Higher-order-structure analysis of proteins by native size-based separations coupled to optical and mass-spectrometric detectors

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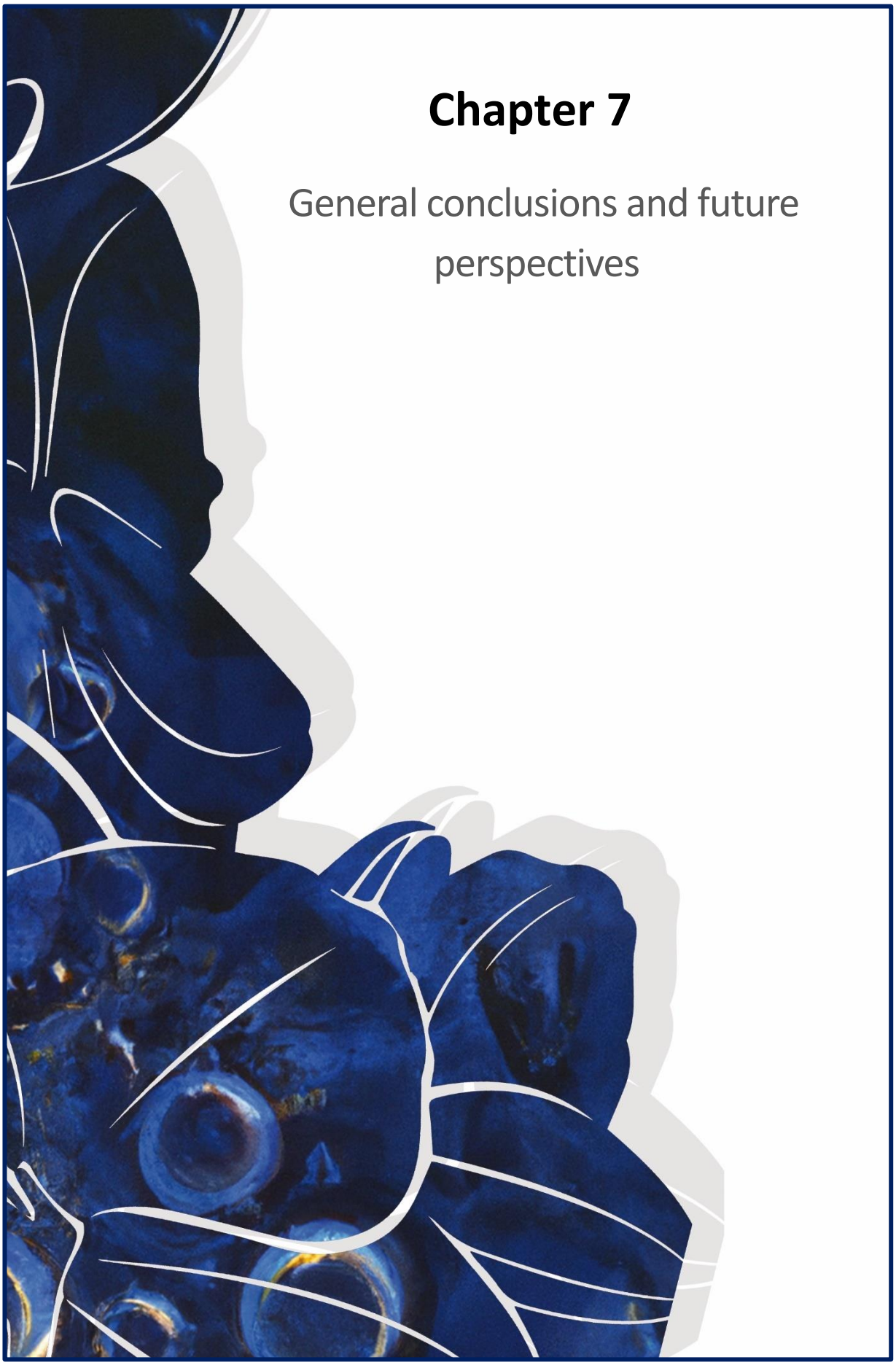

Chapter 7

General conclusions and future perspectives

7.1 General Discussion on AF4 and SEC

In the present thesis, we aimed to establish hyphenated and integrated analytical systems that could – in a single analysis – provide orthogonal and detailed data on size, chemical composition, conformation, and supramolecular structure of intact protein biotherapeutics. Seeking a deeper understanding of the factors that may impact the integrity of HOS during the analysis, we focused on the hyphenation of size-based separations, i.e., size-exclusion chromatography (SEC) and asymmetrical flow field-flow fractionation (AF4), with multi-angle-light-scattering (MALS) detection and high-resolution mass spectrometry (MS). Correlating the conformation in the solution-phase (MALS) with that in the gas-phase (MS) may greatly increase the level of structural information, shedding light on the complex denaturation and aggregation pathways of protein biotherapeutics.

