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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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Publication date
2023

[Link to publication](#)

Citation for published version (APA):

Ventouri, I. K. (2023). *Higher-order-structure analysis of proteins by native size-based separations coupled to optical and mass-spectrometric detectors*. [Thesis, fully internal, Universiteit van Amsterdam].

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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.

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- [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).

Acknowledgements

And like that we reached the end of this life-chapter. Yes, “we” because this task would have been impossible without the support, help, patience, and love from so many beautiful people. In this journey, I learned really a lot about my field and steadily I started feeling like the researcher that I was envisioned to become. This era is coming to an end and what remains are major feelings of gratitude, perseverance, and pride.

Peter, one “thank you” really doesn’t sound enough! It all started by you giving me the opportunity to be part of the MSc+ program. Little did I know that this opportunity would really determine my career and future. Later you would accept my late application for the HOSAna project and include me at the UvA group. I will always be so grateful to you for your support. Your motivational speeches, the responses to my late night and very early morning messages and a solution to every tremendous problem have been incredible. Most importantly, I will always thank you for being there at the dancefloor at the conference parties with us, showing everyone how a great promotor should be.

Govert, you have been such an inspiration and influence at the level of researcher and scientist that I want to still reach. Never compromise quality, be critical, not only look but understand and eventually learn; these are only a few of your lessons that I am taking with me in my next steps. It has been such an honor to have you as my co-promotor and I am utterly thankful to you for allowing me to work at one of your projects. From every critical discussion and feedback, you had given me a lesson, something to improve and work on.

Rob, a paragraph is absolutely not enough to express my gratitude and appreciation to you. You taught me almost everything that I know analytical chemistry and protein characterization-wise. You have been my super-supervisor and I couldn’t have asked for a better one. Your ideas, knowledge, guidance, helped me reach my goals and eventually acquire this PhD. A big part of the success of the HOSAna project is on you. But that is not all, your personality, your support, your shoulder (full of my tears by now) have really helped me to develop as a person. A huge thank you for everything from the bottom of my heart.

Not many people have the chance to have two supervisors, but I was one of them. **Alina**, your support with the field-flow fractionation research but also your motivational speeches were so valuable during my HOSAna journey. My deepest gratitude for your help and support! I am keeping our trip to Vienna for the FFF symposium as such a nice memory of real girl-power moment. Thank you for everything!

Andrea, a huge thank you for your support, advice, feedback, scientific discussions and for giving me the opportunity to work with you. It has been very helpful and interesting to hear your thoughts for improvements and suggestions to the HOSAna projects. We started working on the micro-flow SEC-MS project at a point that I was struggling to find my motivation, but this project kept me going and brought back the sparkle. Thank you for helping me to finish the PhD journey working on a project that I really enjoyed.

Robert, you really taught me a lot without even realizing it. I still wouldn't have chosen anyone else to be the other half of this project. Your determination, endless ideas, perseverance, and presentation skills are something that I will always remember from you and keep being inspired from. I am grateful that we were in this parallel journey together. Robert'n'IrO team!

I would like to extend my warmest thanks to the great collaborators of the HOSAna project. Special appreciation and gratitude to the **Postnova team**, and especially **Florian** and **Roland** for being there, responding to every question, ready to provide support and materials to move forward all the ideas and project proposals. Thank you for making my training and secondment so pleasant. I will never forget my time to Landsberg. I felt so welcomed and you made sure that we also had a lot of fun. I would also like to thank Bruker and especially **Rob** for the valuable feedback and discussions during the biweekly HOSAna meetings. My deepest gratitude and appreciation to **Erwin**, for the collaboration to the biggest and probably the most challenging project of my PhD. I feel that I learned so much from your feedback, and guidance during the HOSAna project and my secondment to DSM.

UvA team, Dorina, Marta, Liana, Mimi, Maria, Alan, Gino, and Bob, thank you for all the memories we created these years, thank you for every discussion, cake, coffee, borrel, conference we had together. Thank you for your great support in this journey, I feel very lucky that you we were together in this. I must also thank the **VU team** and especially my dear friend **Raya** for her support with motivational speeches to actual help in the lab and nice scientific discussions. This is only a thank you for everything we have experienced but I am looking forward to creating more memories together.

Tom, and Aleksandra, thank you! **Tom**, thank you for being there providing technical support and solutions. You had to deal with our stress, our very lows and most of the emergency situations and that was not an easy task. **Aleksandra**, I will miss our conversations in the MS lab, and I will miss your advice and support. You have been there for me when you really didn't have to (coming weekends or late night to Science Park), and I will never forget that.

