mass Spectrometry (CDMS) Instrument for the Analysis of Megadalton-Sized Molecules

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Presenting Author: Jakub Ujma

Advances in Electrostatic Linear Ion Trap (ELIT)-based Charge Detection Mass Spectrometry (CDMS) over the past 10 years have revolutionized its use for analysis of very high molecular weight species such as protein complexes, viral vectors, vaccines, viruses and amyloid fibrils. Nonetheless, ELIT-based CDMS has remained embedded in very few specialized instrumentation groups, predominantly in academia, where the complex hardware is operated by highly skilled scientists. In this presentation, we will discuss the primary challenges that were addressed in the design of a benchtop, prototype ELIT-based CDMS instrument. We will highlight key design aspects of the hardware, acquisition modes and control software, as well as present key performance characteristics such as acquisition speed, sensitivity, mass-to-charge (m/z) and charge (z) resolution.

Various molecular weight samples were used to test the performance of the prototype ELIT-based CDMS instrument, ranging from monoclonal antibody (0.15 MDa) to the chikungunya virus-like particle (50 MDa). All samples were buffer exchanged into ammonium acetate solution and introduced to the instrument using new nano-electrospray interface. Ions enter the vacuum system through a heated capillary and conjoined ion guide to a high-pressure segmented quadrupole (SQ). A subsequent low-pressure SQ accelerates the ions through DC ion optics to the ELIT analyser. Detected time-domain signals are Fourier transformed; the measured frequency and the magnitude correspond to an individual ion's m/z and z respectively, enabling direct calculation of mass values. Data for individual ions are histogrammed to generate a mass spectrum.

The prototype instrument has a novel nESI interface which has re-useable, easy fill-and-flush emitters within a protective sheath. The nESI plume is sprayed into a sealed chamber. Ions are sampled into the vacuum system through a heated capillary which can provide desolvation and/or sample denaturing then into a conjoined ion guide operating around 5 mbar for transport into a SQ operated at around 0.1 mbar. The potential of this SQ defines the ion energy required in the ELIT for operation (currently 130 V/z) and the relatively high pressure is to ensure thermalisation of the very high mass species. This device also houses a signal attenuation device and can be used to trap ions extending the instrument dynamic range and sensitivity (duty cycle) respectively. In the subsequent chamber is another SQ operating at around 10 -4 mbar through which ions are accelerated into the next vacuum chamber operating at 10 -7 mbar. This chamber has einzel lenses and steering optics to focus the ion beam into the ELIT which is in the final vacuum chamber operating at 10 -9 mbar. Preliminary sample analysis shows good ion transmission over the 0.15 to 50 MDa mass range. Typically, adeno-associated virus capsid particles can be detected at solution concentrations of 5x10 10 vp/mL and spectra obtained in <20 minutes. Early data on glutamate dehydrogenase indicate a m/z resolution in the 125-150 range. For most acquisitions trapping times of 100 msec are adequate, however, to reach near baseline separation in the charge domain longer trapping times are required. Initial results indicate that trapping times of 2 seconds or less are sufficient for this. In the presentation we will provide more detailed information around the instrument design and performance characteristics for a broader range of analytes.

Design and performance of a benchtop prototype ELIT-based CDMS instrument for simplified mass determination in native structural characterization of ultra-high mass and heterogeneous biomolecules.

Evolving Mass Spectrometry to Enable its Application in Structural Biology and Biophysics Activities within Small Molecule Drug Discovery

Jon Williams, AbbVie, Inc.; Shaun McLaughlin, AbbVie, Inc.

Presenting Author: Jon Williams

Many technologies are deployed for structural biology and biophysics within small molecule drug discovery programs in large pharma; however, mass spectrometry (MS) is typically not chosen. Structural based drug discovery is dependent on X-ray crystallography, cryo-EM, and NMR all of which consistently provide high resolution structures of protein ligand complexes that enable medicinal and computational chemists to synthesize and design selective and potent molecules. Biophysics techniques such as SPR, NMR and calorimetry are commonly performed at a throughput that enables fast progression of projects. This presentation will discuss situations where MS can be applied to enhance understanding of how small molecules engage with proteins. Concepts to improve MS throughput to enable it to match or potentially exceed the productivity of biophysics technologies will be shown.

This presentation will represent a current perspective of Structural Biology MS use for small molecule drug discovery.