Although SEC is the most commonly used separation technique for the characterization and quantification of aggregates of protein biotherapeutics, our research revealed some undisputable advantages of AF4 regarding the preservation of the HOS during the analysis. Labile, non-covalent complexes and protein oligomers can be preserved during the AF4 separation. In contrast, SEC requires careful optimization of the mobile-phase composition to suppress interactions between the protein complexes and the column-packing material, and the resulting conditions may lead to disruption of the HOS. AF4 covers an extended separation range (covering macromolecules and nanoparticles) and the use of an open channel instead of a packed column causes relative flexibility with respect to the carrier-liquid composition. As a result, AF4 analysis can greatly contribute to the understanding of protein aggregation and/or oligomerization. In the work underlying this thesis we realized an on-line coupling between AF4 and MS. Under appropriate conditions (soft ionization methods, low flow rates, suitable solvent composition) MS can be performed under “native” conditions, i.e., the protein conformation – and possibly the HOS – can be preserved during transfer to the gas phase, ionization and mass separation, and detection. Hyphenation of AF4 to a multi-detection platform including native MS was shown to provide unprecedented information to aid in the understanding of the interaction/association processes and interaction kinetics. Table 1 summarizes the strengths and weaknesses of AF4 and SEC.

Table 1. Strengths and weaknesses of asymmetrical field-flow fractionation (AF4) and size-exclusion chromatography (SEC). Both techniques separate analytes based on the size of molecules in solution.

	Strengths	Weaknesses
AF4	• Application range extends up to ultra-high-molecular-weight polymers and nanoparticles • Very little stress exerted on analyte molecules • Can be used to characterize (weakly bonded) higher-order structures • One single channel can be used for all separations (with a given type of membrane) • Elution conditions can be programmed • AF4 instrumentation may also be used for SEC • Can now be coupled to high-resolution mass spectrometry • High-temperature AF4 has been demonstrated	• Requires careful optimization (flow rates, injection conditions, carrier liquid) • Availability of instrumentation is (still) limited • Possible interactions between membrane and analytes • Limited choice of membrane chemistry (e.g., for polar organic carrier fluids) • Dedicated detectors (MALS, viscometry, differential refractive index) do not allow miniaturization
SEC	• Application range extends to very high-molecular-weight molecules (MDa range) • Widely available and pervasively used • Routinely operated with a very broad range of solvents and temperatures • Very broad range of columns available (pore sizes, chemistry, particle size, column length and diameter, *etc.*) • (very) Fast analysis possible[1] • Miniaturization causes minor losses in performance[2] • Can easily be coupled to (high-resolution) mass spectrometry • Can readily be incorporated in (comprehensive) two-dimensional liquid chromatography systems	• Possibly erroneous results for ultra-high-molecular-weight polymers (> 10MDa) and nanoparticles. • Medium (relatively large particles, low flow rates) to high (relatively large particles, low flow rates) stress exerted on analyte molecules • Different molecular-weight ranges require different (sets of) columns if selectivity is to be maintained • Possible interactions between packing material (with high surface area) and analytes • Dedicated detectors (MALS, viscometry, differential refractive index) do not allow miniaturization

Based on the results described in this thesis, we believe that AF4 and AF4-MS will gain popularity for native analysis. For example, for applications related to the characterization of biotherapeutic medicines and drug-delivery particles. Unravelling the “corona” formation upon adsorption of proteins around drug delivery nanoparticles when in contact with biological fluids is a contemporary challenge of extreme relevance. The nanoparticle-protein complex is a dynamic system and separation of the particles from the biological fluid, while preserving the integrity of the corona formed, is highly challenging. Separation methods, such as AF4, capillary electrophoresis (CE) and hydrodynamic chromatography (HDC), have been proposed as befitting non-destructive techniques, offering interesting possibilities for the characterization of fragile drug-delivery particles. AF4 has been explored to separate the nanoparticles from the other components of the biological fluid and for the identification of nanoparticle-protein corona. However, while the so-called “hard coronas”,

