Protein-Surfactant Interactions in Complex Systems

Study of Physical Stability of Proteins and Peptides

Hjalte, Johanna

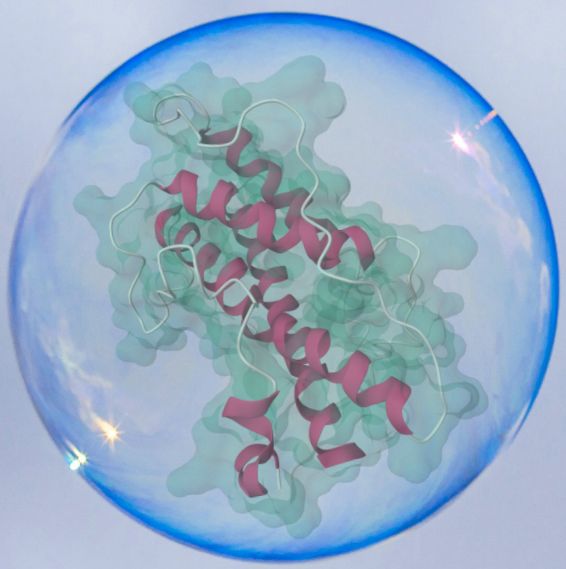
2024

Document Version:
Publisher's PDF, also known as Version of record

Protein-Surfactant Interactions in Complex Systems

Study of Physical Stability of Proteins and Peptides

JOHANNA HJALTE
DEPARTMENT OF PROCESS AND LIFE SCIENCE ENGINEERING | LUND UNIVERSITY



Protein-Surfactant Interactions in Complex Systems

Protein-Surfactant Interactions in Complex Systems

Study of Physical Stability of Proteins and Peptides

Johanna Hjalte

LUND
UNIVERSITY

DOCTORAL DISSERTATION
for the degree of Doctor of Philosophy in Engineering (PhD) at the Faculty of Engineering at Lund University, Sweden. To be publicly defended in lecture hall KC:A, Sölvegatan 39, Lund, on Thursday 23rd of May at 09.00

Faculty opponent
Associate Professor Marco van de Weert, Department of Pharmacy, University of Copenhagen, Copenhagen, Denmark

Organization: LUND UNIVERSITY

Division of Food and Pharma

Document name: Doctoral Dissertation **Date of issue:** 25-04-2024

Author(s): Johanna Hjalte **Sponsoring organization:** Vinnova

Title and subtitle: Protein-Surfactant Interactions: Physical Stability of Proteins and Peptides

Abstract: The objective of this thesis is to characterize the interaction of proteins and excipients in solution. The behaviour of selected excipients, proteins and peptides are studied as they are driven to the verge of instability in different manners; by inducing aggregation, inducing surfactant complexation, or inducing precipitation by increasing ionic strength in the presence of preservative. The response is characterized, and concentration dependent transition points are identified, in the quest of elucidating molecular mechanisms. A through line in the papers of this thesis is an increasing complexity of the systems, from peptides in neat water, to therapeutic proteins in solution with surfactants, preservatives, and salt. Key methods are nuclear magnetic resonance spectroscopy (NMR) and fluorescence titration spectroscopy. Small angle neutron scattering (SANS) is also applied.

Two main themes emerge from this collected body of work. Firstly, by building an understanding of the system at hand, starting from bench-top methods, and using that knowledge to design studies with more advanced methods like NMR, SANS or all atom molecular dynamics simulations, an integrative approach is established that can be applied to study physical stability and excipient interactions in formulation. These studies allow elucidation of molecular mechanisms in the interface of colloidal science and biophysics of proteins and peptides.

In particular, the utility of NMR in characterizing the behaviour of amphiphilic molecules in relevant model systems for pharmaceutical formulation is demonstrated in Papers I, II, III, and V. Unexpected interactions between human growth hormone (hGH) and the non-ionic surfactant dodecyl maltoside (DDM) are observed in Papers II, III, and V. No interaction or change in the protein conformation is observed in the presence of DDM alone. However, if an anionic surfactant like sodium dodecyl sulphate or a preservative like phenol is added, there are indications hGH-DDM interactions. In summary, this goes to show that non-covalent interactions in pharmaceutical formulations are difficult to predict. Nonetheless, by applying the herein developed approach, the driving forces of the observed interactions can be identified.

Key words: Protein and peptide aggregation, protein-surfactant interaction, preservatives, NMR

Language: English

ISBN(e-version): 978-91-8096-039-7

ISBN(print): 978-91-8096-038-0

Number of pages: 88

Signature Date 2024-04-02

Protein-Surfactant Interactions in Complex Systems

Study of Physical Stability of Proteins and Peptides

Johanna Hjalte

LUND
UNIVERSITY

Cover design by Johanna Hjalte
Cover photo of bubble and cloud by Zdeněk Macháček (via unsplash.com)

Faculty of Engineering
Department of Process and Life Science Engineering

ISBN (print): 978-91-8096-038-0
ISBN (e-version): 978-91-8096-039-7

Printed in Sweden by Media-Tryck, Lund University
Lund 2024

Media-Tryck is a Nordic Swan Ecolabel certified provider of printed material. Read more about our environmental work at www.mediatryck.lu.se
MADE IN SWEDEN

Optimist – Someone who figures taking a step backward after taking a step forward is not a disaster, it's a cha-cha (one-two-cha-cha-cha)

Table of Contents

Abstract

The objective of this thesis is to characterize the interaction of proteins and excipients in solution. The behaviour of selected excipients, proteins and peptides are studied as they are driven to the verge of instability in different manners; by inducing aggregation, inducing surfactant complexation, or inducing precipitation by increasing ionic strength in the presence of preservative. The response is characterized, and concentration dependent transition points are identified, in the quest of elucidating molecular mechanisms. A through line in the papers of this thesis is an increasing complexity of the systems, from peptides in neat water, to therapeutic proteins in solution with surfactants, preservatives, and salt. Key methods are nuclear magnetic resonance spectroscopy (NMR) and fluorescence titration spectroscopy. Small angle neutron scattering (SANS) is also applied.

Two main themes emerge from this collected body of work. Firstly, by building an understanding of the system at hand, starting from bench-top methods, and using that knowledge to design studies with more advanced methods like NMR, SANS or all atom molecular dynamics simulations, an integrative approach is established that can be applied to study physical stability and excipient interactions in formulation. These studies allow elucidation of molecular mechanisms in the interface of colloidal science and biophysics of proteins and peptides.

In particular, the utility of NMR in characterizing the behaviour of amphiphilic molecules in relevant model systems for pharmaceutical formulation is demonstrated in Papers I, II, III, and V. Unexpected interactions between human growth hormone (hGH) and the non-ionic surfactant dodecyl maltoside (DDM) are observed in Papers II, III, and V. No interaction or change in the protein conformation is observed in the presence of DDM alone. However, if an anionic surfactant like sodium dodecyl sulphate or a preservative like phenol is added, there are indications hGH-DDM interactions. In summary, this goes to show that non-covalent interactions in pharmaceutical formulations are difficult to predict. Nonetheless, by applying the herein developed approach, the driving forces of the observed interactions can be identified.

Acknowledgement

I would like to take a moment to voice my gratitude to the people, without whom, this thesis would not have seen the light of day.

First and foremost, my main supervisor, Marie Wahlgren. You have been my primary guide and teacher as I've embarked on this academic journey. I've been told that our teachers in life come to us when we need them. I have had many teachers in my scholarly career, but there is a difference between teachers and Teachers with capital T whose lesson changes you to the core. Capital T teachers hold safe spaces and show new perspectives in a way that expands my way of thinking while letting me come to terms with what I am learning in my own way. You have been one of those teachers to me, so from the bottom of my heart, thank you.

I would like to thank my co-supervisors Dan Lundberg, Adrian Sanchez-Fernandez and Lars Nilsson. Dan, your vast knowledge and eye for detail is an immense resource, I will strive to apply the level of your care in my future endeavours. Adrian, going to beam-time with you will never not be memorable, whether it's my first day at work, or during the height of COVID with more nose swabs than I could count. Also, thank you for your handwriting, I don't know if I would have mixed SDS and DDM as early as I did without misreading it, but more importantly, thank you for your bold ambition. Lars, thank you for your sound and grounded advice, your presence in any project provides reliability and a good eye for where energy is best spent.

Thank you Helen Sjögren and Stefan Ulvenlund for your guidance from an industrial perspective. Thank you for sharing your impressive brain power and compassionate nature with me.

To the NMR experts, Carl Diehl and Zoltan Takacs. I don't know why but applying NMR to a problem just makes me happy, and what's even better is that the work I've done with you has resulted in really nice data. Thank you for your time, your knowledge and your kindness.

At the division of Food and Pharma, I would like to thank the people that make division go around. Mattias Alveteg, thank you for your considerate and steadfast leadership. Peter Jensen, thank you for knocking Friday fika out of the park when you bake the bread. Peter Eklöv, thank you for always being helpful. Hilde Skar Olsen, thank you for your contagious energy. Mia Hedin, thank you for your reassuring support. Olexandr Fedkiv and Hans Bolinsson, thank for your help, patience, and answers to all practical things. Thank you to Catalina Fuentes for being my office mate these years, your light and happy presence makes all the difference. To my master thesis students, Anna-Maria Börjesdotter, Johan Sjölund and Karnsinee Viriyayudhakorn, thank you for your curiosity and dedication. To all

the people I haven't mentioned by name, thank you for sharing these past years with me, and an extra thank you to all the PhD students at the department. You have all been so nice and it's been lovely sharing this journey with you!

I also want to thank the NextBioForm consortium and the people behind it. Thank you, Anna Fureby, Ulla Elofsson, Sara Malekkhaiat Häffner, and Randi Nordström for organizing and creating a wonderful research space to partake in. Thank you to my PhD brother and sisters in Stockholm, Amanda Västberg, Daniel Osanloo and Zandra Gidlöf, it's always a treat to meet up with you.

Lingping Zhang, Jade Schut, Eva Rütten, Ingrid Ramm, Shruti Lalwani, Juanita Francis, Anna-Maria Börjesdotter, Shuai Bai. Thank you for being a highlight of my day. I don't want to think that this era is coming to an end. Our routine of lunch and coffee and horoscopes has been a constant when other things have felt uncertain. I feel blessed to know you, you are all such warm, considerate, genuine, and downright fun people. You feel like family and even if we move away from our lunch table, I know that we will share many meals to come.

To my friends in Skåne, Uppsala and Sundsvall, your company is formative, and I really appreciate the impact you have had in my life. Whether it's an occasional fika, or a På Spåret tradition, the companionship and joy you provide is priceless.

To my family, in Canada and in Sweden, I'm lucky to have such a wide net to fall back on, you make me brave and curious. I love you.

Thank you, Ernest and Sarah, my little brother and sister, but all grown up now. You are both the wisest people I know.

Mamma and Daddy, thank you for your kind hearts, your sense of humor and your outlook on life. Your support has instilled in me the sense that I can achieve what I set my mind to, and the values you taught me ensure that my ambitious journeys are warm, a little funny, and never lonely.

Daniel, this has been a challenging journey for me and I'm sure, by extension, it's been a bit of a challenge for the person living with me as well, but if this was a pressure test then I'm blessed to see that what we have is diamond strong. You have been my support, my relief, my jokester, my master chef, my love. I don't know how I would have done this without you. Thank you. I love you.

Populärvetenskaplig sammanfattning

Biologiska läkemedel är viktiga i flera behandlingar, till exempel mot cancer och autoimmuna sjukdomar. Det som definierar ett biologiskt läkemedel är att den aktiva substansen är framställd ur biologiskt material, som levande celler eller vävnad. Denna vida definition leder till ett brett spektrum av molekyler som klassas som biologiska läkemedel, från hormoner, till antikroppar och goda bakterier.

En viktig grupp är proteiner. Proteiner har en peptidkedja som ryggrad och varje peptid bär en sidokedja. Storleken och strukturen på proteiner kan variera, små proteiner har en molekylvikt under 20 kDa medan stora antikroppar (som också är proteiner) kan ha en molekylvikt på flera 100 kDa. I jämförelse så har traditionella läkemedel ofta en mycket lägre molekylvikt, till exempel paracetamol på 0.15 kDa. Proteiners storlek medför en strukturell komplexitet. En proteinkedjas veckning och 3D struktur är essentiell för dess biologiska funktion och denna struktur är väldigt känslig för proteinets omgivning. En god förståelse för proteiners stabilitet är därför grundläggande för att utveckla biologiska läkemedel.

