

Intact Cell MALDI-TOF Mass spectrometry and Top-Down High Resolution Mass Spectrometry to identify biomarkers in animal reproduction sciences

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Intact Cell MALDI-TOF Mass Spectrometry (ICM-MS) is a well-known methodology to identify microorganisms. Over the past years, this phyloproteomic approach was integrated in many clinical diagnostic laboratories as routine standardized procedures. In addition to bacteria identification, the MALDI-TOF MS technology can be used to phenotype isolated mammalian cells. By ICM-MS, endogenous biomolecules < 30 kDa can be detected but not identified. Of all currently available MS-based identification approaches, Top-Down proteomics constitutes the best strategy to identify peptidoforms and proteoforms, providing a complete description of primary structure of the molecular species and its modifications. The targeted identification of the peaks of interest, observed by ICM-MS, remains challenging. Here, we present an overview of our analytical strategy applied to biological models in relation with reproduction physiology (spermatozoa, oocytes, follicular cells) of different domestic animals (avian, ovine, bovine) or human. We describe a high throughput pipeline from ICM-MS differential analyses (UltrafleXtreme, Bruker) to Top-Down proteomics. Combination of pre-fractionation steps using HPLC decreased sample complexity before micro-liquid chromatography. Coupled to high-resolution tandem mass spectrometry (LTQ Velos Orbitrap, ThermoFisher) this approach resulted in construction of a database that integrated all the biomolecules identified within the biologic systems in relation with animal reproduction sciences. By this way, among the 3811 identified molecular species only a minor part corresponded to whole proteins, whereas the majority of the identified peaks were either N- or C-terminal fragments, or internal fragments of original proteins. Many of the identified proteoforms, which were cleaved from the same protein, contain common N-ter or C-ter sequences and constitute a series of cleaved products produced from sequential truncations. Many of them represent specific targets for proteases as kallikreins or caspases. Thus, our dataset contains more protein degradation products than whole proteins. However, the profile of these fragments is specific to type of the cells and their physiologic stages. Therefore, degradome profiling in our biologic systems seems to be highly sensitive to physiological changes, and provides specific signatures containing potential markers related to gamete maturation or fertility, which could be potentially useful for diagnostic using predictive models.

Investigation of top down protein measurements in a sensitive and robust PASEF enabled TIMS-TOF mass spectrometer

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Trapped ion mobility coupled to quadrupole time of flight platform has proven to be a successful configuration for bottom up proteomics application. Parallel accumulation serial fragmentation (PASEF) increases the sequencing capacity at the same time without losing ions in data dependent acquisition scheme (Meier, ..., Mann, JPR 2015, Meier, Brunner, ..., Mann, MCP 2018). This instrument is very robust and sensitive with no need for cleaning of ion optics even after constant data acquisition for more than a year and such a platform should also bear significant advantages for intact protein measurements. We hypothesize that the TIMS device could offer leverage for improved and controlled protein desolvation for very large protein molecules and can also be used for top down protein sequencing. TOF platforms have no inherent limitation on the upper mass limit and therefore the combination of Trapped ion mobility and the quadrupole-TOF should also enable top down protein sequencing measurements and native protein complex analysis. Here we explore the feasibility and benefits of performing protein top down experiments on the TIMS-TOF platform using purified proteins and protein standard mixtures. We believe that this platform should also enable large-scale top down protein sequencing projects.

1. Meier F, Brunner AD, et al. Online Parallel Accumulation-Serial Fragmentation (PASEF) with a Novel Trapped Ion Mobility Mass Spectrometer. *Mol Cell Proteomics*. 2018 Dec;17(12):2534-2545.
2. Meier F, et al. Parallel Accumulation-Serial Fragmentation (PASEF): Multiplying Sequencing Speed and Sensitivity by Synchronized Scans in a Trapped Ion Mobility Device. *J Proteome Res*. 2015 Dec 4;14(12):5378-87.

Selected examples of top-down proteomics-based approaches used within a mass spectrometry facility

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Top-down proteomics including top-down and middle-down mass spectrometry (MS) gain a constantly increasing importance in the field of structural analysis of individual proteins and complex protein mixtures. Rapid technical advancement in this area allows nowadays application of top-down-related approaches not only within research laboratories heavily specialized in the topic, but also in a MS facility within an academic institution.

