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

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Chapter 8

Appendices



Summary

Analysis of intact macromolecules – whether synthetic, natural or biotechnological – and their (supra-) molecular structure is essential to obtain insights in structure-function relationships. Biotechnological proteins are large, complex protein molecules derived from living cells. Detailed characterization of the protein-based products is necessary to guarantee their efficacy and safety. Two major concerns in manufacturing protein biopharmaceuticals are to preserve the active, functional protein during the process and to prevent aggregation of the protein molecules. The higher-order structure (HOS) of protein biopharmaceuticals affects their biological and pharmacological function. Slight changes in the HOS can result in protein denaturation and aggregation, with significant impact on the product efficacy and quality. The relationship between the HOS and the therapeutic function of the protein is complex and not yet fully understood. Particular changes in the HOS are likely to affect the activity of different proteins differently. For these reasons, there is an urgent quest for analytical techniques that provide a detailed picture of the structure of biopharmaceuticals and information on product related impurities and degradants, while preserve the labile HOS during analysis.

The aim of the work presented in this thesis was to explore combinations of size-based separations and state-of-the-art spectrometric techniques to establish analytical platforms that – in a single analysis – can provide accurate and detailed information on the size-variants and HOS of protein biopharmaceuticals in solution. We have been exploring the synergistic advantages of either size-exclusion chromatography (SEC) or asymmetrical flow field-flow fractionation (AF4) with multi-angle light scattering (MALS) and native mass spectrometry (nMS) to identify the factors that can lead to structural changes during the analysis and to understand aggregation and denaturation pathways. We have attempted to correlate information from the liquid state, based on the separation and detection of the various size-variants, with gas-state information from nMS, to gain a clearer and more reliable picture of the protein HOS and aggregates in the sample.

In **Chapter 1**, we elaborate on the necessity of in-depth characterization of the HOS of biopharmaceutical and biotechnological proteins, and we discuss the existing techniques that serve this purpose. The basic principles of the techniques are briefly explained. Gaps in the technology and challenges related to the characterization of protein HOS are also discussed, shedding light on the high demand for continuous advances in characterization techniques. To demonstrate the suitability of SEC and AF4 coupled with either MALS or nMS for the analysis and characterization of protein HOS and aggregates, applications and findings presented in literature are evaluated.

Field-flow fractionation (FFF) and, especially, AF4 have gained considerable attention as tools for the characterization and analysis of complex samples and labile entities. **Chapter 2** provides a comprehensive review of advancements in FFF, with specific focus on interaction studies involving (bio)macromolecules and nanoparticles. The aim of this review is to highlight the possibilities and practical limitations of the FFF techniques for interaction-related studies.

In **Chapter 3**, we demonstrate the suitability of AF4 for studying the dynamic association equilibria between the various oligomeric forms of a biotechnological enzyme. In this study, effects of the

carrier-liquid composition on the equilibria were carefully studied. AF4 and frit-inlet FFF were coupled to a triple-detection system, comprised of UV-absorbance, multi-angle-light-scattering, and differential-refractive-index detectors (UV-MALS-dRI). Various AF4 parameters, including the focusing process, the cross-flow rate, and the injected amount, were demonstrated not to affect the equilibria. In contrast, SEC was found to disturb the dynamic association equilibria of the enzyme, possibly due to physical stress exerted on the molecules.

The need for a clear understanding of whether protein structural features are preserved during analysis, calls for complementary information from high-resolution techniques. In **Chapter 4** we report an online analytical platform based on the coupling of AF4 to nMS in parallel to UV-MALS-dRI detectors for the in-depth characterization of the labile HOS of the biotherapeutic enzyme (anticancer agent) L-asparaginase (ASNase). By combining the structural information obtained from the liquid phase (AF4 and UV-MALS-dRI) and the gas phase (nMS), misinterpretations related to the existence of protein aggregates or oligomers (i.e., monomers, dimers, trimers) in solution can be avoided. More-reliable conclusions can be drawn about the stoichiometry, the dynamic equilibria between the various oligomer assemblies, and the stability of these structures.