[1] Except for very high-molecular-weight polymers (MDa range)
[2] Provided suitable detectors are used.

formed by proteins with a high affinity for the surface of the nanoparticle, can be kept intact during the AF4 analysis, it is yet not clear whether “soft coronas”, formed by proteins with a low affinity for the surface, can be preserved. We believe that hyphenation of AF4 with (ultra-) high-resolution MS may shed light on the presence of protein molecules in the particle fraction and that a careful control strategy may reveal protein adsorption.

The online coupling of non-denaturing separation techniques, such as SEC, AF4, ion exchange chromatography (IEX), and CE with MS provides significant benefits. However, hyphenation is also accompanied by practical challenges. Essentially, the separation techniques that are to be combined with ESI-MS must allow direct ionization of the bio-macromolecules from the separation effluent. Advances in MS instrumentation and ionization techniques have led to a spectacular increase in the applicability of the technique for larger molecules. We are still pushing the limits of the current possibilities of direct couplings of non-denaturing separations with MS and native MS.

One of the most critical concerns is that both the separation conditions and the MS conditions must preserve the protein structure, while optimal compatibility between the two techniques is required. In SEC the presence of interactions between the protein and the column material may lead to protein unfolding and/or disturbance of the protein HOS. High-ionic-strength conditions achieved using volatile salts are necessary to suppress such unwanted interactions and to ensure preservation of the HOS during the analysis. However, the introduction of large amounts of salts into the MS may require harsher desolvation conditions and may compromise the sensitivity. More-bioinert materials for SEC, with high chemical stability, reduced silanol activity are being developed. Such SEC columns are very attractive for hyphenation with MS, as lower ionic strengths may be required to analyze proteins and protein aggregates. Additionally, microbore SEC columns (1 mm I.D.) operated at micro-flow conditions (15 µL/min) directly coupled to MS were evaluated in this thesis. Enhanced sensitivity was achieved when using microbore SEC column, which seemed to outweigh the ionization suppression caused by the volatile salts present in the effluent. Because of the improved detection limits, lower amounts of protein analyte were also required. This is a useful approach for samples of limited availability and low-abundant proteins. However, it is important to consider that this approach can have limitations related to significant loss of resolution in case of increased injection volumes of, for example, highly diluted samples. Addition of a trap column before the micro-flow SEC-MS allowed refocusing of the analyte band, reducing band broadening and peak tailing. This approach showed promising results and yielded picogram detection limits for characterization of protein species.

The combination of AF4 with ion-mobility spectrometry – mass spectrometry (IMS-MS) is still largely uncharted territory, while efforts to explore SEC with native IMS-MS have been reported. In IMS ionized molecules (or assemblies thereof) are separated based on their collision cross section (CCS) when migrating against a flow of low-pressure gas (typically air, nitrogen or helium) under the influence of an applied electric field. The CCS strongly depends on size and conformation of the molecular ions. Therefore, IMS can provide information on molecular conformations or HOS of one protein species, which obviously cannot be discerned by MS only. Nowadays, IMS-MS has been explored for the analysis of proteins and their conformers and aggregates under (near-) native conditions. However, so far, the coupling of native separation techniques with native IMS-MS for protein analysis has hardly been reported.

7.2 Some considerations concerning other applications of field-flow fractionation techniques

The techniques that were applied and developed in the present thesis can be used to advance developments in (bio-) pharmacy and drug delivery, liposomes, viruses and virus-like particles, extracellular vesicles and exosomes. Improved membrane materials for AF4 (broader selection of materials; better defined molecular-weight cut-off) and more-inert column materials for SEC may reduce interactions with the analyte, improve peak shapes and resolution, and enhance sample recovery. Improved materials may reduce demands on the carrier-liquid composition, which is critical if we are to gain reliable and useful insights in structure-function relationships of biotherapeutics, innovative polymer materials, and nanoparticles. Specifically missing for AF4 are membranes suitable for analyzing polymers that are exclusively soluble in highly polar organic solvents. Developments in this direction will extend the range of AF4 applications.