Herein, we briefly present our current service activities as a MS facility in top-down proteomics and related techniques. Main instrument used for these experiments in our facility is QExactive HF Orbitrap (Thermo Scientific) equipped with Protein mode and HCD fragmentation.

One of the most frequently requested service analyses by our users is an accurate structural characterization of individual proteins, *i.e.* precise intact mass measurement, proteoform identification and localization of post-translational/synthetic modifications. As a routine example, we analyzed allergen proteoforms resulting from an *in-vitro* nitration reaction. Due to advantages of top-down MS, product proteoforms of bovine α -lactalbumin, main milk allergen of 14 kDa, were rapidly characterized in a direct infusion experiment and Y₁₈ was confidently assigned as a nitration site. A more challenging task was a targeted top-down MS analysis of 43 kDa stimulator of interferon genes (STING) protein modified with covalent small molecule inhibitors. After accurate intact mass measurements of this highly hydrophobic membrane protein, an amino acid residue binding with inhibitors was identified completing proteoform characterization. To overcome limitations of HCD fragmentation efficiency on relatively large STING protein and increase confidence of modification site detection, we combined data from LC-MS/MS runs with different fragmentation conditions.

Although, requests of top-down proteomic analysis on complex protein mixtures are still rare among our users, we have few interesting projects conducted with this approach. For instance, we qualitatively characterized proteins in exosomes, secreted from *in-vitro* grown human melanoma cell lines. In this case top-down approach revealed more insightful information about sample protein composition than classical bottom-up one, clearly demonstrating its significance for proteomics field.

Various virulence factors of pathogenic and symbiotic *Proteobacteria* species possess amyloid properties

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Amyloids represent specific variant of quaternary protein structure formed by highly-ordered unbranched protein fibrils stabilized by numerous intermolecular hydrogen bonds. This unusual spatial structure gives amyloids resistance to variety of physical and chemical factors including treatment with ionic detergents that solubilizes most non-amyloid protein complexes. Pathogenic amyloids are associated with the development of dozens of incurable diseases in humans and animals called amyloidoses. Functional amyloids are important for vital biological functions in a variety of species from Archaea to humans. In bacteria, amyloids are known to participate in the biofilm formation, toxin storage and overcoming surface tension. Thus, amyloid formation is important in both pathogenic and functional aspects.

Using previously developed bottom-up proteomic assay called PSIA (Proteomic Screening and Identification of Amyloids) we compared potentially amyloidogenic protein fractions resistant to treatment with ionic detergents isolated from common pathogenic *Gammaproteobacteria* species *Escherichia coli* and important nitrogen-fixing legume symbiont *Rhizobium leguminosarum* belonging to *Alphaproteobacteria*. We found that in both cases detergent-resistant protein fractions are rich in proteins involved in the virulence of these bacteria. In particular, outer membrane proteins that are essential for virulence of these species were found to be resistant to treatment with ionic detergents. Of these proteins, OmpA and OmpC of *E. coli* were previously shown to form amyloid fibrils while two porins of the *R. leguminosarum* outer membrane belonging to the Rop family were found to have amyloid properties in our work. In addition, we demonstrated that evolutionary conservative M60 mucin metalloprotease domain of the YghJ protein that is related to pathogenesis of enterotoxigenic *E. coli* is amyloid-forming.

Taking together, our data show that amyloid formation is associated with the virulence of both symbiotic and pathogenic bacteria belonging to the phylum *Proteobacteria*.

The study of amyloid proteins of *R. leguminosarum* was supported by the Russian Science Foundation (Grant No 17-16-01100).

Top-down proteomic approach for the study of basic proteins in the human sperm nucleus

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The protamine 1 (P1) and protamine 2 (P2) family comprises the most abundant basic proteins in human spermatozoa and packs 85-95% of the paternal genome. P1 is synthesized as a mature form, whereas P2 components (HP2, HP3, and HP4) are generated from the proteolysis of the precursor. The particular physical-chemical properties of protamines hinder their identification by the standard bottom-up mass spectrometry (MS) strategy. A top-down MS approach together with a bottom-up approach using proteinase K for enzymatic digestion are therefore proposed to identify human protamines and their post-translational modifications (PTMs) at the molecular level.