En styrka med biologiska läkemedel är att de ofta härmar eller förstärker molekylära mekanismer som finns i kroppen. En utmaning är att dessa känsliga molekyler behöver genomgå flera steg, inklusive produktion, transport, och förvaring, dessutom behöver de ta sig förbi kroppens barriärer innan de når sin verksamma site. Den viktigaste aspekten för att säkerställa ett stabilt terapeutiskt protein är dess formulering. Formulering innefattar det mesta utöver den verksamma molekylen i en läkemedelsprodukt. Eftersom terapeutiska proteiner lätt bryts ner i magen så är injektion, antingen intravenöst eller som injektion i underhudsfettet, den absolut vanligaste administrationsvägen. En formulering för terapeutiska proteiner behöver därför utvecklas med detta i åtanke.

Mänskligt tillväxthormon är ett mindre protein (22 kDa) och en av huvudrollsinnehavarna i denna avhandling. Patienter som tar denna behandling behöver regelbundna injektioner. Det blir en del av vardagen. En produkt som underlättar detta är, till exempel, en injektionspenna som kan användas upprepade gånger. En typisk formulering för detta bruk innehåller (utöver proteinet) vatten, buffertsalter, tensid och konserveringsmedel. En tensid är en ytaktiv molekyl. Även proteiner är ytaktiva och de kan destabiliseras genom interaktion med de ytor som omger proteinlösningen. Tensider har i regel en snabbare inbindning till ytor än proteiner. De används därför i formulering av terapeutiska proteiner för att blockera proteiners tillgång till destabiliserande ytor.

Både tensider och konserveringsmedel är viktiga för en stabil produkt, men det är också känt att tensider och konserveringsmedel kan interagera med varandra. Något som kan leda till partikelbindning, vilket i sin tur leder till fler ytor i lösningen och en instabil produkt. Stabilitet av proteinformuleringar är därför en svår balansgång, varje ingrediens behövs, men blandar man fel kan resultatet bli instabilt ändå. Denna

avhandling studerar proteiner i lösning tillsammans med tensider och konserveringsmedel. Dessa system drivs till sin gräns, de drivs medvetet mot instabilitet. Målet är en bättre förståelse för de mekanismer som driver både partikelbildande och förstörelse av proteinets aktiva struktur. Med kunskap om vad som driver dessa processer, utöver den kunskap som redan finns angående vilka kombinationer som resulterar i instabilitet, hoppas vi kunna underlätta framtida produktutveckling. Detta kan leda till fler nya proteinformuleringar som i sin tur kan underlätta livet för fler människor. Nedan följer en sammanfattning av de mekanismer och interaktioner som vi observerat.

Oladdade tensider interagerar i regel inte med proteiner medan laddade tensider binder till och bildar komplex med proteiner. Det är också känt att tillsatsen av oladdade tensider driver laddade tensider bort från protein-komplexet, för att i stället bilda blandade miceller (sfäriska tensidstrukturer) med den oladdade tensiden. När den laddade tensiden lämnat proteinet så har en återbildning av proteinets nativa (ursprungliga) struktur observerats. När vi försökte efterliknande detta med tillväxthormon, en laddad tensid och en oladdad sockertensid observerade vi något nytt. Vid relativt låga koncentrationer av sockertensid så drivs proteinstrukturen längre ifrån den nativa. Ett komplex med alla tre komponenter bildas och det som driver inkorporeringen av sockertensid är närvaron av den laddade tensiden.

Ytterligare en överraskande interaktion har observerats för tillväxthormon och sockertensid, denna gång i närvaro av salt och konserveringsmedel. Det är känt att specifika oladdade tensider är inkompatibla med konserveringsmedel, men inte sockertensid. I vår studie av konserveringsmedel inkluderades därför sockertensider för att ingå i en stabil referensformulering. En lösning av konserveringsmedel, sockertensid och tillväxthormon är stabil, men tillsats av salt leder till en dramatisk partikelbildning. Denna partikelbilning sker inte om proteinet är utbytt mot serumalbumin. Sammanfattningsvis så är konsten att formulera biologiska läkemedel, med till synes få komponenter, otroligt komplext. Mitt arbete visar att mekanismer i dessa multikomponentsystem kan urskiljas, kartläggas och karakteriseras, och dessutom förankras i teoretiska fysikalkemiska koncept.

Popular science summary

Biologics are important in several treatments, for example against cancer and autoimmune diseases. What defines a biological drug is that the active substance is produced from biological material, such as living cells or tissue. This broad definition leads to a wide range of molecules that are classified as biologics drugs, from hormones to antibodies and good bacteria.

An important group is proteins. Proteins have a peptide backbone chain and each peptide carries a side chain. The size and structure of proteins can vary, small proteins have a molecular weight below 20 kDa while large antibodies (which are also proteins) can have a molecular weight of several 100 kDa. In comparison, traditional pharmaceuticals often have a much lower molecular weight, such as paracetamol of 0.15 kDa. The size of proteins entails structural complexity. The folding and 3D structure of a protein chain is essential for its biological function and this structure is very sensitive to the protein's surroundings. A good understanding of the stability of proteins is therefore fundamental for the development of biologics.

One of the strengths of biologics is that they mimic or enhance molecular mechanisms found in the body. A challenge is that these sensitive molecules need to go through several steps, including production, transport, and storage before reaching a patient. They also need to get past the body's barriers before they reach their active site. An important aspect to ensure a stable therapeutic protein is formulation development. Since therapeutic proteins are easily broken down in the stomach, injection, either intravenously or as an injection into subcutaneous tissue, is by far the most common route of administration. A formulation for therapeutic proteins therefore needs to be developed with this in mind.

Human growth hormone is a small protein (22 kDa) and one of the main protagonists of this thesis. Patients who depend on this treatment need regular injections. It becomes part of everyday life. A product that facilitates this is multi-injection devices that can be used repeatedly. A typical formulation for this kind of use contains (in addition to the protein) water, buffer salts, surfactant, and preservatives. Preservatives keep the product sterile and protects the formulation from microbiological degradation. A surfactant is a surface-active molecule. Proteins are also surface-active, and they can be destabilized by interaction with surfaces surrounding the protein solution. Surfactants usually bind to surfaces more quickly than proteins. They are therefore used in the formulation of therapeutic proteins to block proteins' access to destabilizing surfaces.

Both surfactants and preservatives are important for a stable product, but it is also known that surfactants and preservatives can interact with each other. This can lead to particle formation, which in turn leads to more surfaces in the solution and an unstable product. Stability of protein formulations is therefore a difficult balancing act, every ingredient is needed, but if you mix incorrectly, the result can be unstable

anyway. This thesis studies proteins in solution with surfactants and preservatives. These systems are pushed to their limits, deliberately driven towards instability. The aim is to achieve a better understanding of the mechanisms that drive both particle formation and disruption of the protein's active structure. With knowledge of what drives these processes, in addition to the knowledge that already exists regarding which combinations result in instability, we hope to facilitate future product development. This could lead to new therapeutic products, which in turn could help more people. Below is a summary of the mechanisms and interactions we have observed.

Uncharged surfactants generally do not interact with proteins, while charged surfactants bind to and form complexes with proteins. It is also known that the addition of uncharged surfactants drives charged surfactants away from the protein complex, to instead form mixed micelles (spherical surfactant assemblies) with the uncharged surfactant. When the charged surfactant has left the protein, a refolding of the protein's native bioactive structure has been observed. When we tried this with human growth hormone, a charged surfactant, and an uncharged sugar surfactant, we observed something new. At relatively low concentrations of sugar surfactant, the protein structure is pushed further away from the native one. A complex with all three components is formed and what drives the incorporation of sugar surfactant is the presence of the charged surfactant.

Another surprising interaction has been observed for human growth hormone and sugar surfactant, this time in the presence of salt and preservatives. It is known that specific uncharged surfactants are incompatible with preservatives, but not sugar surfactant. In our study of preservatives, sugar surfactants were therefore included in what was supposed to be a stable reference formulation. A solution with preservative, sugar surfactant and growth hormone is stable, but the addition of salt leads to a dramatic particle formation. This particle formation does not occur if the protein is replaced by bovine serum albumin. In summary, the art of formulating biologics, with seemingly few components, is incredibly complex. My work shows that mechanisms in these multicomponent systems can be distinguished, mapped, characterized, and anchored in theoretical physicochemical concepts.

List of Papers

Paper I

Johanna Hjalte, Shakhawath Hossain, Andreas Hugerth, Helen Sjögren, Marie Wahlgren, Per Larsson, and Dan Lundberg. (2021) Aggregation Behavior of Structurally Similar Therapeutic Peptides Investigated by ^{1}H NMR and All-Atom Molecular Dynamics Simulations

Paper II

Johanna Hjalte, Carl Diehl, Anna E. Leung, Jia-Fei Poon, Lionel Porcar, Robert Dalgliesh, Helen Sjögren, Marie Wahlgren, Adrian Sanchez-Fernandez. (2024) Modulating Protein Unfolding and Refolding via the Cooperative Association of an Anionic and a Nonionic Surfactant

Paper III

Johanna Hjalte, Helen Sjögren, Marie Wahlgren, Zoltan Takacs, Dan Lundberg. (2024) Characterization of Aggregates Formed in Aqueous Mixtures of Anionic-nonionic Surfactants and Protein, using NMR

Paper IV

Marie Wahlgren, Anna-Maria Börjesdotter, Johanna Hjalte, Javier Lagares Martín, LingPing Zhang, Helen Sjögren and Stefan Ulvenlund. (2024) Interactions between Polysorbate and Antimicrobial Preservatives in Aqueous Parenteral Products

Paper V

Johanna Hjalte, Anna-Maria Börjesdotter, Carl Diehl, Stefan Ulvenlund, Marie Wahlgren, and Helen Sjögren. (2024) Excipient Effect on Phenol-induced Aggregation of Human Growth Hormone and BSA

Author's contribution to the papers

Paper I

I participated in the design of the NMR experiments together with Dan Lundberg., I conducted the experimental work and analysis of the NMR experiments. I interpreted the data and wrote the NMR and general sections of the paper in collaboration with my supervisors. The simulations were designed and conducted by Shakhawath Hossain and Per Larsson.

Paper II

I designed the first experiments together with Adrian Sanchez-Fernandez. I conducted all the fluorescence titration measurements and prepared NMR samples that were analysed at the Swedish NMR centre in Gothenburg by Carl Diehl. I participated in the collection of SANS data together with Adrian Sanchez-Fernandez. I wrote the first draft of the manuscript and worked on the finished draft together with my supervisors.

Paper III

I designed the study together with my supervisors. I conducted the experimental work and analysis of data with contributions from co-authors. I wrote the first draft of the manuscript and worked on the finished draft together with my supervisors.

Paper IV

I conducted part of the experimental work and supervised two of the master students involved in the study. I was partially involved in writing the final paper.

Paper V

I designed the study together with my supervisors. I conducted all the fluorescence titration measurements and prepared NMR samples that were analysed at the Swedish NMR centre in Gothenburg by Carl Diehl. I worked on the manuscript together with my supervisors.

Abbreviations

hGH	Human Growth Hormone
BSA	Bovine Serum Albumin
β-LG	β-lactoglobulin
DDM	Dodecyl Maltoside
DM	Decyl Maltoside
SDS	Sodium Dodecyl Sulphate
PS20	Polysorbate 20
PS80	Polysorbate 80
NMR	Nuclear Magnetic Resonance
SANS	Small angle neutron scattering
CD	Circular dichroism

Introduction

Aim

The work of this thesis aims to aid development of liquid protein formulation by studying phenomena related to physical stability. This is further defined by the following points:

- Improve understanding of aggregation of proteins and peptides
- Improve understanding of protein-excipient interactions
- Explore integrative approaches to connect in-house methods to advanced methods like NMR and SANS

The work toward these aims was conducted in three projects. These projects can be defined by the following aims:

- Characterization of differences in aggregation behavior for structurally similar peptides
- Understanding mechanisms of protein unfolding and refolding induced by surfactants
- Identifying driving mechanisms of incompatibility in protein-surfactant-preservative systems

Background

This thesis sets out to investigate physical stability of therapeutic proteins and peptides in solution. The study of physical stability includes phenomena like unfolding, aggregation, precipitation, and interactions with excipients. Some essential classes of excipients in formulation of therapeutic proteins in solution are buffering salts, surfactants, preservatives and tonicifiers (Pramanick, Singodia, and Chandel 2013). In formulation, non-ionic surfactants prevent the adsorption of protein to surfaces and interfaces, thereby restricting protein aggregation induced by interfaces (Manning et al. 2010). The purpose of preservatives is to maintain sterility, which is important in multiple use products (Meyer et al. 2007). Multiple

use products alleviate the logistics of drug administration for patients in need of daily injections. Tonicifiers are added to ensure that the liquid formulation has the same osmotic properties as physiologic fluid.