Thermal field-flow fractionation (TF3 or ThFFF) does not require a semi-permeable membrane. In TF3 a temperature gradient is employed perpendicular to the flow of a carrier liquid through the open, ribbon-like channel. Under certain condition (depending on the chemical composition of the analytes and solvents) molecules will experience movement in the field through a process called thermal diffusion. As usual in FFF, this movement is counteracted by molecular diffusion (depending on the molecular weight of the analyte and on the solvent). The net results in the fractionation of analytes according to size, chemical composition and microstructure. A serious disadvantage of TF3 is that in a one-dimensional fractogram these distributions are confounded. The absence of a membrane is an advantage of TF3. It provides an even greater flexibility on the carrier-liquid composition, as there is no need for optimization to avoid analyte-membrane interactions. Therefore, TF3 may be a attractive alternative for determining the molar-mass distribution of (homo-)polymers soluble in highly polar organic solvents.

In a feasibility study, we explored the possibilities of TF3 using the strong solvent, 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) for the characterization of industrial polyamides. The previous Mylar-spacer was replaced by a carbon-enforced PTFE material. Although, there were some promising results obtained when using binary-solvent systems, for example a mixture of HFIP and ACN, a consistent contamination of undefined origin compromised the analysis, and no reliable analyzes could be performed. The main conclusion of this study was the materials of the TF3 system could not withstand the solvent, so that additional effort was required to select or develop suitable materials. The choice in possible solvents for TF3 is quite limited for practical (compatibility with the channel and the instrument) and fundamental (existence of thermal diffusion). In combination with the confounded results for anything but homopolymers this causes the application range of TF3 to be quite narrow.

7.3 Molecular simulations

Many interesting results have been reported in this thesis, which add to our knowledge, but not (immediately) to our understanding. A case in point is our study into the HOS of β-D-galactosidase reported in Chapter 3. We have clearly demonstrated that monomers, dimers, tetramers and other HOS of the protein occur, often simultaneously, depending on the ions present in the aqueous solution. The composition of the solvent greatly affects the HOS. For example, no tetramers were

formed when NaCl was present even in high concentrations (up to 100 mM), but they were abundant when magnesium ions were present, even at low concentration (about 4 mM). Oligomeric aggregates can quickly fall apart when the conditions promote dissociation, but they can also be formed in remarkably short times if association is favorable. In contrast, it proved difficult to exchange divalent ions, such as Zn^{2+}, once these are incorporated in the dimeric or tetrameric structures. In conclusion, we know a lot more about β-D-galactosidase and its HOS, but we know little about what causes its bahavior. Molecular-dynamics studies may shed light on the bahavior of β-D-galactosidase and they may help to predict the bahavior of other enzymes and biotherapeutics.

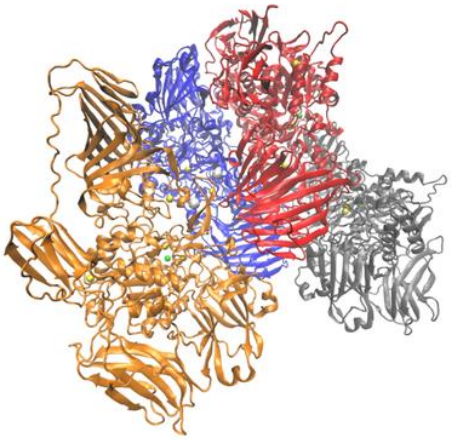

Figure 1. The tetramer form of β-D-galactosidase with the four proteins shown in different colors used to investigate ion binding times. The figure has been provided by Mat Tolladay and Jocelyne Vreede.

The findings reported in this thesis have led to a project at the University of Amsterdam, in which Mat Tolladay and Jocelyne Vreede (HIMS Computational-Chemistry Group) are performing molecular-simulation studies to establish where different metal ions bind on the protein and what effect they have on its conformation and HOS. Preliminary results indicate that the tetramer of β-D-galactosidase occurs naturally, but also that such a “dimer of dimers” will not form if no (metal) ions are present on specific sites on the protein. Sodium is found to have little binding capacity, while both magnesium and zinc ions strongly promote the formation of tetramers. These findings are in agreement with the experimental observations reported in Chapter 3 of this thesis. Using a combination of molecular simulations and an experimental approach involving analytical platforms based on native separation and detection may provide a deeper understanding of the bahavior of biotechnological proteins in complex fluids.