Glycoproteome and Surfaceome Mapping of Epithelial-to-Mesenchymal Transition Reveals Key Ion Channel Regulation and Novel Biomarkers in Cancer Metastasis

Xing Xu, School of Chemistry and Biochemistry and the Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology; Kejun Yin, School of Chemistry and Biochemistry and the Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology; Ronghu Wu, Xing Xu, School of Chemistry and Biochemistry and the Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology

Presenting Author: Xing Xu

The epithelial-to-mesenchymal transition (EMT) is a crucial process where epithelial cells transition into mesenchymal states, driving cancer progression, metastasis, and drug resistance. EMT is not a binary process but comprises intermediate states marked by co-expression of epithelial and mesenchymal markers. While transcriptional changes during EMT have been extensively studied, they alone cannot predict protein-level alterations or modifications like N-glycosylation, which impact protein folding, stability, and interactions. Dysregulated N-glycosylation promotes cancer progression, with evidence linking aberrant glycosylation of proteins such as EpCAM, ITGAV/ITGB3, and IL6 to EMT and metastasis. However, a systematic analysis of protein N-glycosylation and surface glycoproteins during EMT remains unexplored, necessitating comprehensive studies to uncover their roles in cancer metastasis.

To investigate EMT at the molecular level, A549 lung carcinoma cells were treated with TGFβ1 (4 ng/mL) and sampled at nine time points over five days. A multidimensional workflow based on MS-based proteomics enabled simultaneous analysis of the whole-cell proteome (WP), N-glycoproteome (NG), and surface glycoproteome (SG) from the same samples. For WP, lysed cells were digested, TMT-labeled, pooled, and analyzed by nanoLC-MS/MS.NG analysis utilized the DBA enrichment method for N-glycopeptides, while SG analysis employed an enzymatic tagging strategy using neuraminidase, galactose oxidase (GAO), horseradish peroxidase (HRP), and hydrazide beads to enrich cell-surface glycoproteins. Enriched glycopeptides were TMT-labeled, pooled, and treated with PNGase F in $H_2^{18}O$, generating glycosylation-specific heavy-oxygen tags for precise MS-based identification and quantification.

The whole proteome analysis reveals five distinct EMT states with unique characteristics, and these states are further featured with different protein N-glycosylation patterns. Changes in protein N-glycosylation during EMT were driven by alterations in protein expression and the N-glycosylation machinery. Furthermore, surface glycoprotein profiling revealed different dynamic patterns, with differential regulation of sodium, chloride, and calcium ion channels, indicating their roles in promoting cancer metastasis. Finally, we characterized site-specific changes in protein N-glycosylation, which revealed that EMT regulates protein function and localization by altering N-glycosylation at specific domains and aggregation-prone regions (APRs). These changes affect key processes such as calcium ion binding, growth factor binding, signaling receptor binding, and adhesion molecule binding as well as cell-surface glycoprotein trafficking during EMT. The global and site-specific analyses of protein glycosylation and surface glycoproteins advance the understanding of the molecular mechanisms of EMT and provide unprecedented and valuable information for future functional and mechanistic studies of cancer metastasis, ultimately contributing to the development of targeted cancer therapies.

Integration of dynamic proteomics, glycoproteomics, and surfaceomics characterizes EMT states at both the molecular and post-translational modification (PTM) levels, revealing a link between ion channel regulation, site-specific N-glycosylation, and cancer metastasis.

Characterization of Human Cytomegalovirus Surface Protein by Native MS and Single Particle Methods

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Presenting Author: Zhixin Xu

Human cytomegalovirus (HCMV) is a type of beta-herpesvirus that causes end-organ disease (EOD) in immunocompromised individuals. HCMV is sero-prevalent worldwide. Although HCMV infection is asymptomatic and mostly lifelong for healthy individuals, it can cause birth defects such as hearing loss and brain damage (1 in 5 infected) for infants. To date, due to a poor understanding of the infection mechanism, no federally approved HCMV vaccine is available. An assembly on the HCMV surface, “GATE (gH-Associated Tropism and Entry complex)”, has recently been proposed to be involved in the infection process. To facilitate the vaccine development for GATE, here we applied native mass spectrometry (nMS)-based techniques to characterize its glycosylation profile and oligomerization to understand the infection mechanism of HCMV.