The papers of this thesis are ordered by increasing complexity of the studied formulation. From therapeutic peptides in neat water in the first project (Paper I) to therapeutic proteins in solution with surfactants in the second project (Papers II-III), with the addition of preservatives and salt in the third project (Paper V). The peptide or protein in question is characterized as it is driven to either aggregate, to form protein-surfactant complexes or to precipitate. A key advantage with these studies is the implementation of a titration method that allows the study of protein transitions with high resolution in terms of titrant concentration. In Papers II-V, Probe Drum equipment (Labbot, Lund, Sweden), which allows automatic nanolitre titration while collecting fluorescence emission spectra and/or laser scattering data, is applied to study concentration dependent effects of excipients on selected proteins.

Once important transition points are identified, the implementation of advanced characterisation methods allows further development of a detailed model of the interaction between biomolecules and excipients. Nuclear magnetic resonance spectroscopy studies have been designed to achieve an in depth understanding of the protein response, while contrast-variation small angle neutron scattering was employed to study the conformational changes in the structure of the protein. Overall, the goal of these systematic studies is to demonstrate general mechanisms based on non-covalent interactions. A summary of aims and conclusion for each paper, as well as the selected biologic, excipients and methods is found in Table 1.

Summary of Papers

In Paper I, the aggregation propensity of structurally similar peptides was investigated. Using NMR, absolute integral values for a representative proton peak were obtained and compared over the time scale of a week, and over a concentration range of 0-1 – 10 mM peptide. This is compared to aggregation behaviour simulated on a 500 ns time scale, using all atom molecular dynamics, provided by a collaborative research group. Though based on different principles, the obtained ranking of aggregation propensity of the studied peptides is the same for both methods.

In Papers II-III, refolding of proteins unfolded by ionic surfactants is attempted by the addition of a non-ionic surfactant. This kind of refolding has been demonstrated previously (Zardeneta and Horowitz 1994; Doñate et al. 1998; Kaspersen et al. 2017; Saha et al. 2018). For human growth hormone (hGH) and the anionic surfactant sodium dodecyl sulphate (SDS), different complex states have previously

been characterized, and the complex structure was shown to vary with concentration of SDS (Sanchez-Fernandez et al. 2020). We wanted to expand on this by systematically investigating the refolding process starting from different SDS-concentration-dependent-complex-structures (Paper II and III).

The selected non-ionic surfactant was n-dodecyl-β-D-maltoside (DDM), in part because this surfactant does not have polydispersity issues like polysorbates, and because it could be deuterated. This is of great use for advanced methods like contrast matched small angle neutron scattering (SANS). We also hypothesized that a greater refolding potential would be observed with a matching alkyl tail length. In addition, the study involves two model proteins, BSA and β-LG, which allows development towards a general framework to rationalise the protein refolding process. Molecular structures of hGH, SDS and DDM are displayed in Figure 1.

Surprisingly, intrinsic fluorescence titration studies of hGH indicated the involvement of DDM in the hGH-SDS complex, and with increased DDM concentration the refolding process was initiated. In comparison, this complexation behaviour was not observed when polysorbate 20 (PS20) was added to hGH and SDS. Moreover, titration with decyl maltoside (DM) shortened the concentration range of non-ionic surfactant where this complexation was observed. This went against our hypothesis of refolding potential, but it also indicated that interactions of the hydrophobic surfactant tails are essential for this kind of complexation. Papers II-III characterize and investigate the mechanisms of this complex formation with NMR and SANS.

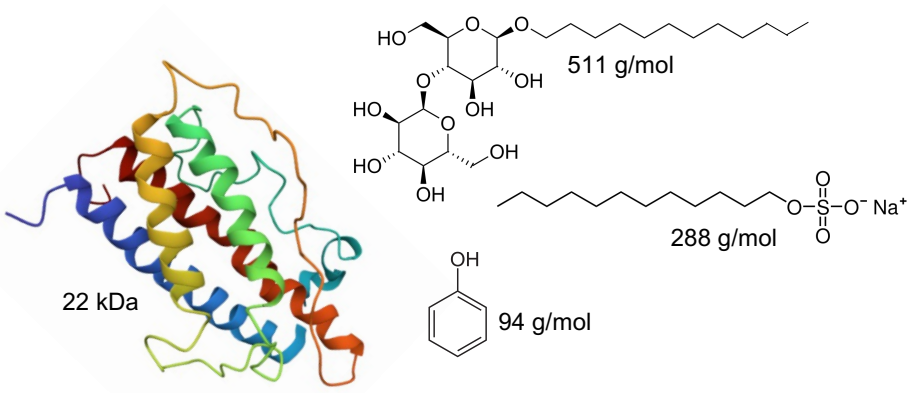


Figure 1. Molecular structures and molecular weight of hGH, PDB 1hgu (Chantalat et al. 1995), DDM, SDS and phenol.

In Paper IV, it is shown that the well-known incompatibility of polysorbates and preservatives is due to a dramatic depression of the polysorbate cloud point in the presence of preservatives like phenol. It is also known that proteins precipitate in the presence of phenol (Hutchings et al. 2013), and in Paper V the interplay of protein, surfactant and preservative is explored.

The driving mechanisms of precipitation are found to vary depending on formulation composition. For hGH, ionic strength of the solution is an important factor. In a solution of hGH-phenol-polysorbate 80, in 10 mM phosphate buffer with no further salt at pH 7, no precipitation formation is observed unless the cloud point concentration of polysorbate is exceeded. However, if salt is added, precipitation is observed below this polysorbate concentration, indicating a different mechanism. Moreover, another surprising hGH-DDM interaction is observed. In the presence of salt and phenol, precipitation occurs at a lower salt concentration with DDM than without. A summary of these clouding propensities is given in Figure 2.

In summary, a general blueprint of the molecular mechanisms driving physical instability in therapeutic protein formulation is far from easy to obtain. Especially as the dominant mechanisms are moving targets when the concentration or identity of specific components is changed. Nonetheless, new insights are available by the means of systematic investigation. This thesis sets out to demonstrate practical examples of the utility of the combination of bench-top techniques and NMR to understand mechanisms of instability in formulation, and to investigate protein-surfactant interactions in complex systems.

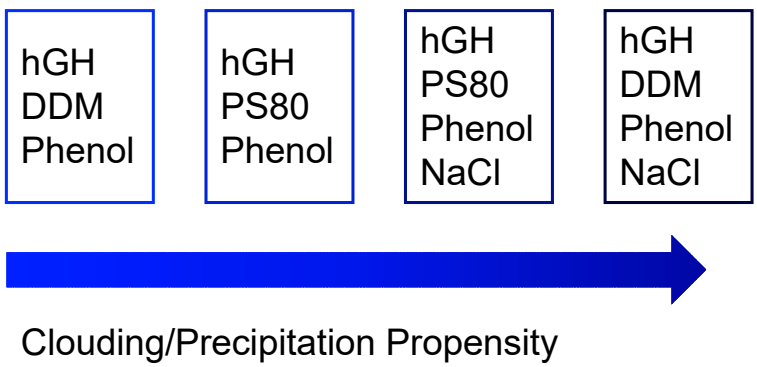


Figure 2. Overview of clouding propensity of hGH and phenol systems, for more specifics see Paper V.

Table 1. Summary of substances, methods, aim and conslusion of the papers in this thesis

	Biologic	Excipient Type	Methods	Aim	Conclusion
1	D-Phe^6-GnRH Ozarelix Cetrorelix Degarelix	-	NMR All atom molecular dynamic simulation	Evaluate applicability of selected methods for investigation of aggregation propensity	The selected methods agree on ranking of aggregation propensity. Only degarelix involves counterion in the formed aggregates.
2	hGH BSA β-lactoglobulin	Non-ionic and anionic surfactant	Fluorescence titration spectroscopy NMR Small Angle Neutron Scattering	Investigate refolding mechanism by the additon of non-ionic surfactant	In a limited concentration interval, there are indications of a DDM-SDS-hGH complex. Complete refolding of hGH is not observed.
3	hGH	Non-ionic and anionic surfactant	Fluorescence titration spectroscopy NMR spectroscopy	Verify the occurrence of a DDM-SDS-hGH triplex and characterize component distribution	The DDM-SDS-hGH complex is verified. Understanding of component distribution within the system is deepened.
4	-	Non-ionic surfactant Preservative Tonicifier	Fluorescence titration spectroscopy	Understand incompatibilities between preservatives, and non-ionic surfactants	Incompatibilities between polysorbates and preservatives can be understood as a cloud point depression.
5	hGH BSA	Non-ionic surfactant Preservative Tonicifier	Fluorescence titration spectroscopy NMR spectroscopy	Understand incompatibilities between preservatives, non-ionic surfactants, and proteins	Precipitation of hGH is observed in presence of NaCl and phenol. This is delayed by presence of polysorbates, but exacerbated by presence of DDM. Without salt, polysorbate driven clouding can be observed.

Chapter 1 – Formulation of Biologics

The skill of preparing medicine is an ancient one. Thousand-year-old medical recipes are found on tablets from Babylon (Geller 2010). Building knowledge on cures and treatments is an age-old craft, and the urge to develop new medicine seems integral to humanity. In this thesis I write my own small contribution to the current field of formulation of biologics.

Biologics

The term biologics covers a wide range of therapeutics, including antibodies, proteins, nucleic acids, viruses, and cells (Sharfstein 2018). Biologics are generally produced by living cells, which allows the assembly of molecules with multidimensional complexity including secondary, tertiary and sometimes quaternary conformation (Castaneda Ruiz et al. 2022). In about two decades, biologics have grown from about 1% of approved drug products to 20% in 2016 (Muralidhara and Wong 2020), and in the period 2020-2022, this number has grown to 30% of drugs approved by the FDA (de la Torre and Albericio 2023).

A growing class of biologics is nucleic acids, like DNA and mRNA (Muralidhara and Wong 2020). The development of mRNA based COVID vaccines was a remarkable display of the capabilities of the pharmaceutical industry. Nonetheless, once an effective and safe product is obtained challenges remain, and for these vaccines, logistics posed a major challenge. In particular, the required cold storage chain. The improvement of these products now depends on formulation development. In general, the development an optimized formulation to keep the active modality stable until it reaches the patient is an essential part of drug development.

In this thesis, liquid formulations of proteins with over a hundred amino acids will be encountered, as well as a series of structurally similar decapeptides. The main model protein is recombinant human growth hormone (hGH) with 191 amino acids (Chantalat et al. 1995).

Proteins as active pharmaceutical ingredient

The advantageous use of protein therapeutics has brought great value to the global healthcare system. Protein therapeutics are generally well tolerated with low immunogenicity because many therapeutic proteins are also produced naturally in the body. The biological action of proteins is highly specific, and this lowers the risk of protein therapeutics interfering with normal biological processes and causing adverse effects. Moreover, the complex set of functions that proteins serve cannot be mimicked by small molecule drugs. Some examples of action mechanism of protein therapeutics include replacing deficient or abnormal protein, augmenting existing pathways, interfering with a target molecule or as vaccines and diagnostics (Leader, Baca, and Golan 2008). However, a risk with protein pharmaceutics are immunogenic effects due to protein aggregation, including severe allergic responses. Strategies to ensure safe therapeutic protein products include screening for protein aggregates, control sources of stress (e.g. temperature), addition of stabilizing excipients, PEGylation and glycosylation (Lundahl et al. 2021).

In 1982, the first recombinant protein, insulin, was approved by the FDA, and this was an important turning point (Leader, Baca, and Golan 2008). Sixty years prior, insulin was first purified from bovine and porcine pancreas and used in life-saving injections for people with diabetes type 1 (Leader, Baca, and Golan 2008). However, widespread accessibility of this therapy faced several challenges: the availability of the animal source, the cost of insulin purification and adverse immunological reaction to animal insulin. These issues are circumvented with recombinant insulin. With recombinant DNA technology, bacteria or mammal cells are engineered to express the desired protein. Recombinant insulin can be produced on large-scale resulting in a therapy that is inexpensive and of low immunogenicity (Leader, Baca, and Golan 2008).