SEC is considered a non-destructive separation technique. It is widely used for the characterization of (bio)macromolecules and their aggregates. However, there are known limitations of SEC, related to unwanted interactions between the protein analytes and the column material. The impact of such interactions on the integrity of the protein HOS was investigated using nMS as a selective tool to reveal conformational changes during analysis. This work is described in **Chapter 5** of this thesis. On-line coupling of SEC with nMS allowed monitoring of structural changes during the separation, while varying the mobile-phase composition (ionic strength, type of salt, pH). nMS provided great insights in protein denaturation due to secondary interactions in SEC. High-ionic-strength conditions achieved using volatile salts allowed coupling of near-native SEC and nMS, providing optimal separation and ionization conditions. Careful optimization of the SEC mobile-phase and nMS conditions was found necessary to preserve the protein HOS during the analysis, depending on the type of SEC column and the protein analyte.

In **Chapter 6** we illustrate the use of SEC columns with an internal diameter (I.D.) of 1-mm operated at micro-flow rates (15 $\mu\text{L}/\text{min}$) and coupled to nMS. These conditions were pushing the limits of online SEC-nMS. The drastic reduction of the SEC column I.D. and, by extend, of the flow rate directed towards MS, resulted in improved ionization efficiency and enhanced MS sensitivity for proteins and protein complexes. Consequently, reduced amounts of protein samples could be injected. The microflow rate directed to the MS allowed softer ionization MS conditions, thus preserving the HOS features. A trap containing an ion-exchange (IEX) material was installed prior to the SEC-MS to reduce the adverse effects of large injection volumes on the chromatographic performance. The enhanced sensitivity attained by the micro-flow SEC-MS along with the on-column focusing achieved by the IEX precolumn allowed picogram detection limits for proteins.

In **Chapter 7** we discuss some considerations regarding size-based separations and their hyphenation with MS. Also, we present an outlook on possible future research. Based on the work and findings described in Chapter 3, a new project was initiated at the University of Amsterdam aiming to explore the binding of various ions to the biotechnological enzyme *b*-D-galactosidase using a combination of molecular simulations and the information to explain the results obtained by

AF4-UV-MALS-dRI. Additionally, the limitations and possibilities of AF4 for studying the formation of protein-corona in drug-delivery systems and the use of thermal field-flow fractionation (TF3) for characterizing poorly soluble industrial polyamides are discussed.

Samenvatting

Analyse van intacte macromoleculen, van hetzij synthetische, hetzij natuurlijke, hetzij biotechnologische oorsprong, en hun (supra-)moleculaire structuur is essentieel als we inzicht willen verkrijgen in het verband tussen structuur en functie. Biotechnologisch geproduceerde geneesmiddelen en enzymen zijn grote, complexe eiwitmoleculen, verkregen uit levende cellen. Nauwgezette karakterisering van op eiwitten gebaseerde producten is noodzakelijk om de werkzaamheid en veiligheid ervan te kunnen garanderen. Twee belangrijke zorgpunten in de fabricage van op eiwitten gebaseerde biofarmaca zijn het behoud van het actieve, functionele eiwit tijdens het productieproces en het voorkomen van aggregatie van eiwitmoleculen. De hogere-orde structuur (HOS) van biotechnologische eiwitten heeft een belangrijke invloed op hun biologische en farmaceutische functie. Kleine veranderingen in de HOS kunnen leiden tot het denatureren en samenklonteren van de eiwitten, hetgeen van invloed is op de werkzaamheid en kwaliteit van het product. Het verband tussen de HOS en de therapeutische of enzymatische functie van het eiwit is ingewikkeld en nog niet volledig doorgrond. Veranderingen in de HOS hebben waarschijnlijk bij verschillende eiwitten een verschillende invloed op de activiteit. Daarom is er een dringende behoefte aan analyzetechnieken die een nauwgezet beeld geven van de structuur van biofarmaca en industriële enzymen, de aanwezigheid van aan het product gerelateerde verontreinigingen en degraandatieproducten, zonder de HOS tijdens de analyse te veranderen.

