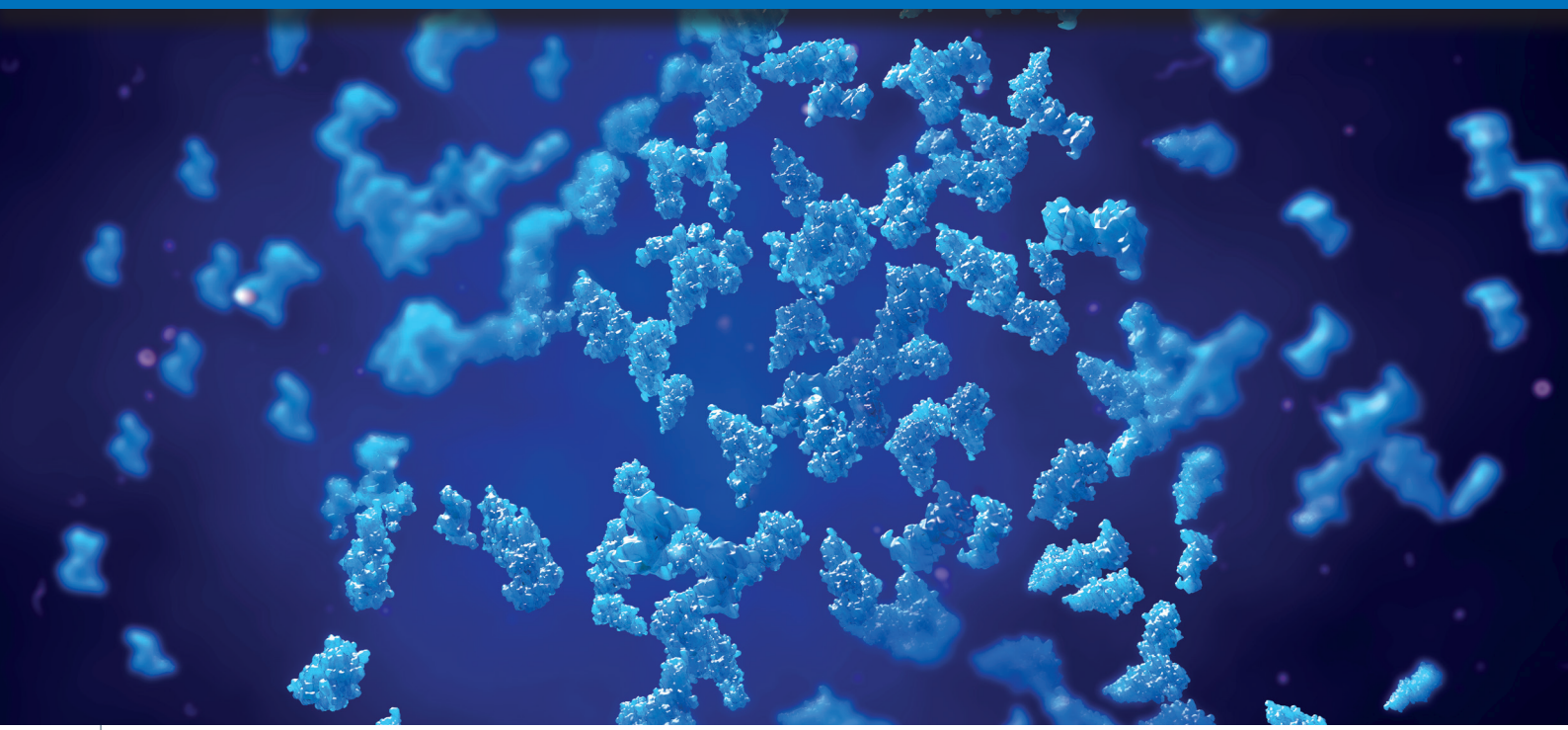

Improve your XL-MS results using the 4D-Proteomics based caps-PASEF acquisition method and the innovative PhoX crosslinker

Crosslinking mass spectrometry is a powerful and widely used technique to study the tertiary and quaternary structures of proteins.