The intact protamine-enriched fraction from a single man was isolated from 15 million purified spermatozoa after histone removal and disulfide bonds reduction. Protamine enrichment was verified through acid-urea PAGE. The protamine-enriched fraction was analyzed by nanoLC-MS/MS using an Orbitrap Fusion Lumos mass spectrometer. Data analysis was performed using Proteome Discoverer 2.1 with Prosight PD 4.0 and Sequest HT nodes, TopPIC and BioPharma Finder software.

The top-down proteomic MS approach mainly allowed the identification of the intact naïve P1, while HP2, HP3 and HP4 were detected with minor peak intensities. A phosphorylation pattern and combinations of other PTMs among the different protamines were detected. The bottom-up strategy led to the identification of an alternative spliced variant of P2 identified at RNA level by our group and others.

The top-down MS approach allows the characterization of protamine PTM profiles at the intact level. The establishment of the normal protamine PTMs profile in fertile individuals and the identification of pattern alterations in different types of infertile patients would provide insights into the role of protamine PTMs code to male fertility and its potential function as epigenetic mark during early stages of preimplantation embryogenesis.

Probing the fundamentals of native LESA mass spectrometry of proteins

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Bioanalytical applications of liquid extraction surface analysis (LESA) have been increasing over the last decade due to its ease of use and high-throughput. Here, LESA in combination with travelling wave ion mobility (TWIMS) was employed for the structural analysis of horse heart myoglobin, ubiquitin from bovine erythrocytes and bovine carbonic anhydrase. For LESA, proteins were dissolved in ammonium acetate or in an acidic, denaturing buffer, dried and sampled from the surface of a glass slide. For direct infusion, samples were introduced without further manipulation for mass spectrometry. Firstly, we examined the native-like conformations of these three model proteins. Secondly, we compared their unfolding characteristics both in LESA and direct infusion modes. Thirdly, we examined whether it was possible to re-fold horse heart myoglobin and ubiquitin upon addition of a native-like buffer, using both sampling techniques. Charge state distributions (CSDs), drift time profiles and collision cross sections (CCSs) are presented here for each protein acquired in infusion and LESA mode to address these questions. We demonstrate that the overall folding landscape of all three proteins is preserved under native conditions when sampled with LESA, however a few minor, conformationally labile populations have been identified in case of horse heart myoglobin. We showed that once myoglobin had been denatured it could not refold to its holo-form, whereas ubiquitin refolded more readily. We found that drying prior to LESA sampling does have an effect, in particular on the conformational families of myoglobin. Overall, our data demonstrate the utility of LESA in conjunction with TWIMS for interrogating proteins with different biochemical (presence or absence of co-factor) and structural properties (predominantly helical, or β -sheet rich) similarly to direct infusion. As illustrated herein, LESA can be successfully incorporated into the toolkit of structural mass spectrometry that opens up new opportunities for studies of protein-ligand interactions and analysis of sub-complexes in a more complex, biological milieu.

Amyloidogenic properties of legume seed storage proteins bearing CUPIN-1 domains

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Amyloids are beta-sheet rich highly-ordered protein fibrils sharing certain distinguishing features including resistance to numerous influences such as detergent treatment or proteolytic digestion. Usually associated with pathological processes in human and animals, amyloids are now ubiquitously discovered to possess beneficial functions in different organisms; however, higher plants have been paradoxically overlooked so far. To reveal presence of amyloidogenic proteins we first performed a large-scale bioinformatic screening of all publicly available plant proteomes covering 75 species with total amount of proteins close to 2.9 million entries. Using two different algorithms, namely SARP and Waltz, we found several potential candidates distributed widely among numerous plant species, of which seed storage proteins were a notably enriched group of proteins. 302 protein entries in 54 of 75 species put into analysis, were identified as seed storage proteins bearing evolutionary conserved across plants CUPIN-1 family barrel domains, predominantly 7S and 11S globulins.

