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

Defining the overall impact of basic scientific instrumentation research, such as the work presented in this thesis, is a difficult undertaking. The effects of such research that will eventually translate into biological applications may not be immediately apparent on short or medium-term timescales. Multiple analytical tools are necessary to understand the omics of complex biological systems, and even with their usage, it is impossible to comprehend all aspects when trying to answer a biological question. Nonetheless, we believe that the technological developments in mass spectrometry accomplished here have pushed forward that envelope and will likely have a long-term impact on molecular/structural biology.

The main focus of this thesis was on the technological advancements in mass spectrometry (MS) for the retrieval of structural features of multiproteoform assemblies (MPCs) at all levels of its molecular complexity using a single instrument platform. **Chapters 2-4** detail the characterization of the imaging systems from the Medipix (MPX)/Timepix (TPX) family for the detection of high-mass native single MPC ions, while **Chapters 5-6** outline the utilization of these TPX-based detection assemblies along with ultraviolet photodissociation (UVPD) in a custom-built Orbitrap/TOF (time-of-flight) instrument for TOF-resolved imaging and high-resolution Orbitrap analysis of MPCs. This innovative Orbitrap/TOF instrument paves the way to resolve the higher-order molecular structure of MPCs in their pseudo-native state in the gas phase. The high-resolution UVPD Orbitrap spectrum is capable of characterizing the molecular structure of the MPCs at all levels of its organization by retrieving information such as proteoform sequence, subunit stoichiometry, conformation, and molecular interactions. The energetics of the MPC dissociation process is revealed by the TOF imaging approach, which also has great potential in elucidating the quaternary structural features of MPCs including 3D geometry and interface area. However, it might take a few more years to reconstruct the 3D conformation of the MPC as this method still has to be validated on a number of oligomers with different conformations and requires the development of computational tools. Once this is accomplished, this instrument will serve as a unique platform for the extensive molecular, structural, and functional characterization of complex MPCs with biological relevance. The successful application of this unique MS-based platform will complement the structural research of cryogenic electron microscopy (cryo-EM), nuclear magnetic resonance (NMR), x-ray crystallography, and other structural MS-based methods by offering new perspectives on a shared goal. This approach will be a great addition to the MS-based structural toolkit and allows researchers to interrogate protein systems that are challenging to conventional high spatial resolution biophysical methods, e.g., intrinsically disordered and membrane proteins for x-ray crystallography, small proteins for cryo-EM, and highly heterogeneous MPCs for NMR.

The innovative Orbitrap/TOF instrument developed here is expected to have a significant impact in both clinical and pharmaceutical research settings. The study and elucidation of the complex MPC structures can be accomplished using

only minute amounts of simple solution-phase samples, which may eventually be applied in clinical settings in which only small amounts of patient samples can be obtained. The MPC structural information is critical in understanding structure-function relationships and drug/ligand binding sites, two essential parameters for pharmaceutical product development. It is foreseen that our novel technologies will draw the interest of pharmaceutical and biotechnology companies. Although of immediate interest to clinical and pharmaceutical research, extensions of the technology presented in this thesis can be applied for the extensive characterization of non-biological materials like nanoparticles as well.

This research emphasizes the importance of collaboration between research institutes and industrial partners as well as among research institutes. During different phases of my PhD, I had the privilege to visit and explore the MS facilities in Fasmatech Science and Technology (Athens, Greece), the provider of the custom-designed and built TOF analyzer used for the studies conducted in **Chapters 5-6**. Working on the ion optics design and testing of the TOF analyzer and associated ion optics with Fasmatech, along with inputs from ThermoFisher Scientific (Bremen, Germany), one of the leading companies in MS instrumentation and research, and the provider of the Q Exactive Orbitrap ultra-high mass range (UHMR) mass spectrometer to which the custom-built TOF analyzer was integrated, was an incredible experience. The joint research between M4i, Fasmatech, and ThermoFisher Scientific led to further collaborations between M4i and these companies, including the integration of a custom-built matrix-assisted laser desorption/ionization (MALDI) ion source (Fasmatech) to an Orbitrap Elite mass spectrometer with an aim of improving the spatial resolution to 1 µm in mass spectrometry imaging (MSI) and coupling an Aquilos cryo-focused ion beam/scanning electron microscope and an Orbitrap Exploris 480 mass spectrometer to enhance the spatial and mass resolution in MSI (ThermoFisher Scientific). Throughout my thesis, I had the wonderful opportunity to work closely with Amsterstedm Scientific Instruments (ASI, Amsterdam, the Netherlands), the supplier of the TPX3CAM (TPX3 based camera), the imaging detector used for the research conducted in **Chapters 4-6**, which facilitated the exchange of knowledge, experience, and insight from both perspectives. While I communicated the added value of position-sensitive detectors in the MS field from a user's point of view, ASI provided valuable resources and expertise in hardware and software development and tailored solutions in TPX-based detection technology. Furthermore, ASI is actively collaborating on a number of projects with M4i, including the integration of TPX3CAM to a BioTRIFT mass spectrometer to enhance the throughput in MSI[152], as well as the employment of TPX3 detectors to Artica and Polara microscopes with an aim to operate EM at a broader range of energies, at higher throughput and higher dynamic range (Department of Nanoscopy, M4i).[357-358] Besides the successful collaborations with the industry partners, I gained hands-on experience on native MS and UVPD implemented on the Q Exactive UHMR and Exactive plus extended mass range (EMR) mass spectrometers from the Hecklab, Utrecht University (Utrecht, the