Abstract

Covalent bonds between residues in close proximity are created by crosslinking reagents under native conditions, to ensure that only relevant structural information is retrieved. By reducing, alkylating, digesting and mass spectrometric analysis these residue-residue crosslinks can be identified, from which distance restraints can be derived with a resolution of 20-30 Å. The identification of the crosslinks is however hampered by the presence of unmodified and monolinked peptides (one reactive group coupled to the protein while the other is quenched), which typically overwhelm the

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signals from the crosslinked peptides of interest. The newly introduced trifunctional crosslinker PhoX in combination with timsTOF Pro and Collisional Cross Section Assisted Precursor Selection (caps-PASEF) boosts the identification rate for crosslinked peptides by depletion of unmodified peptides prior to MS-analysis and by preferential MS/MS acquisition of crosslinked peptides during mass spectrometric analysis. This makes it feasible to readily identify 98 crosslinked peptide pairs for BSA and 364 crosslinked peptide pairs for a HeLa lysate from a single-shot experiment [4].

Introduction

Proteins typically fold into well-defined tertiary structures and interact with other proteins to form homo- and hetero-oligomers to accomplish their physiological functions. These functions have implications on health and disease, but are also relevant for biotechnology applications. This generates significant interest in protein structure analysis, and in methods that can rapidly provide structural information for a diverse set of protein samples. Crosslinking MS has proven to be particularly suitable for this task due to its ability to reveal structural information for protein samples of varying complexity (ranging from purified proteins to cell lysates and intact cells), varying protein size (due to digestion the problem is divided to peptide sets), and flexibility (highly flexible regions in proteins that are difficult to visualize by other techniques are typically well covered). Proteins are crosslinked under native conditions with a reagent that – at a minimum – consists of two amino acid reactive groups separated by a spacer arm. Subsequent enzymatic digestion generates peptides that remain covalently connected by this crosslinker, which allows identification of residues that were in proximity in the native protein state – thus providing information on protein structure and structural dynamics. The identification of crosslinked peptides is however difficult due to the fragmentation spectra containing a mixture of two peptides. Crosslinking is generally carried out so that only one or a few crosslinks are present on each protein molecule to avoid that multiple crosslinks perturb the native state structure. This has the consequence that crosslinks are present as a small fraction of the final peptide sample (typically estimated as roughly 0.1%), thereby further complicating the analysis [1,2,3].

The novel trifunctional crosslinker PhoX allows for enrichment of crosslinked peptides, drastically reducing sample complexity prior to mass spectrometric analysis. Besides two NHS moieties that react with lysine-sidechains, it contains a phosphonic acid group that serves as an affinity tag. PhoX modified peptides are bound by immobilized metal affinity chromatography (IMAC), and can be eluted with ammonia, providing a convenient one-step method for efficient removal of unmodified peptides. The enrichment handle additionally is hydrolysis-stable, which allows for specific removal of phosphorylated peptides. IMAC enrichment has reached a high degree of reproducibility and automation allowing for parallel processing in a 96-well plate format.

The enrichable handle resolves the issue of the massive background of unmodified peptides, however monolinks are affinity captured and remain in the final mixture. Ion mobility separation on the timsTOF Pro with charge state determination based on isotopic patterns on the fly allows for the separation and preferential fragmentation of crosslinked peptides by Collisional Cross Section Assisted Precursor Selection (caps-PASEF), excluding the fragmentation of less informative monolinked peptides and thus enhancing the information obtainable from a crosslinking experiment.

Material and Methods

Proteins were crosslinked with PhoX (available at Bruker **#1881358**). The reaction was quenched by addition of Tris-HCl. Following reduction with DTT and alkylation with IAA, proteins were digested with LysC and trypsin. For the enrichment of PhoX-modified peptides, peptides were desalted with a Sep-Pak C_{18} column, dried, resuspended in 80% acetonitrile, 0.1% TFA, and loaded onto Fe(III)-NTA cartridges (Agilent G5496-60085). After washing with 80% acetonitrile, 0.1% TFA to remove unmodified peptides, PhoX modified peptides were eluted with 10% ammonia. The eluent was neutralized with formic acid, dried, and resuspended in 10% formic acid for mass spectrometric analysis.