Wild-type samples were buffer exchanged into 200 mM ammonium acetate (AmAc) by using 10 kDa molecular weight cut-off Amicon® Ultra Centrifugal Filters (Sigma Aldrich). nMS experiments were carried out on a modified Thermo Q Executive Ultra-High Mass Range mass spectrometer (UHMR) with a custom Electron-based Dissociation-Surface-Induced Dissociation (ExD-SID) device. Single-particle methods, charge detection mass spectrometry (CDMS) and mass photometry (MP), were used to determine the oligomeric states in wild-type samples. Electron capture charge reduction was performed to distinguish the specific glycoforms of GATE at the intact protein level by reducing protein ions' charge states and generating more resolved spectra. nMS, CDMS, and MP data were deconvolved or processed by using the software UniDec, STORIboard, UnidecCD, and DiscoverMP, respectively.

MP and CDMS were used to characterize the assembly of wild-type recombinant GATE samples. MP experiments in both AmAc and Hepes Buffer Saline (HBS) were conducted and compared. Mass distributions were largely buffer-independent, with intact trimer and hexamer (centroids at 200 and 400 kDa) observed under both buffer conditions in MP experiments. A serial dilution experiment was also performed in the CDMS, and due to the relatively high abundance of hexamer compared to the trimer, the Kd is suggested to be in the nM range. Wild-type recombinant GATE is composed of three different glycoproteins: glycoprotein H (gH), UL116, and UL141. Specifically, gH contains 6 N-glycan sites, UL141 contains 3 N-glycan sites, and UL116 contains 14 potential N-linked glycans with ~90 kDa glycan masses. The large percentage of glycan mass on UL116 (60%) makes this UL116 adopt a dynamic range of conformations, and the density of all glycoforms was averaged in cryo-EM single particle analysis, resulting in an unresolved density map for this protein. Although gH, UL116 and UL141 were studied individually, the intact repertoire of GATE’s glycoforms has not been characterized. Given that MP and CDMS can only provide the average mass of all intact glycoform masses for GATE, a method applying narrow quadrupole window m/z selection combined with electron capture charge reduction (ECCR) was performed to understand each intact glycoform mass. By applying ECCR, two mass distributions that reveal specific glycoforms for GATE’s trimer and hexamer were obtained. Combining this with glycoproteomics and future glycan distribution simulations will help to understand the glycan crosstalk and glycosylation in different oligomeric states, further facilitating the quality control of vaccine development.

Applying the single-particle method and nMS-ECCR to a novel surface protein involved in infection process will help unravel the biophysical information of GATE, including structural heterogeneity, oligomeric assembly, and glycoform distributions.

Determining the Structural Consequences of the Specific Distribution of N-glycans on Candidate Vaccines against HIV

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Presenting Author: John Yates

Site-specific N-glycosylation on many highly glycosylated candidate vaccines must be determined. This helps to estimate the impact of changing glycosylation on antigenicity and immunogenicity when developing vaccines and provides a measure for monitoring the consistency of GMP-grade vaccine production. With substantial number of vaccines being designed, it is essential to rapidly select candidates with glycan landscapes that most closely resemble a naturally-occurring immunogen. DeGlyPHER (Deglycosylation-dependent Glycan/Proteomic Heterogeneity Evaluation Report) is developed as a highly sensitive, rapid, and reproducible approach, that perfectly fits these needs.

Using Proteinase K we generate overlapping peptides mapping to every N-glycosylation site, sequentially deglycosylate these peptides with Endo H and then PNGase F, hence creating residual mass signatures that are identified by LC-MS/MS, thus quantifying the degree of glycan occupancy, and the broad characterization of glycan processing – constituting the transformation of high mannose forms into complex forms owing to mannose residues being replaced by other "apical" monosaccharides

DeGlyPHER has evaluated N-glycan distribution on >200 immunogens that are being developed as vaccines against HIV, SARS-CoV-2, Influenza and Ebola. We have DeGlyPHER-ed >150 vaccines being developed using the highly-promising germline-targeting approach against every important immunogenic epitope on HIV Env/spike-protein that elicits broadly-neutralizing antibodies (bNAb). E.g., [1] we scanned boost-vaccine candidates against the V3-glycan epitope that elicits BG18-like antibodies, to inform V1-loop design to cause high glycan occupancy, potentially limiting off-target responses; [2] we discovered that in 10E8 priming-immunogens comprising of membrane proximal external region (MPER) epitope grafted into a protein-scaffold to create a multi-valent nanoparticle, the nanoparticle component has much higher glycan occupancy than the epitope scaffold; and [3] we confirmed the plugging of the N276 "glycan-hole" as part of a "prime-boost" strategy based on VRC01 epitope. We also found that HIV Env gp41 is significantly better shielded with N-glycans when HIV Env is membrane-tethered – its native state on intact virus and in most mRNA vaccine strategies, thus promising a means to reduce unwanted base effects stemming from partial glycan-shielding of gp41. All these findings have contributed substantially to downstream non-human primate (NHP) studies and clinical trials.