Het doel van het in dit proefschrift beschreven werk was om combinaties te onderzoeken van groottescheidingen en *state-of-the-art* spectrometrische technieken om daarmee een analytische platform te creëren dat – in een enkele analyse – juiste en gedetailleerde informatie geven over varianten met afwijkende grootte en HOS van eiwitten in oplossing. We hebben de synergetische voordelen onderzocht van hetzij *size-exclusion* chromatografie (SEC), hetzij asymmetrische *flow field-flow fractionation* (AF4) in combinatie met *multi-angle* lichtverstrooiing (MALS) en natuurlijke (“*native*”) massa spectrometrie (nMS) om de factoren te identificeren die kunnen leiden tot veranderingen in de structuur tijdens de analyse en om de wegen die leiden tot aggregatie en denaturering te doorgronden. We hebben geprobeerd om informatie vanuit de vloeistoffase, gebaseerd op de scheiding en detectie van varianten met verschillende groottes, te correleren met nMS informatie vanuit de gasfase om een duidelijker en betrouwbaarder beeld te krijgen van de HOS en aggregaten van de eiwitten in het monster.

In **Hoofdstuk 1** gaan we dieper in op de noodzaak om de HOS van biofarmaceutische en biotechnologische eiwitten grondig te onderzoeken en bespreken we de hiervoor bestaande technieken. De grondbeginselen van die technieken worden in het kort uitgelegd. Hiaten in de technologie en uitdagingen op het gebied van de karakterisering van de HOS van eiwitten worden ook besproken en de grote vraag naar steeds betere karakteriseringsmethoden wordt verduidelijkt. Om de bruikbaarheid van SEC en AF4 in combinatie met MALS of nMS voor de analyse en karakterisering van de HOS van eiwitten en aggregaten te onderschrijven worden in de literatuur beschreven toepassingen en resultaten geëvalueerd.

Field-flow fractionation (FFF) – en in het bijzonder AF4 – hebben aanzienlijke belangstelling getrokken als mogelijke technieken voor de analyse en karakterisering van complexe monsters en

instabiele componenten. **Hoofdstuk 2** geeft een compleet overzicht van vorderingen in FFF, met speciale aandacht voor interactiestudies die betrekking hebben op (bio-)macromoleculen en nanodeeltjes. Het doel van dit overzicht is om de mogelijkheden en praktische beperkingen van de FFF technieken voor onderzoek op het gebied van interacties voor het voetlicht te brengen.

In **Hoofdstuk 3** tonen we aan dat AF4 bruikbaar is voor het bestuderen dynamische associatie-evenwichten tussen verschillende oligomere vormen van een biotechnologisch enzym. In dit onderzoek zijn de effecten van de samenstelling van het loopmiddel op de evenwichten zorgvuldig onderzocht. AF4 en *frit-inlet* FFF werden gekoppeld aan een drievoudig detectiesysteem, bestaande uit UV-absorptie (*multi-angle*) lichtverstrooiing en differentiële brekingsindexdetectoren (UV-MALS-dRI). Diverse AF4 parameters, waaronder het focussingproces, de grootte van de zijuitstroom en de geïnjecteerde hoeveelheid, bleken geen effect te hebben op de evenwichten. SEC daarentegen bleek de dynamische associatie-evenwichten mogelijk te verstoren, mogelijk als gevolg van de fysieke stress die op de moleculen werd uitgeoefend.

Om een duidelijk beeld te krijgen het al dan niet behouden van de structureigenschappen van eiwitten tijdens de analyse moet aanvullende informatie verkregen worden met behulp van hoge-resolutie technieken. In **Hoofdstuk 4** rapporteren we over een fysiek gekoppeld (*on-line*) platform gebaseerd op de simultane koppeling van AF4 met nMS en met UV-MALS-dRI, bedoeld om labiele HOS van het biotherapeutische enzym L-asparaginase (ASNase, een medicijn tegen kanker) in detail te kunnen bestuderen. Door de structuurinformatie verkregen voor de vloeistoffase (AF4 en UV-MALS-dRI) te combineren met die van de gasfase (nMS) kunnen foutieve interpretaties betreffende het bestaan van eiwitaggregaten of oligomeren (monomeren, dimeren, trimeren) in oplossing worden voorkomen. Betrouwbaardere conclusies kunnen getrokken worden met betrekking tot de stoichiometrie, de dynamische evenwichten tussen de verschillende oligomere complexen en de stabiliteit van deze structuren.