Peptides were analyzed with a nanoElute UHPLC coupled to a timsTOF Pro mass spectrometer (Bruker Daltonics GmbH & Co. KG). Injected peptides were separated on a 25 cm, 75 μm ID IonOpticks C_{18} column using a 68 min gradient from 2% to 34% acetonitrile, 0.1% formic acid.

Data were acquired in PASEF mode. Precursors were selected based on collisional cross section (CCS) and monoisotopic mass with a user-defined polygon for data dependent selection of precursors for fragmentation.

Raw data were converted to .mgf files with FragmentLab (https://scheltemalab.com/software) and analyzed with XlinkX version

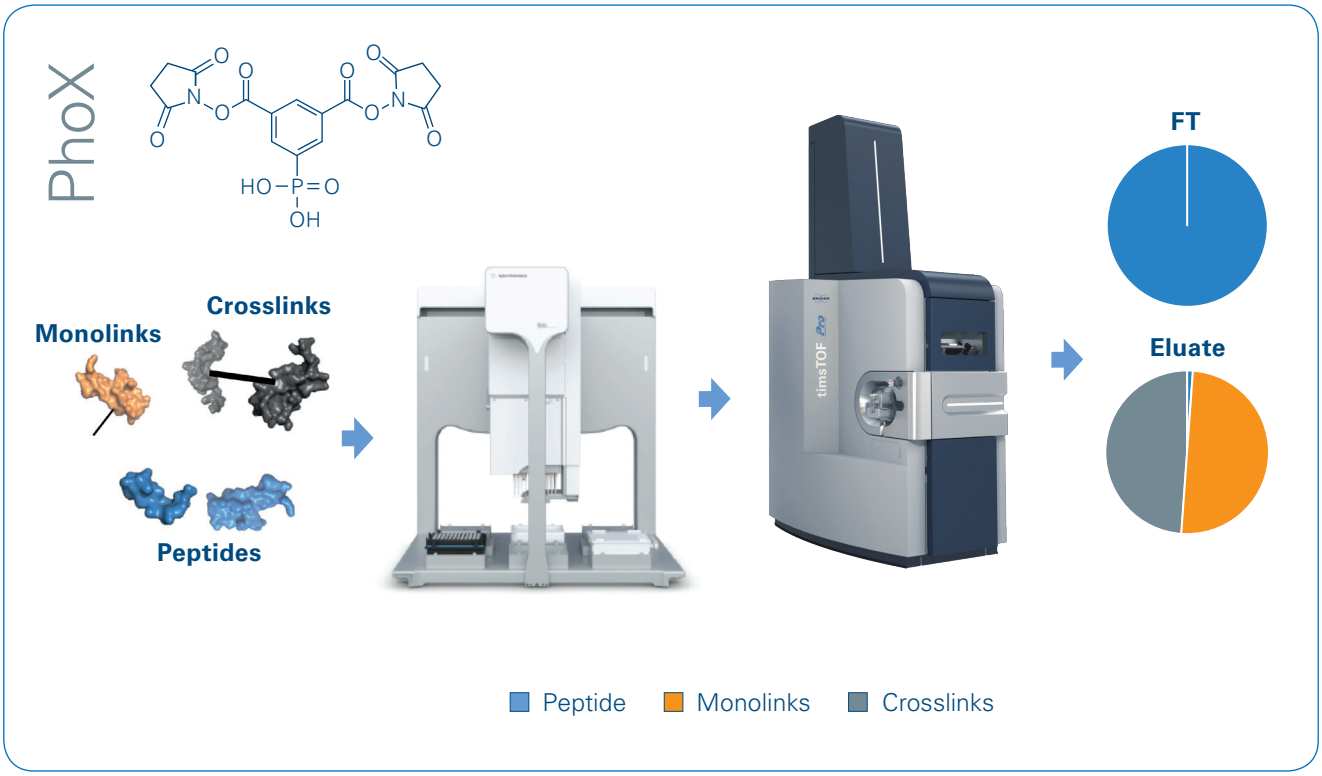

Figure 1: Workflow for the enrichment of PhoX modified peptides. Unmodified peptides are not retained by Fe^{3+} IMAC and are thereby removed.