DeGlyPHER is informing HIV vaccine development in real time, being a much more rapid, sensitive, and robust quantitative alternative to "intact-glycan" glycoproteomic approaches, with excellent potential for adapting it to high-throughput platforms for bulk glycoprotein analysis.

Interactions of the ER Chaperone BiP with Small Molecules and Nucleotides: Insights from Native Mass Spectrometry

John Young, Dept of Chemistry, University of Oxford; Carol Robinson, Dept of Chemistry, University of Oxford

Presenting Author: John Young

The process of protein transport - or "translocation" - across the membrane of the endoplasmic reticulum (ER) is a highly conserved biological process and is essential for life. The resident ER chaperone BiP (Binding Immunoglobulin Protein) – a member of the Hsp70 protein family - is intimately involved in the transport of newly synthesized polypeptides across the ER membrane and performs these functions in an ATP-dependent manner.

Here, we employ native mass spectrometry – an emerging technology for characterizing interactions between proteins and small molecules in unprecedented detail – to probe interactions between BiP, various nucleotides, and calcium. We assess the effects of these ligands on the oligomeric state of the BiP enzyme.

Surprisingly, we find that BiP co-purifies with a novel small molecule ligand, which was not evident in previous high-resolution structural studies. We assess the effect of this ligand on protein function and use structural modelling and site-directed mutagenesis to identify the ligand binding site. Altogether, our results shed valuable light on the biological activity of BiP and highlight the utility of native mass spectrometry for characterizing protein-small molecule interactions.

We identify a novel small nucleotide that co-purifies with the molecular chaperone BiP. We find that this nucleotide influences BiP activity.

Revealing N-Glycans on Antigen-specific Antibodies as Biomarkers for Disease State and Outcome by Sample Sparing Multiplexed MALDI Mass Spectrometry Imaging

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Presenting Author: Hanhao Zhang

N-Glycosylation of pathogen-specific antibodies is an emerging biomarker reflecting the host immune response to infections. While antibody titer often cannot distinguish between current and past infections, N-glycosylation changes in a disease state-specific manner across diseases. Despite its clinical significance, comprehensive antigen-specific antibody glycoprofiling for biomarker discovery remains challenging: LC-MS-based glycomics, the current gold standard, requires purification of low abundance antigen-specific antibodies, which consumes unrealistically large sample volumes. We have earlier developed microscale lectin-binding assays to address this, but these only indirectly profile limited glycan types. Here we develop a platform coupling MALDI-MSI with antigen microarrays enabling multiplexed antigen-specific antibody glycan profiling from limited sample volumes. We demonstrate MALDI-MSI-based quantification of glycosylation changes in viral infections (Hepatitis C and COVID-19).

Capture antigens were immobilized on poly-L-lysine coated glass slides in array format, where each array contains various HCV or SARS-CoV-2 antigens. Each slide was blocked in 1% BSA in PBS, then a laser-patterned polydimethylsiloxane sheet was placed on the slide to form wells for each array. Serum samples from patients and healthy controls were added to the wells. After 1 hour of incubation, the PDMS was removed and samples washed away. 0.01% PNGase in water was sprayed on the slide then incubated at 38°C for 2 hours. Alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA was then sprayed. The slide was then imaged with a Bruker timsTOF Flex mass spectrometer. Results were analyzed with SCiLS Lab and Graphpad Prism.