SEC wordt beschouwd als een niet-destructieve scheidingstechniek. Het wordt veelvuldig gebruikt voor de karakterisering van (bio-)macromoleculen en complexen daarvan. SEC heeft echter erkende beperkingen, die verband houden met ongewenste interacties tussen de te analyseren eiwitten en het kolommateriaal. De impact van dat soort interacties op integriteit van de HOS van de eiwitten werd onderzocht met behulp van nMS, als selectieve techniek om veranderingen in de conformatie tijdens de analyse boven tafel te brengen. Dit werk wordt beschreven in **Hoofdstuk 5** van dit proefschrift. De fysieke koppeling van SEC en nMS maakte het mogelijk om wijzigingen in de structuur tijdens de scheiding waar te nemen bij verschillende samenstellingen van de mobiele fase (ionensterkte, type zout, pH). nMS verschaftte veel inzicht in de denaturering van eiwitten als gevolg van secundaire interacties in SEC. De hoge ionensterkte, die werd bereikt met vluchtige zouten, maakte het mogelijk om nagenoeg natuurlijke SEC en nMS te koppelen zodat optimale scheidings- én ionisatiecondities werden verkregen. Zorgvuldige optimalisering van de SEC mobiele fase en de nMS condities, afhankelijk van het type SEC kolom en de te analyseren eiwitten, bleek noodzakelijk om de HOS van de eiwitten tijdens de analyse in stand te houden.

In **Hoofdstuk 6** wordt het gebruik van SEC kolommen met een inwendige diameter (I.D.) geïllustreerd, met een microdebiet (15 $\mu\text{L}/\text{min}$) en rechtstreekse koppeling met nMS. Daarmee werden de grenzen van de SEC-nMS koppeling opgezocht. De drastische reductie van de I.D. van de kolom – en daarmee het debiet dat naar de MS geleid werd – resulteerde in een hogere ionisatie-

efficiëntie en MS gevoeligheid voor eiwitten en eiwitcomplexen. Daardoor konden kleinere hoeveelheden eiwit geïnjecteerd worden. Het microdebiet richting MS liet mildere ionisatiecondities toe, zodat de HOS behouden konden blijven. Een “trapping” kolom, gepakt met een ionenwisseling (IEX) materiaal werd voor het SEC-MS systeem aangebracht om de negatieve effecten van grote injectievolumina op de chromatografische prestaties te temperen. The verhoogde gevoeligheid die werd bereikt met micro-SEC-MS, in combinatie met de focussing van het monster op de IEX trap, maakte het mogelijk om detectiegrenzen in de orde van picogrammen te bereiken voor eiwitten.

In **Hoofdstuk 7** worden enige overwegingen met betrekking tot op grootte gebaseerde scheidingen en de combinatie daarvan met MS besproken. Ook wordt een blik geworpen op mogelijk toekomstig onderzoek. Op basis van de bevindingen beschreven in Hoofdstuk 3 werd aan de Universiteit van Amsterdam een onderzoek gestart naar de binding van verschillende soorten ionen met het biotechnologische enzym *b*-D-galactosidase, waarin moleculaire simulaties worden gebruikt om de met AF4-UV-MALS-dRI verkregen resultaten te verklaren. Daarnaast worden in Hoofdstuk 7 de mogelijkheden en beperkingen besproken van AF4 om de vorming van eiwit-corona's in medicijnafgiftesystemen te onderzoeken en die van thermische FFF (TF3) voor de karakterisering van moeilijk oplosbare industriële polyamides.

