Technological developments in mass spectrometry towards molecular structural elucidation of macromolecular assemblies

Higher-order molecular structural elucidation of multiproteoform complexes (MPCs) is one of the most attention grabbing applications in the field of mass spectrometry (MS), as the MS-based structural toolbox has the ability to characterize the structure of MPCs at its all molecular complexity levels. MS has demonstrated the ability to retrieve important structural information typically missed by high-resolution biophysical approaches. The work presented in this thesis focused on the technological advancements towards the molecular structural characterization of large MPCs by bringing together soft-ionization techniques, mass-to-charge ratio (*m/z)*-resolved imaging using position- and time-sensitive Timepix (TPX) and TPX3 application-specific integrated circuit (ASIC) based detection assemblies. Nano-electrospray ionization (nESI), matrix-assisted laser desorption/ionization (MALDI), axial/orthogonal reflectron time-of-flight (TOF) and high-resolution Orbitrap MS, top-down proteomics (ultraviolet photodissociation (UVPD)) were all explored to evaluate the different elements of our approach.

Although the TPX ASIC has been successfully implemented in biomolecular MS in combination with microchannel plate (MCP) amplifiers in the past, it has only been used in the detection of relatively small biomolecules. No native, non-covalent large MPCs were detected using TPX based detection systems. The MCP-TPX quad detection assembly equipped with nESI-orthogonal reflectron TOF LCT MS employed for the studies conducted in **Chapter 2** enabled and demonstrated the detection and imaging of the multiply charged non-covalent 14-mer (GroEL) with a molecular weight in excess of 800 kDa. Moreover, GroEL was measured by TPX as well as conventional detector on two LCTs under identical ion optics conditions, and the results were compared and contrasted. The better quality GroEL TPX spectrum supports the previous investigations conducted on TPX based systems, where the TPX detector provided spectra with enhanced signal-to-noise (S/N) ratio and sensitivity compared to conventional detectors. The potential of the TPX to detect and image individual MPC ion events demonstrated in this Chapter forms the foundation of the investigations performed in Chapters 3 and 6. The ability of the TPX detector to provide both the TOF spectrum and the spatial distribution of the ions striking at the detector offers a more detailed insight into the functionality of each ion optical element within the mass spectrometer as well as the corresponding ion trajectories. This makes TPX a suitable tool for the optimization of newly developed MS instruments and allows for more direct comparisons between the ion optics simulations and experimental data. For instance, in the LCT system, a strangely behaved secondary, low resolution, low *m/z* signal at the TOF spectrum of high mass species was observed. Similar, low *m/z* signals when spraying high mass species were previously observed in

quadrupole (Q)-TOF I, II and LCT MS from Waters MS instruments. However it was impossible understand the origin of the signal merely by examining the mass spectrum. Here, using the unique capabilities of TPX detectors and ion optic SIMION model of the LCT, we were able to identify this unknown signal as "secondary electrons formed by the interaction of intact precursor MPC species with the TOF housing", which appear as a secondary distribution in both time and space domain.

The ability of the TPX positioned at the back of the MCP to measure the pixel cluster size corresponds to individual ion events was utilized to understand the response of the MCP to the ion optics parameters and ion properties (**Chapter 3**). This allowed us to characterize the MCP's performance in the high mass low velocity regime, where the MCP has reduced detection efficiency. The pixel cluster size of the secondary electron cloud generated by the MCP on the TPX for each individual ion event is analyzed as a measure of MCP performance at each *m*/z value for the following measurement ranges; ion mass = 195 to 802,000 Da, ion velocity = 8.4 to 67.4 km/s, and ion charge = 1+ to 72+, and resulted in a Poisson distribution. The MCP detector performance is shown to improve with an increase in ion charge, velocity, and energy and deteriorates with an increase in ion mass, *m*/z, and TOF. The dependence of ion optical parameters such as TOF tube voltage, MCP bias voltage, and potential gradient between back plate of the MCP and TPX on the MCP charge cloud footprint can be utilized to achieve optimum image quality when MCP is used in imaging systems, as well as to improve native macromolecular ion detection efficiency in the high mass low velocity regime. We were able to derive an MCP performance equation for individual TPX registered single ion events based on two independent ion properties, ion mass and charge; pixel cluster size/MCP performance $\propto m^{-0.49}q^{0.77}$. This equation indicates the oligomers of MPCs that appears at same *m*/z values in the mass spectrum are expected to have different pixel cluster sizes. A separation based on pixel cluster size can be benefitted for the analysis of highly heterogeneous native MPCs samples and complements the single particle MS methods such as charge detection mass spectrometry (CDMS).