To validate our predictions, we focused our attention on legume seed storage proteins stored in intravacuolar organelles commonly referred to as protein bodies. These proteins are abundant in maturing seeds while after germination their quantity decreases rapidly. Indeed, proteins in question were identified by previously published PSIA (Proteomic Screening and Identification of Amyloids) bottom-up proteomic assay including several steps of purification and solubilization of detergent-resistant protein fraction of respective plant seeds followed by trypsinolysis, HPLC separation of tryptic peptides and protein identification by MALDI TOF/TOF mass-spectrometry. Further testing of amyloidogenic properties included analysis of the resistance to ionic detergent treatment, C-DAG (Curli-Dependent Amyloid Generator) fibril formation assay, and amyloid-specific stains (e.g. Congo Red and thioflavin T) binding assay for seed storage proteins in isolated protein bodies. Taken together, these results confirm natural amyloidogenicity of at least some of seed storage proteins containing CUPIN-1 barrel domain in legumes, though physiological meaning of these properties remains elusive. It is also unclear whether storage proteins form fibrils solely or co-aggregate with other proteins. Future top-down proteomic assay of respective aggregates isolated from seeds might shed light on both structure and proteinaceous content.

This work was supported by the Russian Science Foundation (Grant No 17-16-01100).

Characterizing iron loading pattern of ferritin through native MS

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Ferritin is a principal iron storage protein in most living organisms, usually formed by a 24-subunit protein shell and an iron core. The concentration of ferritin in serum has long been considered as a primary measure of iron status and can be easily quantified with immunochemical methods. However the iron loading pattern of ferritin was suggested to give a more comprehensive view than measuring only ferritin protein concentration, but the iron loading status of ferritin is less studied due to the lack of suitable method. Recent advances of mass spectrometry (MS) have expanded the observable m/z range, making the direct measurement of ferritin possible with MS.

In this study, a MALDI-TOF mass spectrometer equipped with a high-mass detector was employed to measure the iron loading distribution of ferritin. An average 2773 ± 1584 iron atoms were determined for a commercial holoferritin sample, and a coefficient of variation below 5 % was calculated from 12 technical replicates. To find optimum guidelines for sample preparation, the stability of holoferritin and apoferritin were assessed under different conditions, including various buffer pH values, cross-linking agents and MALDI laser intensities. The subunit composition of ferritin was also examined by ESI-MS and a H/L ratio of 11.5 was determined from the commercially obtained ferritin sample. This work provides a MS-based toolbox for measuring ferritin stoichiometry, which is meaningful for analysis of iron loading status. By integrating the iron loading status data into iron metabolism studies, interesting biological findings are expected.

Improved and Direct Detection of Antibiotic Resistance Proteins

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The number of antibiotic resistant bacterial infections is rising globally which makes many diseases that were thought to be in check again very harmful. Global travelling and dense populations support the development and spread of resistance and both of these factors are expected to rise within the next decades. The spread of resistance can lead to difficult nosocomial infections and complications of many surgeries, thus, routine medical interventions can again be very troublesome and sometimes deadly.

Rapid diagnostics of infection and resistance followed by targeted administration of antibiotics reduces pathogen burden in the patient, the use of antibiotics, fatality rate and health care cost and in the end shorten hospitalization. Reliable and fast detection of antibiotic resistance is thus absolutely crucial to reduce spread of resistance and manage existing infections efficiently.

In many clinical microbiology laboratories, identification of bacterial species is achieved by measuring a mass spectrum of the cells and subsequent database comparison and scoring. This MALDI-TOF mass spectrometry based method is very fast and reliable, however does not yet give additional information.

I will present a strategy to unambiguously identify bacterial resistance proteins by protein sequencing using a state-of-the-art MALDI-TOF mass spectrometer. The data I acquire using the developed methodology will then be incorporated in available species identification databases and will enrich the species information with additional information about the presence of antibiotic resistance proteins or virulence factors. This will enable infectious disease diagnostics centers to provide information about the identity and the antimicrobial resistance pattern of bacteria in a single, fast experiment. Using the expanded database and the resulting information, I expect to give medical practitioners a competitive advantage in treating antibiotic resistant infections.

Proteogenomics for the MALDI-TOF MS TDS-based rapid identification of novel bacterial isolates from the environment - TDP2019

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Sulfur sources and sulfurous lakes harbor a highly diverse microbial community. Within sharp gradients of sulfur and light, dense population (up to 10⁶ cells per ml) of mainly anaerobic phototrophic sulfur bacteria (PSB) of the genera *Chromatium*, *Lamprocystis*, *Thiodictyon*, *Thiocystis*, and the green sulfur bacteria (GSB) *Chlorobium* thrive.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been used to identify bacterial species in combination with *m/z* spectra libraries (MS fingerprint). However, the wealth of available bacterial (meta-) genomes now allows to expand the databases with predicted *m/z* signals to identify novel isolates.