2.4.0.193. Details on experimental procedure and data analysis can be found in Steigenberger, B. et al., Molecular & Cellular Proteomics 2020 [4].

Results and discussion

Crosslinking and subsequent enzymatic digestion results in complex peptide mixtures even for single proteins and protein complexes. These peptide mixtures generally consist of four products: unmodified peptides, peptides connected to a partially quenched linker (monolink), intra-peptide crosslinks (looplinks), and two peptides covalently connected by the crosslinking reagent (crosslinks). The latter provide information on protein tertiary structure in the form of intra-links (two peptides from the same protein) and protein quaternary structure in the form of inter-links (two peptides from different proteins). Frequently, those mixtures are fractionated by size-exclusion or ion-exchange based chromatography prior to mass spectrometric analysis to reduce sample complexity and increase the probability for identification of low-abundant crosslinked peptides. PhoX alleviates the need for time-consuming fractionation. The phosphonic acid group allows for batch-based and automated IMAC enrichment for efficient removal of unmodified peptides. A comparison of non-enriched and enriched PhoX crosslinked BSA-derived peptide sample revealed that the affinity chromatographic step led to a 300-fold enrichment of crosslinked peptides with 97% enrichment specificity in the eluate, while almost all of the unmodified peptides end up in the flow-through (Figure 1) [3]. The reduction of unmodified peptides with concomitant relative increase in abundance of crosslinked peptides drastically improves the identification of crosslinked peptides by reduction of ion suppression and by reduction of fragmentation spectra for unmodified peptides.

The timsTOF Pro offers the capability of ion mobility-based separation prior to mass spectrometric analysis, which is particularly beneficial for the analysis of crosslinked peptide samples. Lower mobility ions with larger collision cross sections are eluted first, making it feasible to separate larger, crosslinked peptide ions from smaller, monolinked peptide ions (Figure 2A). To be successful, the instrument additionally needs to generate informative fragmentation spectra for precursors containing two separate peptides connected by the crosslinking reagent, for which

the stepped collision energy settings provide excellent support (Figure 2B). $1/K_0$ and *m/z* were transformed to collisional cross section and monoisotopic mass using charge states determined from isotopic patterns to define a polygon filter in the collisional cross section versus monoisotopic mass dimension (Figure 2C). Collisional Cross Section Assisted Precursor Selection (caps-PASEF) based on a polygon including only ions larger 500 Å and 2 kDa was successfully used to preferentially select crosslinked peptide ions for fragmentation (Figure 2D – left panel). Such a selection resulted in a reduction of fragmented monolinked peptide ions by 86% and an increase in fragmented crosslinked peptide ions by 23% in case of a PhoX crosslinked BSA sample (Figure 2D – right panel) [4]. This highlights the utility of ion mobility selection for improved fragmentation of crosslinked peptides over monolinked peptides.