Recombinant protein production systems include bacteria, yeast, and mammalian cells (Leader, Baca, and Golan 2008). The choice of cell line, species origin and culture conditions all affect the final product (Lagassé et al. 2017). For example, glycosylated proteins must be produced by eukaryotic cell lines. In 2006, one third of approved biologics were glycoproteins (Walsh and Jefferis 2006). Until 2008, over 130 proteins and peptides had been approved for clinical use by the US Food and Drug Administration (FDA), and out of these, over 70% were produced recombinantly, while a small number of protein therapeutics were derived from their native source, for example pancreatic enzymes from porcine pancreas (Leader, Baca, and Golan 2008). In the period 2011-2016, 62 recombinant therapeutic proteins were approved, and the treatment area for over half of these was either oncology or hematology (Lagassé et al. 2017).

Human Growth Hormone

The main model protein used in this thesis is recombinant human growth hormone (hGH), interactions between hGH and selected excipients is studied in Papers II, III and V.

In 1922, it was determined that the pituitary gland regulates growth, and in 1956 human growth hormone was first isolated. Recombinant human growth hormone has been used since 1985 (Cohen 2016). It is mainly used in treatment for children with growth hormone deficiency, Turner syndrome, Prader-Willi syndrome, and chronic renal insufficiency, but it is also used in adults for HIV-related wasting and fat accumulation associated with lipodystrophy (Franklin and Geffner 2011; Cai et al. 2014; Hindmarsh and Dattani 2006). Treatment consists of daily subcutaneous injections (Fisher and Acerini 2013; Takeda et al. 2010).

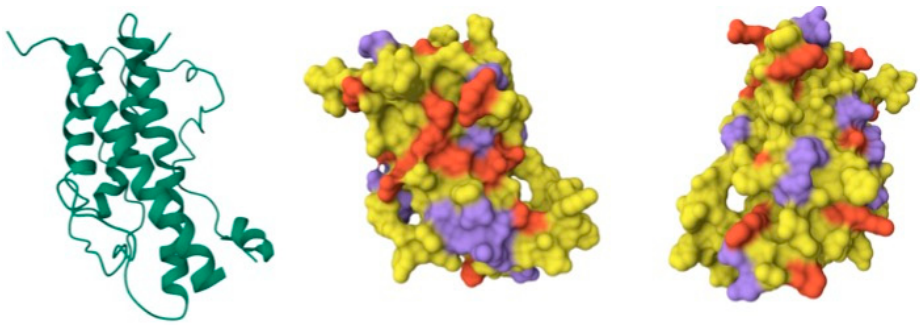


Figure 3. Molecular structure of hGH, PDB 1hgu (Chantalat et al. 1995) . Far left shows secondary structure and the next two views show a molecular surface representation with positively and negatively charged amino acids in red and purple, respectively.

The molecule has 191 amino acids, and a molecular weight of around 22 kDa (Chantalat et al. 1995). The isoelectric point of recombinant hGH is around pH 5 and at pH 7 the net charge of the protein is -5 (Gellerfors et al. 1989). The molecular structure is a bundle of four alpha helices, see Figure 3, with two disulfide bridges (Chantalat et al. 1995). The protein has two sites for zink ion binding (His18, His21 and Glu174). Zink binding results in dimerization of the protein which might be important for aggregation and storage in secretory granules (Petkovic et al. 2013).

The rigidity of the molecule is strongly affected by solutions conditions, and backbone flexibility increases at acidic pH, compared to neutral (Kasimova et al. 2002). Mobility of residues depends on their location in the secondary structure units. One end of the bundle forms a stable nucleus and this end has been shown to be important for receptor binding, whereas an increased water penetration occurs in the other, where a majority of aromatic residues are found (Kasimova et al. 2002).

Therapeutic Peptides

In Paper I the differences in aggregation propensity of structurally similar peptides is studied. The peptides in question are therapeutically relevant analogues of the human gonadotropin releasing hormone, GnRH. These peptides have ten residues and the molecular structure of one of them, cetrorelix, is displayed in Figure 4.

Peptides and proteins can be differentiated in different ways. Peptides have a shorter amino acid chain, and the line is commonly drawn at 50 amino acids. With this definition, insulin is one of the smallest proteins at 51 amino acids. Another rule of thumb is whether the molecule in question has a well-defined tertiary structure, making natively unfolded proteins an exception to this rule (Hovgaard, Frokjaer, and van de Weert 1999).

A practical difference between the two is that while recombinant methods are the most common for proteins, the prevailing method for preparing peptides is chemical synthesis (Hovgaard, Frokjaer, and van de Weert 1999). The development of chemical synthesis of peptides on a solid matrix by Robert Bruce Merrifield was awarded the Nobel Prize in chemistry in 1984 (Merrifield 1967; 'The Nobel Prize in Chemistry 1984'). Peptides prepared by chemical synthesis are therefore not produced by living organisms, and depending on the definition of biologics, this may place them outside that umbrella term. Nonetheless, therapeutic peptides and proteins can be classified together as biomolecules.

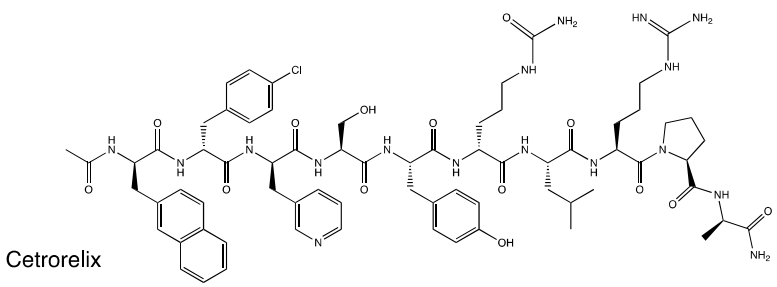


Figure 4. Molecular structure of one of the decapeptides, cetrorelix, studied in Paper I.

Compared to other biologics, peptides show lower potential to elicit immune responses and production costs are lower. Worldwide sales of peptide drugs increased more than two-fold between 2013 – 2019, exceeding $70 billion (Wang et al. 2022). In the body, therapeutic peptides bind to cell surface receptors and trigger intracellular effects with high affinity and specificity. Thus, peptides commonly act as hormones, growth factors, neurotransmitters, or anti-infective agents. However, drawbacks include membrane impermeability and poor in vivo stability.

In particular, the general lack of a defined secondary or tertiary structure makes the amide bonds more exposed to be hydrolysed or destroyed by enzymes, resulting in faster elimination in vivo (Wang et al. 2022). The lack of a defined tertiary structure results in solvent exposure of hydrophobic residues, which could also result in aggregation (Zapadka et al. 2017). Essential aspects in formulation development of therapeutic peptides are protection against both chemical and physical degradation, while retaining the therapeutic function of the peptide, and compliance with regulation.

Formulation of Therapeutic Proteins

As mentioned, therapeutic proteins have revolutionized the pharmaceutical industry. In 2016 about 74% of biologics were proteins. Production of therapeutic proteins in a highly complex process, and a typical protein drug requires many times more critical production steps than small molecule drugs (Lagassé et al. 2017; Schellekens 2009). Therapeutic proteins are subject to a variety of environmental stresses throughout the product life cycle including mechanical stresses, temporary sub-optimal solution conditions, freeze-thaw, interaction with different surfaces, variation in temperature and exposure to light (Muralidhara and Wong 2020). The question of therapeutic protein stability is intimately linked to the safety and efficacy of the product, and the development of an optimized formulation is crucial to bring a product to the market and to patients in need.

Formulations are developed according to administration route. Most biologics are delivered parenterally, that is without passing mouth, stomach, and intestine, because of enzymatic degradation and poor permeation (Muralidhara and Wong 2020). Common excipients include (i) salts and sugars for stabilizing purposes and as tonicifiers (giving the formulation similar osmotic properties as physiologic fluid), (ii) surfactants to protect the biologic from interfacial instability, and (iii) preservatives ensure sterility (Baird, Hodges, and Denyer 2000).

In general, a liquid formulation of therapeutic protein is preferred. When this is not possible due to chemical and physical instabilities, the protein is freeze-dried and reconstituted prior to injection (Ko et al. 2023; Wang 2000). This solid formulation entails additional production steps (freeze drying) as well as additional cryoprotective excipients (Seifert and Friess 2019). Moreover, reconstitution results in a more complex administration procedure.

A key consideration in formulation development is patient compliance, a product will have little value if no patient wants to use it. When high frequency dosing is required, subcutaneous injections with prefilled syringes, autoinjector or pens, can improve quality of life by allowing self-administration at home. In the period of

1995 – 2018, approximately 30% of liquid biological drugs approved in the EU were multiple use and formulated with preservatives (Gervasi et al. 2018).

Moreover, multidose-products are connected to a more cost-effective production process and generate less packaging waste. However, proteins are biodegradable and therapeutic protein formulations can provide sufficient nutrition for bacteria, yeast, and fungi. Thus, for patient safety, multi-dose drugs must show effectiveness against microbial growth (Stroppel et al. 2023), and the formulation must be optimized to reach required shelf life (Muralidhara and Wong 2020). Common preservatives include phenol, benzyl alcohol and metacresol (Meyer et al. 2007).

The condition sensitive, structural complexity of therapeutic proteins can result in a variety of possible degradation paths before the protein is administered. These will be covered in more detail in the next chapter. An understanding these mechanisms is important for the formulation scientist. It is easier to optimize a formulation if one is familiar with the potential pitfalls and the underlying molecular mechanisms associated with, for example, specific excipient choices.

Chapter 2 – Physicochemical Aspects of Protein Formulation

Colloid science involves pretty much everything -on one condition, a characteristic dimension must be in the nanometre range. For example, a glass of milk or smoke from a fire are both considered colloidal systems, despite the obvious differences between them. Thus, any heterogeneous dispersion of "particles" with one or more dimensions between 1 nm and 1000 nm, regardless their composition (organic, inorganic, biologic, or a combination) can be described by colloid science. These systems are governed by a common set of rules, and colloidal interactions are essential for biological life. In this chapter you will find general physicochemical aspects of colloid science that are relevant for this thesis, and for liquid formulations of biomolecules.

Protein Instability

Proteins consist of amino acid chains with residues of a varying degree of hydrophobicity. Residues may also be charged, and the net charge of a protein depends on the pH of the solution. Globular proteins generally fold in solution in such a way that hydrophobic residues are protected at the core of the protein structure (Tanford 1997). The three-dimensional arrangement of the protein is essential for biological function. This structure can be disrupted in different ways, and in general this is classified as four types of instability: (i) chemical instability, (ii) interfacial instability, (iii) conformational instability, and (iv) colloidal instability (Manning et al. 2018).

Chemical instability refers to degradation of the protein by breaking of chemical bonds, for example, oxidation, deamidation and isomerization (Muralidhara and Wong 2020). The other instability types don't alter the protein primary structure and can be grouped together as forms of physical instability. Both interfacial and conformational instabilities result in unfolding of the protein structure. In solution, unfolded proteins species can associate and form aggregates. Aggregation can be the result of colloidal instability When aggregates grow to such extent that they can no longer be suspended in solution, this is precipitation (Hovgaard, Frokjaer, and van de Weert 1999). Also, proteins or peptides, without a secondary or tertiary fold

to lose, can aggregate. Thus, interfacial and conformation instabilities may lead to colloidal instability, whereas a protein unfolding event is not strictly necessary for aggregation to occur.

There are two major issues with aggregation of therapeutic proteins. First, there is the safety of the patient. If aggregated protein is administered to the body this can trigger an immunogenic response. Second, efficacy is lost when bioactive proteins are aggregated. Thus, tracking and understanding aggregation events is of utmost importance for the industry (Zapadka et al. 2017). However, aggregation can also be used to the advantage of a pharmaceutical product. An example of this is the aggregation prone peptide, degarelix, that is used to treat prostatic cancer. When injected, degarelix aggregates and forms a depot in the subcutaneous tissue. From this depot bioactive degarelix is continuously released over a period of up to three months (Pharmaceuticals 2011; Ozono et al. 2017) and this results in a significantly reduced injection frequency for patients.

Physical stability is also impacted by the presence of excipients and co-solutes. An excipient may adsorb to a protein and thereby effect protein conformation, which in turn could lead aggregation, and co-solutes may change the solvating properties of the solution, which could also result in protein conformation changes (Hovgaard, Frokjaer, and van de Weert 1999). Again, these kinds of interactions can be used to the advantage of a protein formulation. For example, phenolic ligands are added to zinc-containing insulin. This protects the protein from unfolding because for protein unfolding to occur the ligand would first need to be removed and this in an energy-consuming process (Huus, Havelund, Olsen, Sigurskjold, et al. 2006).