Dilutions of human monoclonal Immunoglobulin G (IgG), used as positive control, were first directly spotted. Intensity from each spot correlated with the concentration of IgG. High signal to noise ratio was observed across the entire dilution with lowest concentration at 8µg/ml. A linear fit of log(IgG concentration) over mean MALDI intensity returned an average R = 0.853 over the three main N-glycans (m/z = 1485.5077, NGA2F; 1647.5563, NA2G1F; 1809.6083, NA2F) on IgG, indicating a strong correlation between mean MALDI intensity and quantity of N-glycans. Successful capture of antibodies specific to SARS-CoV-2 Nucleocapsid (N) and four HCV antigens (E1, E2, Core1a Core1b) were verified with imaging using a fluorescently tagged anti-IgG probe. From N-specific antibodies, higher levels of N-glycan intensity were measured in patient sample compared to healthy controls, indicating the ability of the platform to analyze N-glycans on antigen-specific antibodies. Intensity close to zero was observed on spots coated with negative control antigen, proving the specificity of the method. Difference in multiple glycans, both fucosylated and afucosylated between antibodies from HCV+ patients and healthy controls were observed. Specifically, on Core1a-specific IgG, N-glycan of m/z 1485.5077 (NGA2F), 1647.5563 (NA2G1F), 1809.6083 (NA2F) and 2122.6819 (A1F+Na) showed elevated intensity from HCV+ patients, suggesting an increase in fucosylation for patients in HCV infection. A similar trend in increased fucosylation was observed from the above listed N-glycans on to Core1b-specific IgG. On Core1a-specific IgG, N-glycan of m/z 1976.6284 (A1+Na) also showed elevated intensity from HCV+ patients, suggesting an increase in sialyation for patients in HCV disease state. The above four antigens were spotted as 1mm adjacent spots and incubated with the same drop of HCV sample (<20µL serum), which returned four 1.5mm spots of distinct intensity, indicating the ability of this platform to perform multiplexed N-glycan analysis on antigen-specific antibodies.

Developed novel MALDI-MSI-based platform where N-glycans on antigen-specific antibodies can be quantitatively measured and visualized in multiplexed form with sample volume usage >50X lower than LC-MS. Discovered elevated fucosylation of N-glycans on IgG from patients in HCV disease state.

Comprehensive and High-throughput Characterization of Therapeutic Monoclonal Antibodies using an Integrated Orbitrap Mass Spectrometry Platform

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Presenting Author: Yu Zhou

Therapeutic monoclonal antibodies (mAbs) have significantly advanced the treatment of cancer, autoimmune disorders, and infectious diseases. Maintaining precise structural integrity is essential for therapeutic proteins, as even minor variations, including aggregation, glycan heterogeneity, oxidation, and charge variant shifts, can adversely impact safety and efficacy, alter pharmacokinetics, and increase the risk of immunogenicity. Traditional analytical methods such as gel electrophoresis, capillary electrophoresis (CE), enzyme-linked immunosorbent assay (ELISA), chromatographic techniques, and spectroscopic techniques such as ultraviolet-visible (UV/Vis) spectroscopy, circular dichroism (CD), and Fourier-transform infrared (FTIR) spectroscopy, have been widely used for antibody quality assessment. However, these methods often fall short due to their limited resolution, reliance on indirect readouts, and inability to accurately quantify subtle structural variations or site-specific post-translational modifications (PTMs).

To overcome these limitations, we present a robust, high-throughput, high-resolution integrated mass spectrometry (MS) platform. This platform features a 3-minute dilute-and-shoot native size-exclusion chromatography mass spectrometry (SEC-MS) workflow as the primary analytical technique, complemented by a 10-minute middle-down MS method and a 30-minute bottom-up MS strategy for confirmatory and site-specific analysis. This integrated approach offers high data confidence and sub-picomole sensitivity, enabling comprehensive characterization of intact mAbs, detailed structural assessments, and precise PTM localization.

We applied this platform in in-depth and high-throughput antibody forced degradation studies, providing time-course or real-time monitoring of critical quality attributes (CQAs), including aggregation, degradation, glycoform shifts, charge variants, and oxidation, all in a single run with minimal sample preparation. Enhanced by site-specific sulfenic acid tagging through click chemistry and advanced data interpretation pipelines, this system delivers molecular and kinetic insights into redox-sensitive modifications. It also supports antibody N293 glycan structural comparability assessments, facilitates predictive stability modeling, and enhances the overall reliability and safety of therapeutic mAbs. Additionally, the platform's speed and modularity make it ideally suited for rapid screening of antibody formulations, enabling high-throughput evaluation of formulation conditions, stress responses, and how stabilizing additives influence the structural stability.

The method described here establishes a new benchmark for next-generation antibody characterization, with particular relevance to public health preparedness and the accelerated development of biologics for emerging medical and formulation needs.