Sundries

Overview of co-authors' contributions

List of publications [1-8]

Overview of co-authors' contributions

Chapter 1. General introduction

| | |
|---------------------------|---|
| I. K. Ventouri | Wrote the chapter. |
| P. J. Schoenmakers | Edited and reviewed the chapter and made suggestions for improvement. |

Chapter 2. Field-flow fractionation for molecular-interaction studies of labile and complex systems: A critical review

| | |
|--------------------------|--|
| I. K. Ventouri | Conducted the literature research and wrote the manuscript. |
| S. Loeber | Assisted in the literature research and composition of the manuscript. |
| G. W. Somsen | Reviewed the manuscript and made suggestions for improvements. |
| P.J. Schoenmakers | Edited and reviewed the manuscript and made improvements. |
| A. Astefanei | Overall supervision of the project. |

Chapter 3. Asymmetrical flow field-flow fractionation to probe the dynamic association equilibria of β -D-galactosidase

| | |
|---------------------------|--|
| I. K. Ventouri | Developed and performed the experiments. Processed the data and wrote the manuscript. |
| A. Astefanei | Assisted with suggestions throughout the study. |
| E. R. Kaal | Overall supervision of the project. Made significant contribution with advice and suggestions throughout the study and composition of the manuscript. |
| R. Haselberg | Reviewed the manuscript and made scientific suggestions for the study. |
| G. W. Somsen | Overall supervision of the project. Reviewed the manuscript and made suggestions for improvements. |
| P. J. Schoenmakers | Overall supervision of the project. Made significant contributions with advice and suggestions throughout the study. Edited and reviewed the manuscript and made improvements. |

Chapter 4. Characterizing non-covalent protein complexes using asymmetrical flow field-flow fractionation on-line coupled to native mass spectrometry

| | |
|---------------------------|---|
| I. K. Ventouri | Co-developed and realized the hyphenation of AF4 to native MS. Processed the data and wrote the manuscript. |
| W. Chang | Performed initial experiments on the aggregation of the biotherapeutic L-D-asparaginase. |
| F. Meier | Provided advice and suggestions regarding the hyphenation of AF4 to MS. |
| R. Drexel | Consulted on various aspects of FFF instrumentation and performance, and supported the development and optimization of the AF4 methods |
| G. W. Somsen | Overall supervision of the project. Reviewed the manuscript and made significant suggestions for improvements. |
| P. J. Schoenmakers | Overall supervision of the project. Reviewed the manuscript and made significant suggestions for improvements. |
| B. de Spiegeleer | Supplied the samples. Provided advice and insights regarding the behavior of the biotherapeutic proteins. |
| R. Haselberg | Overall supervision of the project. Co-developed the hyphenation of AF4 to native MS. Processed data. Reviewed the manuscript and made significant suggestions for improvements throughout the study. |
| A. Astefanei | Overall supervision of the project. Co-developed the hyphenation of AF4 to native MS. Reviewed the manuscript and made significant suggestions for improvements throughout the study. |

Chapter 5. Probing protein denaturation during size-exclusion chromatography using native mass spectrometry

| | |
|--------------------------|--|
| I. K. Ventouri | Co-developed and performed the SEC-UV-MS experiments. Processed the data. Wrote the manuscript. |
| D. B. A. Malheiro | Performed initial SEC-UV experiments. |
| R. L. C. Voeten | Developed the MATLAB script for the data processing. |
| S. Kok | Supplied the columns. Assisted with scientific suggestions and advice. |
| M. Honing | Overall supervision and funding of the project. Made significant suggestions for improvements. |
| G. W. Somsen | Reviewed the manuscript and made significant suggestions for improvements. |
| R. Haselberg | Overall supervision. Co-developed the SEC-UV-MS experiments. Edited and reviewed the manuscript. |