Surfactants

The term surfactant is an abbreviation of surface-active agent. Surfactant activity, in solution, involves adsorption to all interfaces of the solution, like the liquid-vapour surface and interfaces of the solid container. Surfactants are amphiphiles, which can be directly translated to “both-liking”. Surfactant molecules consist of at least two parts, and these parts are referred to as a hydrophobic and hydrophilic part. The hydrophobic part is typically an alkyl chain, referred to as the tail. The duality of the surfactant molecule and the fact that adsorption of the hydrophobic tails at solution interfaces reduces surface tension explains the surface activity (Holmberg et al. 2002). The hydrophilic part is called the head, and there are different types of head groups, they can be charged or uncharged.

Surfactants in Formulation of Biologics

Surface active agents used in parental formulation are the surfactants polysorbate 20 and 80 and the polymer poloxamer 188. In liquid formulation, surface active agents provide two main mechanisms of stabilization. First, interfacial instability is prevented as surfactants are preferentially adsorbed to the interfaces. Second, surfactants may associate with proteins and thereby hinder aggregation (Lee et al. 2011). Polysorbate 20 and 80 are mixtures of different fatty acid esters, and the monolaurate and monooleate fractions make up 40-60% and 58% of the mixture, respectively. Moreover, polysorbates undergo chemical degradation by autooxidation, cleavage at ethylene oxide subunits and hydrolysis of the fatty acid ester bond (Kerwin 2008).

Critical Micelle Concentration

Surfactants tend to form aggregates, called micelles, above a critical surfactant concentration, called the critical micelle concentration (cmc) (Wennerström and Lindman 1979; Israelachvili, Mitchell, and Ninham 1976). In this thesis surfactant molecules that are not part of a micelle or adsorbed at interfaces will be referred to as monomers. The formation of micelles can be regarded as the association of surfactant tails to form oil droplets, with the surfactant head directed toward the solution (Holmberg et al. 2002). Micellization is an equilibrium phenomenon (Mukerjee 1967), and if the surfactant concentration of a micelle solution is diluted below cmc, the micelles will disperse.

The cmc value depends on the chemical structure of the surfactant in question. For example, the cmc of a charged surfactant is often higher than that of an uncharged, due to the electrostatic repulsion of the head groups in micelle formation and the concentration of counter ions by the interface. Micellar size of ionic surfactants is sensitive to co-solutes. Addition of salt or aromatic compounds (like preservatives) promotes micelle growth (Holmberg et al. 2002). For example, for sodium dodecyl sulphate (SDS), an increased sodium chloride concentration lowers the cmc and the aggregation number (Holmberg et al. 2002). Meanwhile, for uncharged sugar-based surfactants, cmc is also affected by salt, and the impact depends on the ion species (Zhang, Somasundaran, and Maltesh 1996).

Micelles are considered as a separate (pseudo-) phase in the phase separation model, where an increased surfactant concentration effects the micelle concentration, while the free monomer concentration won't exceed the cmc value. This approximation is useful in analysis, as the output of many experiments describe the number average of available states to the surfactant molecule (Holmberg et al. 2002). For example, in diffusion NMR, observed self-diffusion coefficients are population-based averages. When plotting these values as a function of the reciprocal concentration, the cmc can be determined as the point where the trends of a constant monomer

diffusion coefficient and a decreasing diffusion coefficient with higher concentration (lower reciprocal value) meet, see Figure 5.

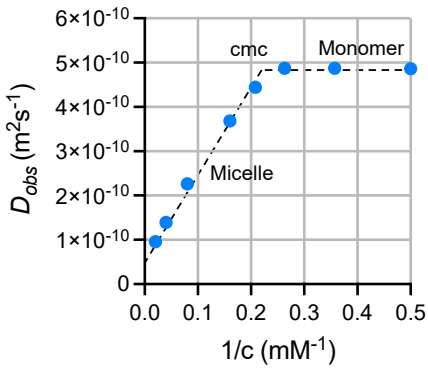


Figure 5. Observed diffusion coefficients, from diffusion NMR, for SDS in a 2 – 50 mM concentration range, plotted against reciprocal SDS concentration (Paper III).

Clouding

Clouding is a phenomenon that occurs for polyethene oxide-based surfactants and polymers. It is a phase separation into one surfactant-rich and one surfactant-poor phase and occurs at elevated temperatures (Mukherjee et al. 2011). This phenomenon can be distinguished by the naked eye, the surfactant solution is no longer clear once the cloud point is reached. In Figure 6, this is demonstrated for a solution of PS80 at 1 w% and $NaNO_3$ at 150 mM.

It is known that the combination of polyethene oxide-based surfactants and polymers with preservatives like phenol can lead to turbidity, and concentration ranges that result in turbidity have previously been identified (Zhi et al. 2015; Gilbert et al. 2022; Ford et al. 2023). In Paper IV it is demonstrated that the addition of phenol results in a depression of the cloud point and at a critical phenol concentration clouding occurs at room temperature.

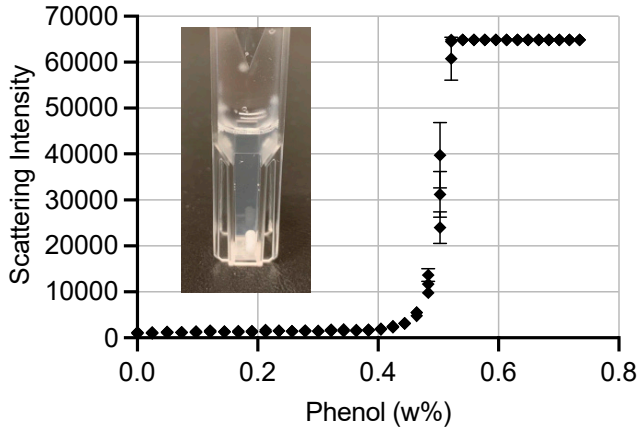


Figure 6. 1 w% PS80 and 150 mM sodium nitate titrated with a 4 w% phenol solution. The data represents 90° scattering intensity at 637 nm, and the insert a photograph of the cuvette after the final addition of phenol. The dectector cannot measure intensities above 64800.

Clouding is affected by co-solutes, and salts can either increase or decrease the temperature where clouding is observed (Dave and Joshi 2018; Schott 1984). For example, PS20 has a cloud point of 80°C in water and 73°C in 0.2 M NaCl (Chawla and Mahajan 2011). The effects of salts and organic compounds has been found to be additive in formulation (Dave and Joshi 2018; Chawla and Mahajan 2011; Rahman Khan et al. 2019). Moreover, if the surfactant binds to protein (e.g. bovine serum albumin), an increase in the cloud point surfactant concentration is observed (Wahlgren, Kedström, and Arnebrant 1997).

Mixed Micelles

In a surfactant mixture, mixed micelles are formed where all surfactant species are included. If there is no net interaction between the different head groups, ideal mixing occurs, and the cmc and mixed micelle composition can be calculated based on the cmc and molar fractions of the individual surfactants in the mixture (Holmberg et al. 2002). In the case of a mixture of charged and uncharged surfactant, the incorporation of uncharged head groups in the micelles shields the repulsion between the charged group, resulting in a net attractive interaction, and a non-ideal mixing (Holmberg et al. 2002; Gerber et al. 2005; Yuan and J. 1982). This effect can be demonstrated by experimentally determined cmc values for an SDS and dodecyl maltoside (DDM) mixture, at varying bulk composition, see Figure 7 (Hines et al. 1997; Zhang, Zhang, and Somasundaran 2004).

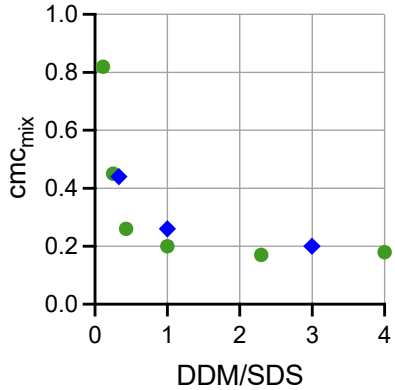


Figure 7. Literature values of experimetally determined critical micelle concentration for mixtures of DDM and SDS with varying composition (Hines et al. 1997; Zhang, Zhang, and Somasundaran 2004)

The cmc of SDS and DDM in pure water at 25°C is 7.95 and 0.17 mM, respectively (Hines et al. 1997). In a mixture where the concentration of SDS is ten times that of DDM, the cmc is lowered by almost a factor ten, compared to SDS alone. In this example, the expected micelle composition is two DDM to every SDS, a twenty-fold difference to the bulk composition (Hines et al. 1997). In a surfactant mixture, the composition of micelles and free monomers will vary with concentration. For SDS and DDM, at cmc, the micelle composition is expected go toward that of the bulk when the bulk fraction of DDM is increased (Hines et al. 1997).

Moreover, the micelle composition will go toward that of the bulk, at a set bulk fraction of DDM, with increasing total surfactant concentration, see for example Paper III. The increase in surfactant concentration will result in an increased concentration of micelles and the overshoot of DDM in micelles (and of free SDS) will diminish. Lastly, in a surfactant mixture, the free surfactant concentration can exceed the cmc of the mixture. For example, if the bulk composition of an SDS-DDM mixture is heavily skewed toward SDS (ten SDS to every DDM) and if the total SDS concentration is below the cmc of SDS, but above the cmc of the mixture, then a considerable fraction of the SDS molecules will be monomers, see Paper III.

Protein Surfactant Interaction

Proteins and surfactants are both amphiphilic, and as such, interactions between the two can be expected. In 1939, Anson noted that conformational changes are driven by the surfactant protein ratio, and specific interactions differ with surfactant identity (Anson 1939). Charged surfactants bind to and unfold proteins, which is utilized for protein separation in SDS-page (Shapiro, Viñuela, and V. Maizel 1967).

Uncharged surfactants, as mentioned, are used in protein formulation to prevent interfacial and colloidal instability. For example, PS20 binds to hGH when the protein structure is both native and in a molten globule state, in both cases the protein aggregation is prevented (Bam, Cleland, and Randolph 1996; Bam et al. 1998).

Surfactants can complex with proteins either as individual monomers or in micelle-like clusters. The surfactant concentration where new hydrophobic domains, in the form of micelle-like clusters, forms on protein is termed the critical association concentration (CAC) (Ghosh and Banerjee 2002). Because the free surfactant monomer concentration is depleted by the presence of protein, the formation of pure micelles can occur at a concentration that is higher than the CAC, and higher than the cmc of the surfactant alone (Ghosh and Banerjee 2002; Sanchez-Fernandez et al. 2020).

Protein-surfactant complexes show a rich variety of conformation, structure, and dynamics (Stenstam, Topgaard, and Wennerström 2003; Sun et al. 2015; Sanchez-Fernandez et al. 2020; Nielsen et al. 2007; Mehan, Aswal, and Kohlbrecher 2015; Saha et al. 2018; Sjögren et al. 2005), that is tuned by the surfactant to protein ratio. For example, the complexation of hGH and SDS has been studied with a wide array of methods. First, at low SDS concentration, individual SDS is adsorbed and there is a loss of tertiary structure (Sanchez-Fernandez et al. 2020). Above the CAC, the secondary structure is affected, and as the SDS concentration is increased a decorated micelle is formed. A decorated micelle refers to a micelle structure decorated with a protein shell. As hGH is a small protein, the decorated micelle is half-covered by a monomeric protein. Decorated micelles are well documented in the literature for a variety of proteins with SDS (Otzen et al. 2022).

Protein complexation with charged surfactants has been shown to be reversible by addition of uncharged surfactants (Zardeneta and Horowitz 1994; Doñate et al. 1998; Kaspersen et al. 2017; Saha et al. 2018). The general explanation is that SDS is incorporated in mixed micelles, leaving the protein free to refold (Otzen et al. 2022). The population of refolded protein increases with increased uncharged to charged surfactant ratio (Pedersen et al. 2020). It has also been observed that proteins that interact with uncharged surfactants, like fatty acid interacting proteins, refolds into a bound state with the uncharged surfactant (Kaspersen et al. 2017).

In this thesis, a refolding process, where the fold of the protein changes toward the native bioactive state of the protein, regardless of whether that state is reached or not, will be referred to as refolding. If the native state is reached, this will be referred to as complete refolding. Similarly, the term unfolding will be used to reference an unfolding process that does not necessarily lead to complete random coil unfolding.