Chapters 2 and 3 utilized earlier generation TPX chip from TPX detector family that was limited by a moderate time resolution (at best 10 ns) and single-stop detection for each pixel that hampered the detection of ions with high *m*/z values at high pixel occupancies. In **Chapter 4**, a Silicon (Si)-coated TPX3 chip, the successor of TPX, has been employed as the linear detector in a MALDI-axial TOF Ultraflex III MS instrument. The enhanced time resolution (1.5625 ns), simultaneous measurement of time-over-threshold (TOT) and time-of-arrival (TOA), and multihit capabilities of the TPX3 chip,

allowed the generation of the TOF/mass spectra with a better S/N ratio and improved the sensitivity of high *m/z* detection in the presence of low *m/z* ions at high count rates and detector voltages. In contrast to the previous detector setup discussed in Chapters 2 and 3, where the TPX chip is directly coupled to the MCP, a P47 phosphor screen is placed in between the MCP and Si-coated TPX3 chip in Chapter 4. This allowed the TPX3 assembly to be installed at atmospheric pressure that brings considerable flexibility, elimination of several technically challenging elements, and flexible mapping between phosphor screen and sensor area compared to the previous MCP-TPX setup. This experimental setup significantly extended the *m/z* range previously detected with the TPX family by the measurement of the intact singly charged IgM ions of *m/z* approaching 1,000,000 Da. In addition, the ability of the TPX detector to provide both TOF spectrum and spatial distribution of the ions striking at the detector along with the application of deflector and retarding voltages allowed the examination of the metastable fragments formed at different parts of the flight tube. The unique ability of space and time-resolved detection to distinguish metastable fragments formed at different locations along the flight path provides an interesting avenue to study the kinetics of metastable ion decay. Additionally, the influence of both MALDI and parameters such as laser fluence, MALDI matrix, and extraction conditions on the metastable decay rate can be investigated with this approach.

Next, we have developed a unique Orbitrap/TOF MS based instrument for the extensive structural and molecular characterization of MPCs at different levels of its organization. **Chapter 5** focused on the detailed design, development and evaluation of the QExactive ultra-high mass range (UHMR) Orbitrap/TOF MS instrument with integrated UVPD and TPX detection assemblies– MCP-TPX quad (used for studies in Chapters 2 & 3) and MCP-P47-TPX3CAM (used for study in Chapter 4). This instrument has four modes of operation that enable the high mass resolution measurement and mass-resolved imaging of UVPD generated fragments from the native MPC ions using the Orbitrap mass analyzer and TOF analyzer-TPX3 imaging assembly, respectively. UVPD on high-resolution Orbitrap and TOF MS instruments has already been employed by several groups to obtain the tandem MS spectra that allow high-level structural and functional characterization of MPCs by extracting information on amino acid sequence, conformation, subunit stoichiometry and molecular interactions. However, the TOF imaging approach implemented with the TPX3 detection assembly allows the visualization of the 3D spatial UV dissociation event of the MPCs. This is a direct result of the unique capability of the imaging TOF analyzer that was designed to maintain the relative positions of the expanding UVPD fragment subunits from UVPD event to

TPX3 detector. There, both arrival time and position are recorded simultaneously with the ultimate goal to reconstruct the 3D geometry of the MPC prior to the UVPD event.

In **Chapter 6**, the *m*/z-resolved TPX3 images of UV generated subunit fragments generated from non-covalent protein complexes at the middle of the pusher have been explored using the TOF imaging approach implemented in the custom-developed Orbitrap/TOF instrument described in Chapter 5. The *m*/z-resolved TPX3 images were utilized to interpret energetics of the 3D MPC dissociation event. The relative distance and angular distribution of the product ions with respect to the impact position of the precursor MPC ions from the *m*/z-resolved TPX3 images is determined. This information will be crucial in retrieving higher-order structural characteristics of the MPC, such as bond strength, conformation, etc., as well as the influence of charge state on the behavior of the MPCs in the gas phase. In addition, the evaluation of the *m*/z-resolved TPX3 images after the integration of other fragmentation methods such as electron capture dissociation (ECD), electron transfer dissociation (ETD), surface-induced dissociation (SID) and infrared multiphoton dissociation (IRMPD) to the Orbitrap/TOF system is expected to yield significant information on various fragmentation mechanisms. We were also able to show the spatially- and temporally (*m*/z)-resolved detection of the subunit fragments produced from single precursor MPC ions of dimeric β-lactoglobulin (36.5 kDa) and tetrameric concanvalin A (102 kDa). The potential of this instrument to probe the fragmentation process at single molecule level can be used to gain a thorough understanding of the fragmentation pathways. Most importantly, the TPX3 images generated by the UVPD of single precursor tetrameric concanavalin A ions to four monomer subunits at high laser energy resemble the projection of concanavalin A's 3D geometry. The analysis of thousands of these types of single ion UVPD fragment, *m*/z-resolved images might allow the reconstruction of the 3D molecular structural model of MPCs. However, the shift in the impact position in one direction due to the ion acceleration in the axial direction prior to the fragmentation process has to be deconvolved before the reconstruction process as this shift might introduce some distortions to the conformation of the MPCs. Also, better computational tools need to be developed in order to fit various geometries to the 2D detector images. This study must be extended to larger oligomers with different conformations in order to demonstrate the effect on the 3D conformation observed with this method.