**THESIS TITLE:** ANALYTICAL CHARACTERIZATION OF BIOLOGICAL PRODUCTS

**Abstract**

The term "biological products" refers to the therapeutic products that are manufactured by living organisms. Currently these biologicals, also called biotherapeutics, dominate the pharmaceutical market. However, these are sensitive to perturbations in their immediate environment which can affect their potency, efficacy, and at times, even safety. Analytical characterization of these biological products is thus paramount, both for the designing of new drugs and for analyzing those already available in the market. Depending upon the information that requires detailing, several analytical tools and techniques can be employed for in-depth characterization of the molecule of interest. These include enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), mass spectrometry (MS), dynamic light scattering (DLS), and cryo-electron microscopy (cryo-EM).

The current work demonstrates how we can utilize some of the aforementioned technologies for in-depth and fast characterization of a variety of biological products, including monoclonal antibodies (mAbs) and their biosimilars, host cell proteins (HCPs), antivenoms, and virus-like particles (VLPs).

The first objective involves utilization of different cyclodextrins (CDs) for the improved detection of mAb charge variants. Charge heterogeneity is a critical quality attribute (CQA) for mAb-based therapeutics. Its characterization involves identification of charge variant species in the final formulation. Capillary zone electrophoresis (CZE) is a valuable tool to assess charge heterogeneity, but typically offers incomplete separation of the different product's isoforms. CD-mediated chiral CE is a well-established electromigration method. We demonstrate how we can use CDs as modulators for CZE-based separation of mAb charge variants (biosimilars of Rituximab and Trastuzumab). Both the negatively charged carboxymethyl-beta-cyclodextrin (CM$\beta$CD) and uncharged (2-Hydroxypropyl)-beta-cyclodextrin (HP$\beta$CD) have been studied. The effect of CD concentration has been found to impact the extent of modulation of resolution of charge variants. Addition of 10 mM CM$\beta$CD not only improved resolution but also resulted in separation of two additional basic and one additional acidic species for Rituximab. Similarly, besides improvement in the resolution, one additional basic variant was observed with 2.5 mM CM$\beta$CD with Trastuzumab. No

improvement in resolution was, however, observed with HP$\beta$CD, suggesting the selective utility of CDs for analysis of mAb charge variants.

The second objective of this project was to establish a method to improve CE throughput for the analysis of mAbs. The complexity of biotherapeutic products implies an ever-increasing list of product quality attributes that need to be monitored and characterized. In addition, the growing interest in implementing process analytical technology (PAT) in biopharmaceutical production has further increased the testing burden, together with the need for rapid testing that can facilitate real-time or near-real-time decision-making. CE has made a place in biopharmaceutical analysis but is regarded as a low-throughput method, with the instrument dead time comprising 80 % of the total analysis time. In this study, the dead time of CE was utilized to analyze 3 mAb samples in a single-CE run. This approach resulted in an up to 77 % reduction in the total analysis time and increased the productivity by up to 300 %, compared to traditional single CE-ultraviolet (UV) runs, without compromising the resolution or the relative peak areas. Additionally, good method reproducibility was observed. The compatibility of the method has been demonstrated with protein A eluate and cation exchange chromatography fractions.

The third objective was the establishment of an offline multidimensional method using liquid chromatography (LC) in conjunction with CE and tandem mass spectrometry (MS/MS). LC is the most frequently used technique in the industry for characterization of biotherapeutics. It does, however, have significant drawbacks, such as a reduced sensitivity to hydrophilic or small peptides. CE is an emerging tool and offers results that are complementary to those of LC but has limited loading capacity which limits its sensitivity. Thus, a combined platform that harnesses the power of both chromatography and electrophoresis has been developed and tested for peptide mapping analysis of mAbs. For this, the trypsin digested mAb was first fractionated on LC, and each fraction was analyzed by CE-MS in an offline manner. This method, called LC-CE-MS, identified more peptides of varying properties, and greatly improved the sequence coverage and post-translational modifications (PTMs) analyses compared to the traditional one-dimensional (1D) LC and CE methods. In fact, the system performed better than even the combined datasets of LC-MS and CE-MS. Hence, this two-dimensional (2D) LC-CE-MS method was proposed as an addition to the analytical tool box for primary characterization of mAbs.

The fourth goal was to use the previously established LC-CE-MS method to identify host cell proteins (HCPs) in the culture supernatant of a Chinese Hamster Ovary (CHO) cell line that is commonly used for production of mAbs. Although many of these HCPs may be identified using LC-MS or CE-MS alone, it can be challenging to identify all of them due to the complexity of the material, and the limited peak capacity of the system. Accordingly, LC-CE-MS identified more than twice the number of HCPs compared to LC-MS and CE-MS alone. Furthermore, a greater abundance of peptidases was also detected with LC-CE-MS. The method was found to be better for CHO secretome analysis than the conventional 1D methods.

The fifth goal was to analyze the interactions between an antivenom therapeutics candidate and snake venoms. A functional characterization of recombinant lethal toxin neutralizing factor (rLTNF) against cobra venom (CV) and Russell's viper venom (RVV) was performed using LC-MS. The LTNF is a peptide-based antivenom candidate which is known to be active against a number of snake venoms. In this study we investigated the interactions of this rLTNF peptide with cobra and Russell's viper venoms. Besides the monomeric peptide, the potential of rLTNF oligomers was also investigated. The rLTNF oligomers were found to severely affect the chromatographic profiles for both CV and RVV, indicating their potential to be used as antivenom therapeutics.

The creation of an LC-ESI-MS-based approach for the detection of conjugated Flock house virus (FHV) virus-like particles (VLPs) was the final goal of this study. Conjugated peptides have been used to improve the specificity of therapeutic VLPs by guiding them to their intended targets. However, deciphering conjugation is analytically challenging. In this instance, based on the masses, and retention times changes, we utilized an LC-ESI-MS-based method to distinguish between conjugated and non-conjugated VLPs. Peptide conjugation resulted in significant changes and shifts in the chromatogram and MS spectra. To the best of our knowledge, this is the first report on the use of ESI-MS for such analysis.

Together, we demonstrated the use of different analytical tools for improved characterization of different classes of biopharmaceuticals. These aforementioned methods could constitute useful additions to the analytical toolbox for better and faster characterization of biotherapeutics.