Phenol Effects on Proteins and Surfactants

The most common preservatives in formulation of therapeutic proteins are benzyl alcohol, phenol, and meta-cresol. These preservatives can decrease the folding stability of the protein, which might trigger aggregation (Hutchings et al. 2013; Maa and Hsu 1996; Zhang et al. 2004; Arora et al. 2017). This destabilization can be explained as a reduction of polarity of the solvent, which reduces the hydrophobic effect (Hovgaard, Frokjaer, and van de Weert 1999). Some proteins also have specific binding sites for these aromatic compounds (Huus, Havelund, Olsen, van de Weert, et al. 2006; Alford et al. 2011).

In the case of micelles, phenol adsorbs in the palisade layer, for both charged and uncharged surfactants (Schott 1984; Lu and Somasundaran 2007). For sugar-based surfactants, this adsorption results in a reduced curvature of the micelles and micelle growth. Moreover, the adsorption of phenol to these micelles depends on pH, and in the pH range of 4 to 10 an increased phenol association has been observed at lower pH, indicating a dependence on the phenol charge state (Lu and Somasundaran 2007).

Hofmeister Series

In 1888, Franz Hofmeister investigated series of anions and cations, and their ability to stabilize protein in solution (Kunz, Henle, and Ninham 2004; Hofmeister 1888). Hofmeister demonstrated ion specific effects, and measured by concentration, he could order the ions according to their effectiveness (Lo Nostro and Ninham 2012). The series spans two kinds of observed effects, salting-in or salting-out. A salting-out anion, like SO_4^{2-}, stabilizes the native fold of the proteins, whereas as a salting-in anion, like NO_3^-, tends to facilitate protein unfolding, and for an ion that falls in an intermediate position of the series like Cl- (or Na+ in the cation series) neither tendency is observed (Kang et al. 2020; Zhang and Cremer 2006). For uncharged surfactants, cmc is decreased by salting-out salts and increased by salting-in (Khatory et al. 1993). Further, a decrease in micelle size for uncharged sugar surfactants has been observed with salting-in salts (Ericsson et al. 2004). The Hofmeister series also has a systematic effect on clouding of surfactants (Dave and Joshi 2018; Schott 1984).

Chapter 3 – Characterization Methods for Protein Formulation

For the formulation scientist working with therapeutic protein, there is a plethora of characterization methods at the ready, where each method can provide unique insight. To get to the core any mechanism, an approach that involves orthogonal methods is required.

For investigation of protein size, aggregation and/or dimerization one could apply dynamic light scattering (DLS) (Hassan, Rana, and Verma 2015; Patil, Keire, and Chen 2017) or field flow fractionation (FFF) (Wahlund and Nilsson 2012). Small angle scattering methods (SAS) (Pokorski and Hore 2019; Glatter 1977) and nuclear magnetic resonance spectroscopy (NMR) (Bramham et al. 2021; Blommers and Cerletti 1997) can also provide this information.

To investigate chemical stability and degradation products, high performance liquid chromatography (HPLC) (Aguilar 2004) and mass spectrometry (MS) (Standing 2003) can be applied. Size exclusion chromatography (SEC) (Hong, Koza, and Bouvier 2012) can also investigate these matters as well as dimerization. For thermal stability, differential scanning calorimetry (DSC) can be applied (Cueto et al. 2003), and another calorimetry method, isothermal titration calorimetry (ITC) can be applied to investigate binding and ligand interaction (Perozzo, Folkers, and Scapozza 2004).

Regarding protein structure, the crystal structure of a protein can be provided by X-ray crystallography, and for the secondary structure of proteins, circular dichroism (CD) (Greenfield 2006) or Fourier transform infrared spectroscopy (FTIR) (Byler and Susi 1986) can be applied. Small angle scattering and fluorescence, or NMR spectroscopy can also provide structural insights. Examples of microscopy methods include Flow Imaging Microscopy, atomic force microscopy (AFM) and transmission electron microscopy (TEM) (Hovgaard, Frokjaer, and van de Weert 1999).

Accessibility of methods varies from bench-top methods to international science hubs like Max IV or ESS. This thesis aims to find a general approach that integrates bench-top and more advanced methods. In the following section the applied methods of this work are described. Two methods that have worked well in tandem are Probe Drum and NMR. Small angle neutron scattering (SANS), and CD have also provided key insights to the systems at hand.

Circular Dichroism (CD)

Due to the chirality of protein molecules, there is a difference in adsorption of left-handed and right-handed circularly polarized light. This can be used to study protein structure (Greenfield 2006). A far-UV CD spectrum (below 250 nm) provides information on the secondary structure of the protein. Signature signals arise, for example, for alpha-helix or beta-sheet structures. In a near-UV CD spectrum (above 250 nm), only the aromatic residues and cysteine adsorb light, this provides information on the tertiary structure of the protein and ligand binding (Jiskoot and Bloemendal 2005). Combined, far- and near-UV CD can be used to study protein unfolding (Sanchez-Fernandez et al. 2020) and refolding (Kaspersen et al. 2017).

Small Angle Neutron Scattering (SANS)

Small angle scattering experiments with X-rays or neutrons provide similar insights. However, neutron scattering has specific advantages when studying biological systems. Neutrons are highly penetrating and weakly interacting. This means that samples will not be heated or destroyed during measurement (Pokorski and Hore 2019). Moreover, with neutrons, scattering from a specific part of samples can be enhanced or supressed. This is a considerable advantage for multi-component samples. This technique is called contrast matching. Neutrons scatter differently from hydrogen and deuterium, so by strategically deuterating components of the sample to either match or contrast with the scattering length density (SLD) of the environment, access to hidden features can be unlocked (Pokorski and Hore 2019). Without requiring assumptions on shape of the scattering objects, Indirect Fourier Transformation techniques (IFT) yield real-space pair-distance distributions from fitting scattering intensities in reciprocal space (Pokorski).

Contrast matching has, for example, been used to characterize hGH-SDS complexation by matching the surfactant to either the protein or the solvent to depict the complex and the protein within the complex (Sanchez-Fernandez et al. 2020), but also to determine the appropriate model to describe the configuration of polyethylene glycol (PEG) in mono-PEGylated lysozyme and hGH (Pai et al. 2011).

Probe Drum

Probe Drum, developed by Labbot (Lund, Sweden) is a machine, not much larger than a cooking pot, that can measure many things at once, see Figure 8. A key feature is the programmable automatic titration, that allows nanolitre titration steps. During a titration, up to four different measurements can be taken at each titration step

('Automate and combine proven biophysical techniques in a new way'). The first measurement can be either pH or conductivity with the appropriate electrode. The Probe Drum also allows for absorption spectroscopy, fluorescence spectroscopy and measurement of laser scattering intensity. These spectroscopic techniques apply light at specific wavelengths and measure spectra in the range of 240-760 nm.

Absorption occurs when the energy of incident light equals the energy difference between a ground and excited state of a target molecule. For proteins, all three aromatic residues (tryptophan, tyrosine and phenylalanine) absorb near-ultraviolet light, between 250 and 320 nm (Mach et al. 1995). The absorbed energy can be dissipated as light of lower energy resulting in fluorescence or phosphorescence. The aromatic residues of proteins give rise to intrinsic protein fluorescence, see next section. Laser scattering occurs when the sizes of molecules or aggregates in solution are comparable to the wavelength of incident light (Mach et al. 1995). This is utilized in the Probe Drum and the 90° scattering of red laser light (637 nm) can be measured.

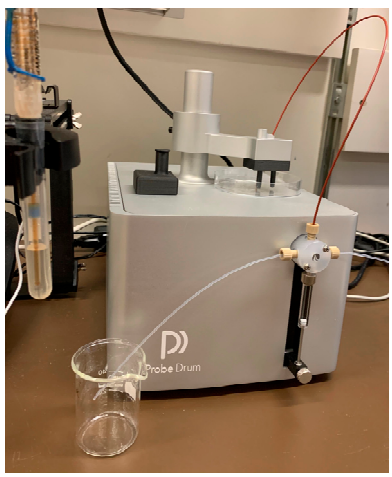


Figure 8. Photograph of Probe Drum equipment.

Intrinsic Fluorescence Spectroscopy

In intrinsic fluorescence measurements, the sample is excited with ultraviolet light (280 nm on Probe Drum) and aromatic residues of the protein emit fluorescent light. Of the aromatic residues, tryptophan residues are the dominant source of UV-absorbance and emission. The emission of tyrosine is often quenched in native proteins, possibly due to peptide chain interactions or energy transfer to tryptophan, and protein unfolding can result in increased tyrosine emission (Lakowicz 2006).

For tryptophan, protein unfolding often results in a red shift, towards longer wavelengths (Liu, Zhang, and Jin 2013). However, local electric charge can impact the shift as well. In a hydrophobic environment, like a protein core, the emission maximum of tryptophan can be as low as 308 nm, whereas tryptophan exposed to aqueous environment have an emission maximum of about 350 nm. An intermediate emission maximum is usually observed for partially solvent exposed tryptophan (Eftink 2002).

Application of Probe Drum

In this thesis the Probe Drum equipment has been utilized for many purposes. Fluorescence measurements have been applied to determine surfactant cmc (Paper III), by tracking the fluorescence emission peak position shift of a fluorescent probe (pyrene-1-aldehyde). A blue shift is observed for this probe when it is incorporated in a hydrophobic environment, like micelles.

Intrinsic protein fluorescence measurements have been applied to track the evolution of the protein folding state during addition of surfactants, addition of salt, or when lowering the pH of the solution (Papers II, III, V). In tandem, the red laser scattering has been tracked. This allows characterization of aggregation events, but also with surfactant addition, a linear increase in scattering with concentration indicates the expected increase in micelle concentration.

In Figure 9, selected intrinsic fluorescence spectra of hGH, and evolution of the maximum peak position, as well as red laser scattering intensity, during titration with SDS and DDM is shown. Data is based on two replicates. As can be seen, the addition of SDS results in a blue shift which indicates SDS complexation, whereas the fluorescence emission peak maximum does not move during addition of DDM. After 30 µl, addition of SDS results in a constant level of laser scattering. The scattering value differed for the two replicates, but the fact that the degree of intensity does not change indicates that the complexes in solution are not increasing in size or number with addition of SDS. Whereas for DDM, an increase in scattering intensity is observed, which can be linked to an increased concentration of DDM micelles. This is also seen in the spectrum at 13 mM DDM, where a shoulder at the excitation wavelength, 280 nm, appears.

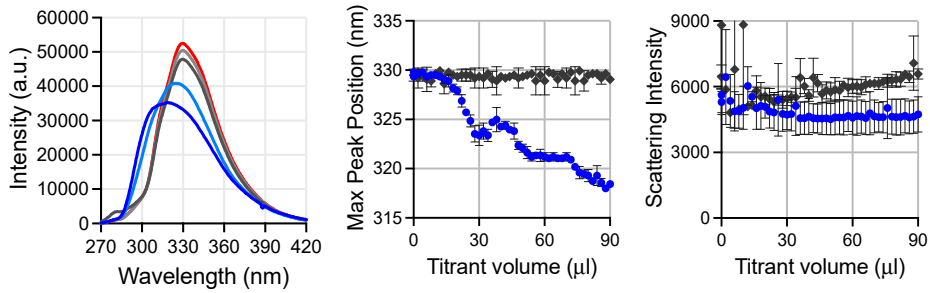


Figure 9. Left panel: Intrinsic fluorescence protein spectra, native (red), with 2 mM DDM (light grey) with 13 mM DDM (dark grey), with 2 mM SDS (light blue) and with 6 mM SDS (dark blue). Centre panel: maximum peak position of intrinsic fluorescence during titration with SDS (blue circles) or DDM solution (grey diamonds). Right panel: red laser scattering intensity (637 nm at 90 °) during the same titration. The titrant concentrations are 72 mM SDS and 144 mM DDM. The starting protein concentration and volume is 0.065 mM and 0.9 ml.

The red laser scattering can also be applied to determine clouding of polysorbate solutions, as the Probe Drum allows for temperature ramping. However, in Paper IV a cloud point depression for polysorbates was observed in the presence of phenol, and at a critical phenol concentration clouding occurs at room temperature. Probe Drum allows for precise characterization of the phenol induced cloud point depression. With controlled automated titration and red laser scattering measurements, the onset of clouding as a sudden increase in scattering can be determined. This approach was applied in Papers (IV-V).

Nuclear Magnetic Resonance Spectroscopy (NMR)

A basic NMR measurement consists of three parts. First, when a sample is injected in the magnet of the spectrometer, the magnetic moments of the sample nuclei will equilibrate in a strong magnetic field. Next, a resonant perturbation in the form of a short, high power, radio frequency pulse excites the nuclei and signal known as the free induction decay (FID) is acquired. This time domain signal is Fourier transformed which results in an NMR spectrum (Keeler 2010b), see Figure 10. This process is repeated and the resulting FIDs are added together to ensure adequate signal to noise ratio. The intensity of a signal will then be increased by the number of scans, n, while the random noise is increased by $\sqrt{n}$ (Keeler 2010a; Keeler 2010b).