We used this MALDI-TOF MS top-down sequencing (TDS) platform to characterize novel PSB isolates of the Lake Cadagno chemocline and a sulfur source within hours. Light harvesting proteins have been found to be the ideal biomarker to identify novel isolates.

This concept can be easily adapted to other microbial ecosystem with minimal preparative work and is a valuable and cost-effective tool for environmental microbiology.

Keywords: purple sulfur bacteria, sulfur cycle, biomarker, proteogenomics, MALDI-TOF MS, top-down sequencing

The big wedge for the small chunk - protein molecular ions split apart by soft X-rays

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Mass spectrometry (MS) methods have become integral parts of biochemical and biophysical studies during the last decades. Deliberate fragmentation is found more and more in the focus for increasing the information yield of the measurements with respect to composition, stoichiometry and stability of complex analytes. This holds true for bottom-up approaches as well as for top-down methods and, similarly, a variant of the latter, native MS. The most commonly used means to achieve fragmentation in these approaches has so far been collision-induced dissociation (CID). Although time-honored, CID is ‘channeled’ in a sense, which in turn promotes certain products in favor of others. The augmentation of CID by another, alternative fragmentation technique will, therefore, yield different and further fragmentation products and thus increase the information yield of the experiments in question.

The measurements were conducted as in house research at PETRA III P04 Beamline at DESY Campus in Hamburg in April 2018. Our mobile hybrid quadrupole / time-of-flight mass spectrometer extensively modified by MS Vision for transmission of high mass ions was further customized for coupling with synchrotron and FEL sources. We connected it to the open port at the beamline via internal and beamline-provided differential pumping stages; sample flow is orthogonal to X-ray beam. Several proteins and non-covalent protein complexes were subjected to soft X-ray radiation under native-like and denaturing conditions.

Spraying native-like oligomeric proteins, yielded the most striking results. Whereas without irradiation most of the selected protein ions remained intact, beam exposure increased fragmentation product formation tremendously. The major finding was increasing release of intact monomeric products from hemoglobin complexes. This observation is especially promising as the data hint at an alternative dissociation mechanism to CID and, thus, at the desired additional fragmentation products.

Top-down Characterization of the Biosimilar Filgrastim by Multimodal Tandem Mass Spectrometry

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An increased focus on development and testing of novel protein therapeutics and their biosimilars drive the need for advanced analytical methods for safety and quality assessments. Filgrastim is a recombinant form of human granulocyte colony-stimulating factor (G-CSF), indicated for prevention of chemotherapy-induced febrile neutropenia and treatment of severe chronic neutropenia. Our aim is to: 1) optimize a top-down mass spectrometry method for detailed characterization of filgrastim and 2) test the applicability of the topdownr software tool recently developed by us (Shliaha et al, 2018) ¹ to therapeutic proteins. We systematically tested different fragmentation conditions (CID, HCD, ETD, ETciD, EThcD and UVPD) using the Orbitrap Fusion Lumos platform. Filgrastim biosimilar samples were electrosprayed at both denaturing and non-denaturing conditions. As methionine oxidation is a modification that can negatively impact the protein product quality, we also analyzed oxidized filgrastim after incubation with hydrogen peroxide. By combining information from MS spectra and optimized top-down MS and MS/MS conditions, we assessed the impact of non-optimal storage conditions, such as increased temperature, on filgrastim's stability and degree of oxidation. We were able to obtain higher amino acid sequence coverage (>40%) than previously reported for top-down experiments on this protein (25%)² and to assign methionine oxidation sites in the oxidized samples. We expect that similar approaches can be developed for analysis of other therapeutic proteins.

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Antibody analysis by 2D MS

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Recombinant monoclonal antibodies and derivatives are widely used as therapeutic drugs. They are susceptible to posttranslational modifications (PTMs) that could occur during the manufacturing process and storage, resulting in product-related impurities. PTMs can change the efficacy, toxicity and the clearance of the antibody; therefore, they need to be well monitored and characterised.