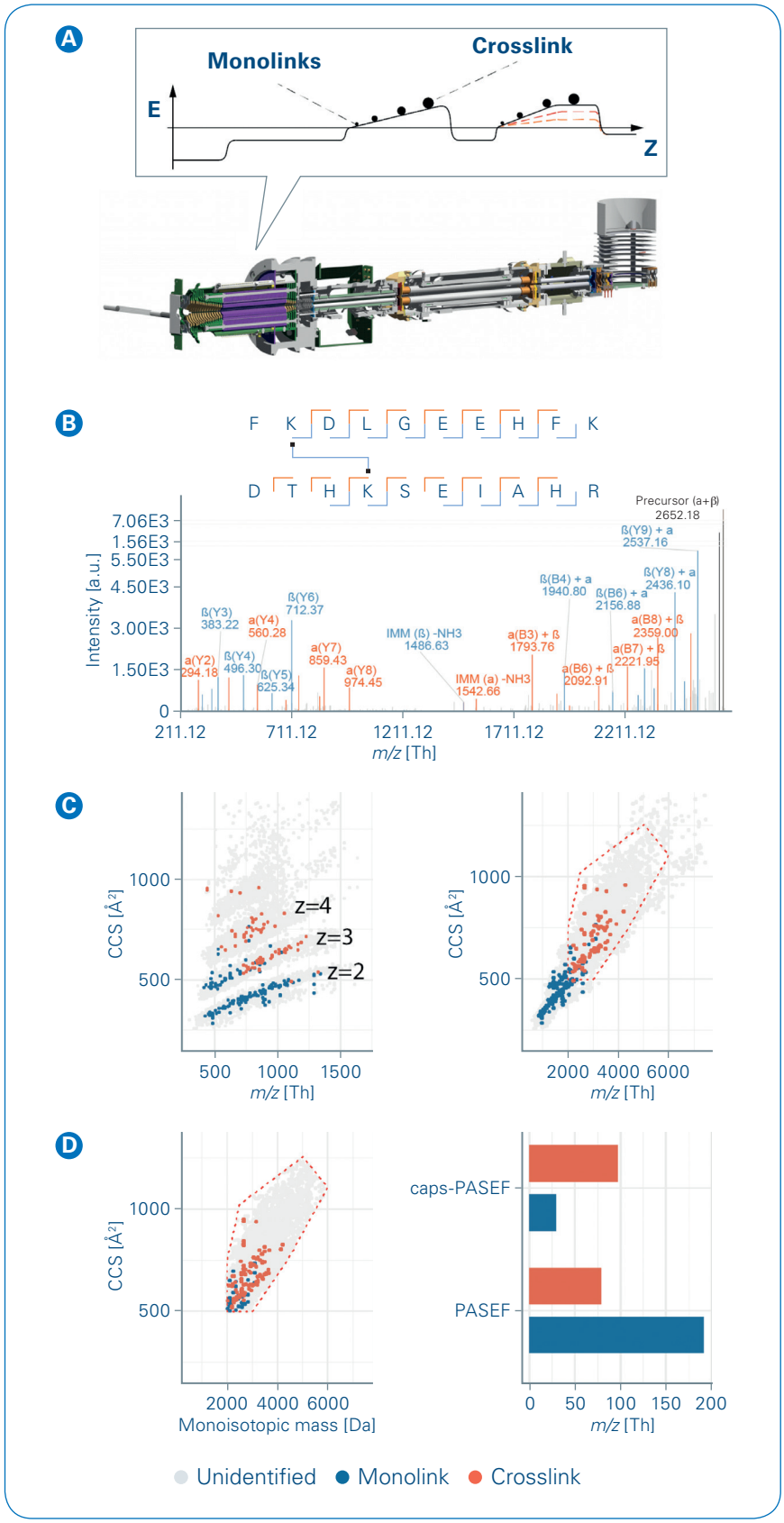

Figure 2: Monolinked and crosslinked peptides are separated by ion mobility prior to fragmentation. Selection of peptide ions with CCS larger 500 Å and mass larger 2 kDa allows for preferential fragmentation of crosslinked peptide ions, resulting in a reduction of monolinked peptides being scheduled for fragmentation. Adapted from Steigenberger, B. et al., Molecular & Cellular Proteomics 2020.

Conclusion

PhoX enables efficient and automatable enrichment of crosslinked peptides prior to mass spectrometric analysis. Mono- and crosslinked peptide ions can be partially mobility separated using the timsTOF Pro mass spectrometer. This makes it feasible to define a characteristic precursor collisional cross section (CCS) versus monoisotopic mass dimension for preferential data-dependent fragmentation of crosslinked peptides, thereby increasing the depth of analysis for XL-MS.

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PhoX (available at Bruker **#1881358**)

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