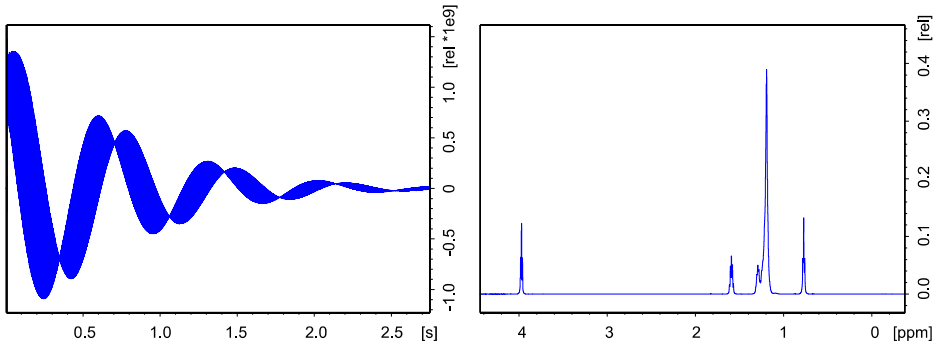


Figure 10. The time domain FID (left) and the Fourier transformed NMR spectra (right) of 2 mM SDS in a 10 mM phosphate buffer in D_2O, pD 7.0.

^{1}H and ^{13}C

NMR signal is acquired for atomic nuclei with a net spin, suitable isotopes are ^{1}H, ^{13}C and ^{15}N with spin ½ (Marion 2013). ^{1}H is the most common hydrogen isotope, abundant in water and biological molecules. ^{12}C is the most common carbon isotope, while about 1% of carbon on Earth is ^{13}C. Water will give rise to a strong signal for any aqueous sample, depending on circumstance this can be mitigated by using D_2O as solvent instead, or applying specific pulse sequences to suppress the water signal, by either selectively eliminating the water signal, or by selectively exciting or refocusing the signals of interest (Giraudeau, Silvestre, and Akoka 2015)

In the acquired NMR spectrum, the positions of the detected absorption frequencies are called chemical shifts. The chemicals shift of each nucleus is sensitive to the electronic environment and chemical shifts therefore provide insight to covalent and non-covalent structures of selected molecules (Wishart 2005; Keeler 2010b). The width and shape of NMR signals depends on the rate of reorientation in solution, which in turn depends on molecular size and mobility. When the rate of reorientation in solution is decreased, the relaxation rate of the transverse magnetization will increase, which results in a broadened NMR peak (Foster, McElroy, and Amero 2007; Keeler 2010c; Jacobs, Anderson, and Watson 1971). For large molecules and aggregates, local rigidity can also cause broadening.

Evaluation of changes in line shape and position with varying sample composition and conditions has been applied to the investigation of amphiphiles for half a century (Tehrani-Bagha et al. 2013; Wennerstrom and Ulmius 1976; Söderman and Stilbs 1994; Drakenberg and Lindman 1973; Lundberg et al. 2007; Söderman, Stilbs, and Price 2004; Söderman and Guering 1987). In Paper I, similar approaches are used to evaluate peptide aggregation behavior.

Two-dimensional NMR spectra can be recorded, where resonances are represented as cross-peaks. The position of these cross-peaks depends on connections between nuclei, either bonded or in close proximity, and sophisticated pulse sequences are applied to generate these spectra. For example, heteronuclear multiple quantum correlation spectroscopy (HMQC) reveals bonds between different nuclei, like ^{1}H and ^{13}C (Kiss, Fizil, and Szántay 2018; Keeler 2010d). Each molecule will give rise to a specific pattern of cross-peaks, and for proteins this can be used as a fingerprint, a signature that specifically depends on primary and higher order structure. Another example of 2D NMR is total correlation spectroscopy (TOCSY), where distinct patterns can be used to identify individual amino acids (Wishart 2005).

Diffusion NMR

Diffusion NMR can be used to offer insight to molecular properties like size, shape, aggregation, and complexation (Zubkov et al. 2017; Stilbs 1987). NMR-based diffusion measurements rely on application of field gradients to characterize the diffusion along the direction of the applied field gradient (Claridge 2009). The measured self-diffusion coefficient is a measure of the rate of mean square displacement in one direction and has a unit of m^2s^{-1} A large molecule will diffuse slower and have a lower diffusion coefficient.

In a basic diffusion experiment, the nuclei are first excited, next a field gradient pulse will impose a spatially dependent phase on the magnetisation vectors, this is flipped by a 180°pulse and refocused by a second field gradient pulse. However, the condition of complete refocusing is only met if the nuclei are in the same physical locations when the two gradient field pulses are applied. There is a delay, Δ, between these pulses and if the molecule diffuses during this delay, the detected signal will be attenuated in proportion to the displacement of the molecule (Claridge 2009). For investigation of biomolecules with short transverse relaxation time, a stimulated echo sequence is often preferred (Merboldt, Hänicke, and Frahm 1991; Frahm et al. 1985).

For a solute with an isotropic self-diffusion coefficient D, the intensity of the pulse field gradient stimulated echo (PFG-STE) can be described by (Hrabe, Kaur, and Guilfoyle 2007; Stejskal and Tanner 1965):

$$I = I_0 \exp\left[-\gamma^2 G^2 \delta^2 \left(\Delta - \frac{\delta}{3}\right) D\right] = I_0 \exp(-kD) \qquad (1)$$

where I is the intensity of the stimulated echo, I_0 is the intensity of the stimulated echo in the absence of pulsed field gradients, γ is the gyromagnetic ratio of protons, G and δ the strength and duration of the gradient pulses, and Δ the diffusion time.

Under the condition of fast molecular exchange, with residence times much shorter than the diffusion time, Δ, the observed self diffusion coefficient, D_{obs}, is the weighted average of the diffusion coefficients at the different residence sites, according to:

$$D = \alpha_1 D_1 + \alpha_2 D_2 + \alpha_3 D_3 + \ldots \quad (2)$$

where $\alpha_{1,2,3\ldots}$ are populations of the molecule residing at sites 1, 2, 3… and $D_{1,2,3\ldots}$ are the corresponding diffusion coefficients. Diffusion NMR data for two structurally similar peptides, D-Phe^6-GnRH and degarelix, is shown in Figure 11.

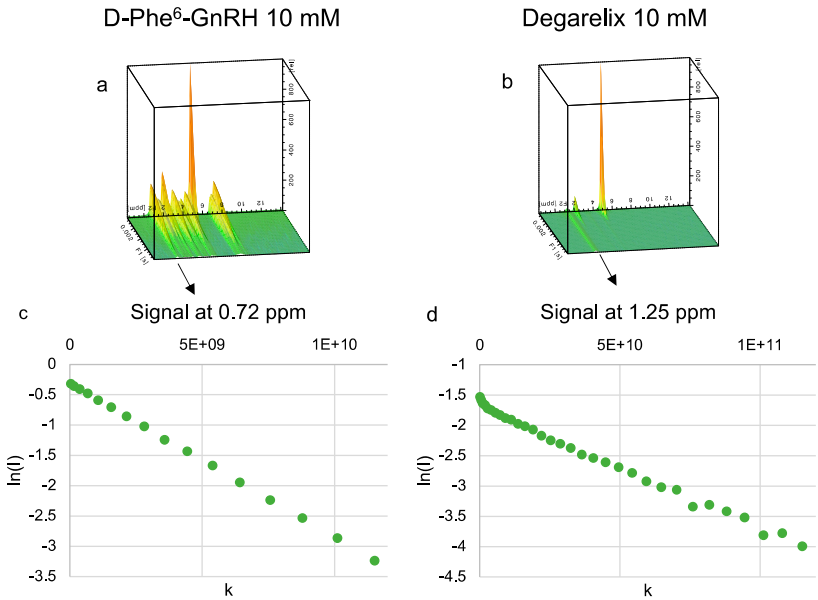


Figure 11. (a-b) Development of signal intensity, over the studied ppm range, with increasing gradient strength. The signal at 4.8 ppm is trace amount of HDO. (c-d) Echo decay at a single ppm value.

For D-Phe^6-GnRH, the diffusion experiment indicates the presence of mainly monomeric peptide. Whereas for degarelix, extensive line broadening is observed due to aggregation. Moreover, the fact that the echo decay of the signals of degarelix is multiexponential indicates the presence of aggregates of different sizes with slow exchange, for more details see Paper I.

Chapter 4 – Application of NMR in Protein Formulation

Fingerprints

NMR spectroscopy can be a powerful tool in formulation science. The use of NMR fingerprints, that is, comparing 2D NMR spectra with a selected reference spectra, (Aubin, Gingras, and Sauvé 2008; Blommers and Cerletti 1997) has been demonstrated as a sensitive approach to control batch-to-batch consistency and to compare protein production processes. In the same manner, 2D NMR fingerprints can be used to investigate whether the presence of specific excipients affect the protein structure. Figure 12 shows HMQC spectra for hGH in the presence of 1 w% polysorbate 80 (PS80) or 0.7 w% phenol.

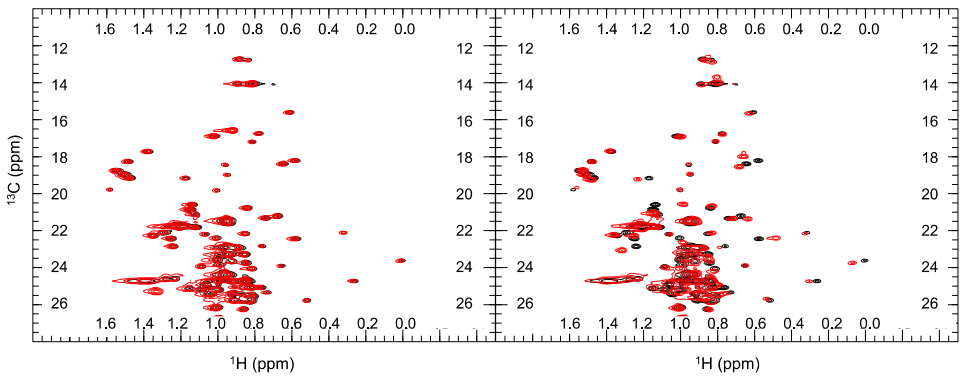


Figure 12. 2D ^{1}H-^{13}C HMQC NMR spectra of 8 mg/ml hGH with 1 w% PS80 (left panel) and 0.7 w% phenol (right panel). In both panels black represents the native protein and red with the excipient.

In the presence of PS80, the fingerprints overlap. Thus, PS80 has no effect on the structure of hGH. In contrast, shifts in specific peaks are observed in the presence of phenol. This indicates specific binding. As the shifts occur outside of the central cluster of peaks, this further indicates that the effected sites reside in regions of lower relative mobility, like a helix. With further analysis of the hGH TOCSY spectra some effected sites are identified; a valine, an isoleucine and two leucine. With this information at hand, one can try to identify possible binding sites on the

protein, by identifying sites where these residues are found together. Valines are found in two regions of the primary sequence, but only one of these regions also has isoleucine and leucine in the vicinity. Thus, comparison of 2D fingerprints is a powerful and accessible technique that can allow for detailed characterization of changes in the protein structure.

1D ^{1}H NMR spectroscopy can also be implemented to characterize protein degradation and folding states in the presence of excipients (Bramham et al. 2021; Malmendal et al. 2010). As an example, the use of NMR in the investigation of the ternary hGH-SDS-DDM system is described below.

Analysis of Single Surfactant Addition

In Figure 13, effects of the presence of the individual surfactants, SDS and DDM, on the aromatic region of hGH is shown. The surfactant ratios for a given experiment are displayed as DDM:SDS:hGH, and the hGH concentration is constant.