The poster will investigate the potential of 2D MS for proteomics, and especially for the analysis of antibodies. 2D MS is a powerful technique, which enables the correlation between the precursor and its fragments in a single experiment without prior isolation. The trypsin digest of an antibody was analysed by 2DMS using IRMPD on the 12T FT-ICR mass spectrometer. The analysis of posttranslational modifications was investigated for the analysis of phosphorylation on proteomics samples. Finally, the use of 2D MS in a top down procedure for the analysis of the antibody will be discussed around the MS/2DMS analysis of the light chain.

Chromophore Renewal in Fluorogen-Based Reporters: Finding the Missing Link between Fluorescence Loss and Protein Oxidation

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Overcoming photo-bleaching is arguably one of the major challenges in bioimaging. This irreversible loss of fluorescence hinders imaging during long periods and more importantly, the accurate quantification of cellular processes in microscopy experiments. Renewable fluorogen-based reporters are composed of a genetically-encoded protein that binds a fluorogenic chromophore, which activates the fluorescence. In this study we propose to characterize the photo-bleaching properties of Y-FAST (Yellow-Fluorescence Activating and Shifting Tag), a fluorogen-based protein displaying fast exchange dynamics with its exogenous fluorogen, HMBR (4-hydroxy-3-methylbenzylidene-rhodanine). Early spectroscopic studies indicate that fluorogen renewal reduces the apparent photo-bleaching rate of the Y-FAST:HMBR duo through fluorogen exchange, with the photodegradation of the protein being 6.5x slower than its counterpart. The loss of fluorescence seems to be linked to two distinct oxygen-dependent photo-bleaching mechanisms affecting both the protein and the fluorogen counterpart.

To better elucidate the different mechanisms of Y-FAST:HMBR photo-bleaching, we performed a top down proteomics analysis of Y-FAST before and during irradiation. The analysis allowed to detect and to follow the kinetics of different oxidative events. They are localized on amino acids in the loop 94-101 that gates the entrance of HMBR to the binding pocket of Y-FAST.

This structural analysis provided agrees with a link between the loss of fluorescence, ROS production by irradiation and amino acids modifications. It opens the way towards the development of more photostable renewable fluorogenic systems (1).

1 Pimenta FM, Chiappetta G, Le Saux T, Vinh J, Jullien L, Gautier A. Sci Rep. 2017 Sep 26;7(1):12316

Sheathless CE-MS for the characterization of new therapeutic antibody formats

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Introduction/Background/Aim of the Work: Antibodies and related proteins are the most growing class of pharmaceuticals. The intrinsic complexity of these molecules and the large variety of antibody-derived proteins opens a demand for improved analytical methods that provides their reliable characterization. We have explore the capabilities of low-flow sheathless CE-MS for the characterization of various novel antibody-derived therapeutics including bispecific antibodies and new fusion proteins.

Instrumentation/Methods: Sheathless integrated capillary electrophoresis electrospray ionization was carried out on a CESI 8000 instrument (Sciex, USA) coupled to either an Impact qTOF mass spectrometer or a Maxis HD from Bruker Daltonics equipped with a nano-electrospray source. Protein separations were performed using neutral pre-coated or in-house polyethylenimine (PEI) coated capillaries with a porous tip. Solutions of 1-20% acetic acid containing 0-5% isopropanol were used as background electrolytes.

Digestion of the antibodies was performed using the endoproteinase IdeS. Bifunctional fusion proteins were reduced using DTT. Sample buffers were exchanged for water using Vivaspin 10kDa MWCO filter tubes (from Sartorius, Germany) prior injection.

Results & Discussion: In this work we have demonstrated the usefulness of sheathless CE-MS for the characterization of novel therapeutic antibody formats. Characterization have been performed after partial digestion of the antibodies with IdeS endoproteinase or after DTT reduction. For IdeS-digested mAbs, all charged variants were nicely separated in less than 20 min, including glycoforms containing sialic acids and other PTMs as loss of C-terminal lysine or deamidation of Asn. IdeS digestion and reduction of a bispecific antibody results on six separated chains allowing independent characterization of their PTM. The bifunctional fusion protein exhibited six N-glycosylation sites (three per HC) with different site occupancy. Analysis of DTT-reduced samples using a PEI-coated capillary allowed examination of the glycoform heterogeneity of the HC. HC containing 2- and 3 N-glycans per chain were separated permitting full assignment of specific glycoforms in the molecule.

Novel Aspect: Application of sheathless CE-MS to new antibody formats