Chapter 6. Micro-flow size-exclusion chromatography for enhanced native mass spectrometry of proteins and protein complexes

| | |
|---------------------------|--|
| I. K. Ventouri | Co-developed the experimental approach and performed the micro-flow trap-SEC-MS experiments. Processed the data. Wrote the manuscript. |
| S. Veelders | Performed initial experiments on micro-flow SEC-MS. |
| M. Passamonti | Consulted on various aspects of micro-flow LC instrumentation and supported the coupling of the trap and micro-flow SEC. |
| P. Endres | Consulted on aspects of SEC and provided resources. |
| R. Roemling | Consulted on aspects of SEC and provided resources. |
| P. J. Schoenmakers | General support and supervision. Reviewed the chapter and made suggestions for improvement. |
| G. W. Somsen | Reviewed the chapter and made suggestions for improvement. |
| R. Haselberg | Consulted on various aspects of the method development. Reviewed the chapter and made suggestions for improvement. |
| A. F. G. Gargano | Developed the idea, helped with experimental design, supported, and supervised the project, reviewed the manuscript, and made suggestions for improvement. |

Chapter 7. General conclusions and future perspectives

| | |
|---------------------------|--|
| I. K. Ventouri | Wrote the chapter. |
| P. J. Schoenmakers | Guidance for chapter structure, reviewed the chapter and made suggestions for improvement. |
| G. W. Somsen | Reviewed the chapter and made suggestions for improvement. |
| R. Haselberg | Reviewed the chapter and made suggestions for improvement. |

List of publications

- [1] Tenchiu, Alia-Cristina, Iro-Konstantina Ventouri, Georgia Ntasi, Dimitrios Palles, George Kokotos, Dimitra Kovala-Demertzi, and Ioannis D. Kostas. "Synthesis of a palladium complex with a β -D-glucopyranosyl-thiosemicarbazone and its application in the Suzuki–Miyaura coupling of aryl bromides with phenylboronic acid." *Inorganica Chimica Acta* 435 (2015): 142-146.
- [2] Voeten, Robert L.C., Iro K. Ventouri, Rob Haselberg, and Govert W. Somsen. "Capillary electrophoresis: trends and recent advances." *Analytical chemistry* 90, no. 3 (2018): 1464-1481.
- [3] Ventouri, Iro K., Daniel B.A. Malheiro, Robert L.C. Voeten, Sander Kok, Maarten Honing, Govert W. Somsen, and Rob Haselberg. "Probing protein denaturation during size-exclusion chromatography using native mass spectrometry." *Analytical chemistry* 92, no. 6 (2020): 4292-4300.
- [4] Ventouri, Iro K., Alina Astefanei, Erwin R. Kaal, Rob Haselberg, Govert W. Somsen, and Peter J. Schoenmakers. "Asymmetrical flow field-flow fractionation to probe the dynamic association equilibria of β -D-galactosidase." *Journal of Chromatography A* 1635 (2021): 461719.
- [5] Ventouri, Iro K., Susanne Loeber, Govert W. Somsen, Peter J. Schoenmakers, and Alina Astefanei. "Field-flow fractionation for molecular-interaction studies of labile and complex systems: A critical review." *Analytica Chimica Acta* (2021): 339396.
- [6] Ventouri, Iro K., Daniel B.A. Malheiro, Robert L.C. Voeten, Sander Kok, Maarten Honing, Govert W. Somsen, and Rob Haselberg. "Probing protein denaturation during size-exclusion chromatography using native mass spectrometry." *Analytical chemistry* 92, no. 6 (2020): 4292-4300.
- [7] Ventouri, Iro. K., Wayne Chang, Florian Meier, Roland Drexel, Govert W. Somsen, G, Peter J. Schoenmakers, Bart De Spiegeleer, Rob Haselberg, Alina Astefanei, (2022). Characterizing non-covalent protein complexes using asymmetrical flow field-flow fractionation on-line coupled to native mass spectrometry. In preparation.
- [8] Ventouri, Iro K., Sharene Veelders, Marta Passamonti, Patrick Endres, Regina Roemling, Peter J. Schoenmakers, Govert W. Somsen, Rob Haselberg, and Andrea F.G. Gargano. "Micro-flow Size-Exclusion Chromatography for enhanced native Mass Spectrometry of proteins and protein complexes." (2022). In preparation (Pre-proof submitted).

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Words have a different connotation in our native language, so I would like to thank my most important people, my family, in Greek.

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