Netherlands). Without this knowledge transfer, it would have been challenging to implement native MS (**Chapters 2, 3, 5 and 6**) and UVPD (**Chapters 5-6**) at M4i with the same level of ease and success. In addition, for the studies conducted in **Chapter 2** two similar LCT TOF mass spectrometers were utilized; one modified with the TPX detector at M4i and the other a standard LCT at Hecklab. The incorporation of TPX imaging detectors to LCT and Ultraflex III mass spectrometers for the visualization of time-resolved images of the electrospray ionization (ESI)/MALDI ion cloud provides insights into the ion trajectories and ion optical processes (**Chapters 2-4**), which led to the in-depth discussions with the leading MS instrument manufacturers such as Waters Corporation (Wilmslow, UK), MS Vision (Almere, the Netherlands) and Bruker Daltonics (Bremen, Germany).

Even though the focus of my Ph.D. was on the technical aspects of MS, I devoted considerable time performing native MS to address some relevant biological questions that were not included in this thesis. Through an ongoing collaboration between the Technical University of Munich and both divisions of M4i - Mass Spectrometry and Nanoscopy, native MS and cryo-EM have been utilized as tools to characterize exosome samples from different breast cancer cell lines and SARS-CoV2 Spike protein transfected and non-transfected HEK cells, in addition to flow cytometry. Native MS has been a valuable tool for the structural and functional characterization of ESX1 (one of the molecular machinery of Mycobacterium tuberculosis)-secretion system related proteins, in addition to the biophysical techniques such as x-ray crystallography, cryo-EM, and circular dichroism spectroscopy. These studies were carried out in collaboration with the Department of Nanoscopy, M4i. Additionally, I had the opportunity to explore various ionization sources and solvent conditions to characterize the molecular structure of some of the challenging MPCs that include intrinsically disordered proteins, membrane proteins etc. in their pseudo-native state in the gas phase. These projects were conducted in collaboration with various research institutes and companies, including the National Research Council (Catania, Italy), Indian Institute of Technology (Kanpur, India), University of Amsterdam (Amsterdam, the Netherlands), and Cristal Therapeutics (Maastricht, the Netherlands).

The majority of the studies presented in this thesis are published in open-access journals to enable fellow researchers and industry entities to benefit from them for their own purposes. In addition, these findings have been presented at various academic meetings, which have attracted individuals from diverse scientific backgrounds.

From an industry perspective, the development of innovative and powerful tools for the structural elucidation of MPCs is one of the most active areas of research for MS manufacturers due to the rapidly growing market demanding this technology. The novel Orbitrap/TOF instrument platform developed from this research will open new markets and revenue streams for both MS instrument manufacturers such as ThermoFisher Scientific and SMEs including Fasmatech Science and Technology and Amsterdam Scientific Instruments.

From a fundamental science perspective, the research conducted here has utilization in many different research fields. Specifically, the structural proteomics research will be accelerated both with the instrumentation platform and corresponding algorithms which will be developed for the reconstruction of the 3D structure of MPCs. The 3D structural data will benefit investigators working in proteomics, specifically by providing structure-function relationships. The technological enhancements achieved through the utilization of the active pixelated detectors from the Medipix (MPX)/Timepix (TPX) family (**Chapters 2-6**) will benefit diverse and broad research fields including fast mass microscopy, structural biology, applied physics, and engineering, where the MPX/TPX detection technology has been applied.[140, 145, 147, 152, 357-367] Through the strong embedding of M4i's detector research within the Medipix consortium at the European Organization for Nuclear Research (CERN, Geneva, Switzerland), the future research will provide new outlets for the expansion of technology developed within this large, international consortium. For instance, the future studies will be benefited from the deployment of Timepix4[138], the latest detector from the MPX/TPX family, which is the first large area (448 x 512) high-resolution pixel detector readout application-specific integrated circuit (ASIC), which provides significantly higher time-of-arrival (TOA) and time-over-threshold (TOT) resolutions than its predecessors TPX[71, 135, 290] and TPX3[137, 322] ASICs used in this research.