My sweet **Noor(etaki)**, you have been such a great gift. There are really not enough words to thank you and not enough space to mention our funny and not so funny moments together. Where to start and where to finish? I cannot even imagine these MSc and PhD years without you. I am keeping the memory of the day that our friendship started so close to my heart, and I am looking forward to the memories we will keep creating together. I am already missing our coffee breaks, our philosophical discussions about life and future. Thank you for everything my dearest friend.

For the academic people or PhD people is somehow easier to understand this journey, and what a PhD candidate is going through, even though everyone experiences it differently. Seeing or experiencing this journey from outside I now understand that is very difficult. So, I want to truly thank my friends and my family for their patience of me missing out their important moments, for not being always there.

The little Greek village in Amsterdam (**George, Katerina, Danai, Maria(s), Lefteris, Haris**) has been a big support and thank you very much for being patient with me and for being there to support the big disasters and celebrate the small victories. A huge thank you to the creative, artistic soul, and

dear friend **Nikoleta** who designed the cover of this book. Nobody else could have dealt better with my indecisiveness and abstract ideas and actually make such a great cover!

A one thousand thank you must go to my paranymph-aki and best friend **Giota**. Thank you for being the joy to the cold and dark days, thank you for being this kind soul, thank you for your patience, thank you for all the hugs, tissues and wines. You really made me feel not alone when I needed it the most. I am so curious what our next parallel adventure will be.

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

Μανού και Ντουρίνι, μπορεί η απόφαση να με στηρίξετε στο να φύγω να μην ήταν εύκολη αλλά θέλω να ξέρετε ότι εγώ σας ευχαριστώ κάθε μέρα που μου δώσατε αυτή την ευκαιρία. Σας ευχαριστώ που με στηρίζετε και πιστεύετε σε εμένα και που μου δίνετε το χώρο να είμαι εγώ και να φτιάχνω το δρόμο μου. Χάρη σε εσάς φοβάμαι λιγότερο, και προσπαθώ περισσότερο. Σας αγαπάω πολύ και σας ευχαριστώ πολύ για όλα όσα έχετε κάνει για εμένα.

Αγαπημένο μου **Μπό (Όλγα)**, χωρίς έσενα η ζωή είναι ζαμπόν δίχως λιπαρά, περιοδικά και λόγια ντεκαφεϊνέ (χεχε), χωρίς εσένα η ζωή είναι μια ανούσια παράσταση, ένα reality show. Σε ευχαριστώ που με έπεισες να στείλω την αίτηση για το διδακτορικό. Σε ευχαριστώ για τη δύναμη που μου δίνεις, για τη στήριξη όταν δε ξέρω ούτε εγώ τι θέλω, και για την υπομονή σου. Σε ευχαριστώ για τις ατελείωτες ώρες στο τηλέφωνο, για όλους τους άλλους δρόμους που μου δείχνεις και που μου θυμίζεις τι πραγματικά έχει σημασία. Σε αγαπάω πάρα πολύ και μου λείπεις συνέχεια.

Δε φτάνουν οι σελίδες και τα λόγια για τα τόσα ευχαριστώ! Ευχαριστώ το υπέροχα ιδιαίτερο και μοναδικό μου σόι γιατί όλοι τους έχουν συμβάλει στο δικό μου σήμερα. Σας ευχαριστώ, **Αγαπάκι, Βιβή, Δημήτρη, και Θεωρή, Τζοαννάκι, Χαρούλι, Κωστίκο, Άλκη, Τίμο** και καλέ μου **Ντάκο**. Και πολλά πολλά ευχαριστώ στο **γιαγιουλάκι Όλγα** και τον **παππού Άλκη** που είναι φωτεινά παραδείγματα πείσματος και σκληρής δουλειάς, σας αγαπώ πάρα πολύ!

Last but absolutely not least, I want to thank **Giannis**, my most important companion in this roller-coaster journey! Thank you for being there to make me feel safe when I was at my high stress moments, thank you for cooking your secret pasta-recipe when you understood that I needed it, thank you for all the sleepless nights. Thank you for all the things you are teaching me and for making me understand what is important in life and how I want to live my life. Thank you for your immense patience and persistence during these years. Moving to another country and living in 25 m², with me doing a master and a PhD brought definitely some great challenges. But we managed to stay together and grow stronger together. I cannot say that without you, I wouldn't have managed to complete the PhD, but I am certain that without you everything would have been so much more difficult. I will be utterly grateful that x-years ago we met, and you decided to take a leap of faith and join me in this journey to go abroad. I am so looking forward to the next x-years together and to what the future holds for us. Σε ευχαριστώ πολύ και σ'αγαπώ ακόμη πιο πολύ.



ISBN/EAN: 978-94-6421-992-0