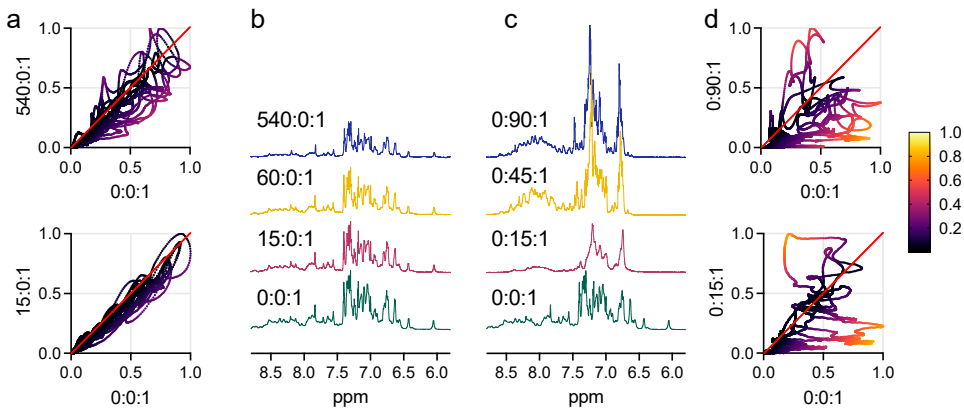


Figure 13. ^{1}H NMR spectra of the protein aromatic region at 8 mg/ml hGH with varying concentartion of DDM (b) and SDS (c). Surfactant-to-protein ratios are written as DDM:SDS:hGH. Correlation plots, comparing the signal of native hGH and hGH in the presence of surfactant, are shown at two selected surfactant-to-protein ratios for DDM (a) and SDS (d). The diagonal line in the correlation plots represents self-correlation of the native spectra (R=1). Colour grading of corrlations plots relate to the distance from this line.

The presence of the non-ionic surfactant DDM has very little effect on the peak pattern (Panel B) even at very high ratios of DDM to hGH, whereas the presence of SDS has significant effect (Panel C). These results are according to expectation and the data provides an important basis for investigation of the ternary hGH-SDS-DDM system. Interestingly, the broadest peaks are observed a the lowest SDS concentration. This is also in line with the expected process of SDS complexation

with hGH. SANS measurement and analysis show that the highest values for maximum dimension of the scatterer and aggregation number, are obtained at 0:8:1. These values decrease as the concentration of SDS increased to 0:45:1 (Paper IV). These changes in complex structure are also accompanied by changes in the protein conformation (Sanchez-Fernandez et al. 2020).

Addition of sufficiently high concentrations of DDM to an SDS-complexed protein is expected to result in refolding (Kaspersen et al. 2017; Pedersen et al. 2020). From a sample set up perspective, designing and analysing ternary samples introduces a new dimension and expands the sample series into a matrix. One could for example consider a series with constant hGH and SDS concentration and an increasing DDM concentration, then repeat this series at different SDS-concentrations. This is the sample set up we selected to investigate the impact of the SDS-hGH complex state on the refolding process.

Evaluation of spectral appearance with increasing surfactant concentration is complemented by correlation plots (Panels A and D). These are obtained by plotting intensity values of a selected ppm range from a selected experiment, say 15:0:1 DDM:SDS:hGH, against the intensity values of a selected reference spectrum, in this case the native spectrum, 0:0:1. The overall distribution around the self-correlation line provides a complementary qualitative comparison of the selected experiments. These plots are an additional representation of the NMR data, and with a complex sample matrix, which is easily achieved when investigating the interplay of different excipients, these plots display the data in a manner that is easy to overview.

For example, the correlation plot of 60:0:1 (not shown) is practically identical to 15:0:1, but a broader distribution is observed at 540:0:1. When comparing the corresponding spectra, an intensity loss is observed. This could be attributed to a reduced mobility of the protein in the presence of high contents of DDM (10 w%). A broad and irregular distribution is observed in Panel D. This indicates significant differences from the native spectrum, which are indeed observed in Panel C. Moreover, the correlation plots indicate that the manner in which the spectra of 0:15:1 and 0:45:1 differ compared to the native spectrum differs as well. This is also observed in Panel C. In summary, these plots provide a complementary way to view the data and indicate key transitions in the sample series.

Analysis of the ternary hGH-SDS-DDM system

As mentioned, addition of DDM is expected to result in refolding of hGH in the presence of SDS. In Figure 14, partial 2D HMQC fingerprints of different combinations of hGH, SDS and DDM are shown. The region displayed corresponds to a methionine region and the panels show experiments with; (i) hGH, (ii) hGH and DDM, (iii) hGH and SDS, and lastly, (iv) hGH, SDS and DDM.

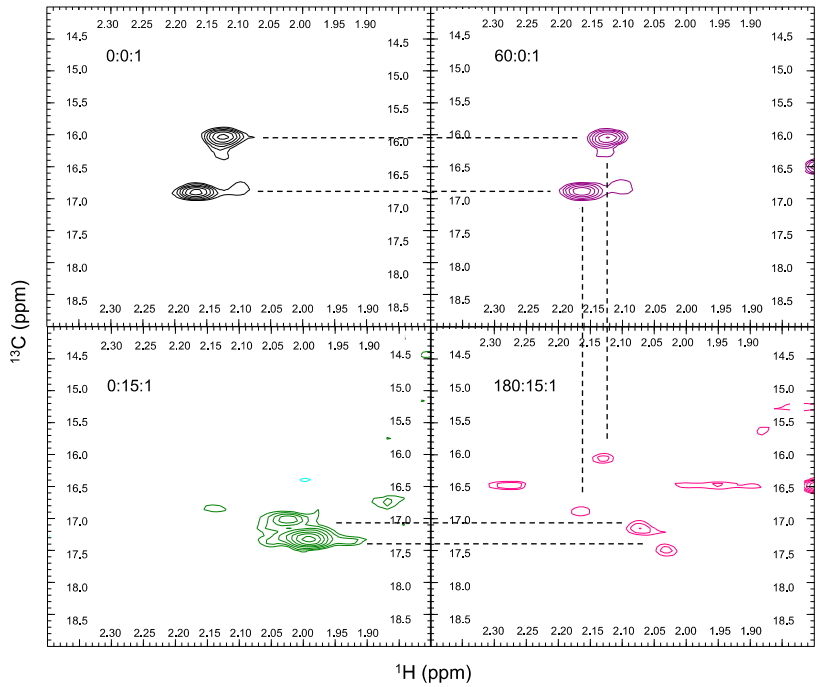


Figure 14. 2D ^{1}H-^{13}C HMQC spectra of the protein methionine region at 8 mg/ml hGH. Surfactant-to-protein ratios are written as DDM:SDS:hGH. Dashed lines indicate the overlap between the selected spectra.

As can be seen, the fingerprints for (i) hGH and (ii) hGH and DDM overlap. This is in line with the previous notion of DDM not interacting with the protein. Meanwhile, SDS changes the conformation of hGH, as seen in Figure 14. The effect of adding DDM to hGH and SDS was first monitored by tracking the maximum position of the protein intrinsic fluorescence peak during DDM titration. The line shape and peak position of the native protein was retrieved at 180:15:1. However, the NMR fingerprint in Figure 14 indicates that the protein is not fully refolded.

There appears to be two protein populations, one that is refolded and one that is complexed with SDS. Changing the mixing order in the ternary system has no impact on the resulting intrinsic fluorescence line shape or position. This indicates that the observed processes are in equilibrium. Thus, the occurrence of different protein populations, refolded and complexed, is not surprising. This has also been observed by modelling of SANS data in a similar system with beta-lactoglobulin (Pedersen et al. 2020). What I wish to highlight is the sensitivity of NMR and how this phenomenon is directly observed by 2D fingerprints. 1D spectra can also be informative, and this is exemplified in Figure 15, where partial 1D ^{1}H NMR spectra

of the ternary system, corresponding to the aromatic region of the protein, at 180:15:1 and 540:45:1 are presented.

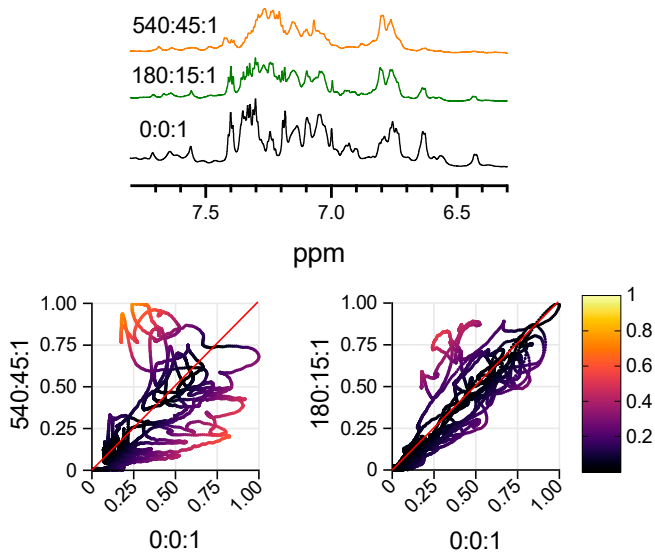


Figure 15. ^{1}H NMR spectra of the protein aromatic region at 8 mg/ml hGH with varying concentartion of DDM. Surfactant-to-protein ratios are written as DDM:SDS:hGH. Correlation plots, comparing the signal of native hGH and hGH in the presence of surfactant, are shown at two selected surfactant-to-protein ratios. The diagonal line in the correlation plots represents self-correlation of the native spectra (R=1). Colour grading of corrlations plots relate to the distance from this line.

The pattern of the spectrum at 180:15:1 clearly resembles that of the native, but they are not entirely identical. Thus, an indication that the protein fold is not fully recovered is also obtained from the 1D spectrum. Moreover, comparison of 180:15:1 and 540:45:1, shows that the peak pattern at a higher SDS concentration, with equivalent DDM to SDS ratio, is even further from the native. The correlation plots also indicate this dependence on the SDS concentration to retrieve the native fold. In summary, the application of 1D and 2D NMR fingerprints is very useful when investigating the interplay of excipients and their effect on the protein fold.

Absolute Integral Values

Obtaining absolute integral values from NMR measurements can allow direct comparison, sample to sample, when studying a concentration series. An example of this concerns the aggregation propensity of structurally similar peptides in Paper I. To extract absolute values, the receiver gain of the instrument and the number of scans collected must be constant (Holzgrabe 2010). For a concentration series, it is advised to adjust the receiver gain according to the sample of highest concentration and to adjust the number of scans according to the sample with the lowest concentration. The absolute intensities and/or integral values of a well isolated peak is expected to increase in proportion to concentration (Holzgrabe 2010; Keeler 2010b). However, any deviation from this expectation can be quite telling of the behavior of molecule in question. For example, the peak in question might be broadened.

Line broadening in a concentration series can occur for many reasons including changes in chemical exchange rate, formation of ordered aggregates or formation of amorphous aggregates. Chemical exchange refers to the exchange between available conformations or sites of a proton. If the exchange rate is lower than the frequency separation of the corresponding peaks in the NMR spectrum, then both peaks will appear. If the exchange rate is higher, one peak will appear, whose position will be the weighted average of the different sites. At intermediate exchange rates, broadening will occur, and either two broad peaks at intermediate-slow exchange rates or one broad peak at intermediate-fast exchange rates will be observed (Keeler 2010c). The formation of ordered aggregates results in an entity that tumbles slower in solution, and this change in mobility will result in broadened peaks (Wennerstrom and Ulmius 1976). In the case of unordered aggregates, size and mobility will have the same effect, but in addition, protons with identical positions in the molecule will have slightly different local environment. This results in slightly different chemical shifts and thus a broader distribution, which appears as a broadened peak (Olsson, Söderman, and Guering 1986).

The plotting of absolute values for a concentration series can be revealing regarding broadening events. For example, for a series of structurally similar peptides, absolute values were plotted to compare aggregation propensity. A subsection of the peptides in question are known to form fibrils, and thus line broadening due to formation of ordered aggregates is expected. A series of samples with increasing concentration, for each peptide, was prepared and 1D ^{1}H NMR measurements were performed with identical gain and scan settings. A well isolated peptide peak was selected and integrated. Each obtained absolute integral value was normalized to concentration, and each peptide series was normalized to the value of the sample with the lowest concentration, see Equation 3. A plot of data normalized in this manner will stay at unity if no broadening occurs, and any deviation is indicative of line broadening.

$$CNAI = \frac{Int_C / C}{Int_{C_{min}} / C_{min}} \quad (3)$$

CNAI is the abbreviation of concentration normalized absolute integral values. Int_C is the absolute integral value at any given concentration (C), and Int_{Cmin} is the absolute integral value at the lowest concentration (C_{min}) of the series. The results of the peptide study are shown in Figure 16. The studied peptides are D-Phe^6-GnRH, ozarelix, cetrorelix and degarelix. Measurements were performed on the same samples at the time of preparation, two hours later and two days later. To complement the NMR data, all atom molecular dynamics simulations were performed by research partners in Uppsala. Snap shots of the simulated systems at the end of the simulation time, 500 ns, are included in the figure as well.

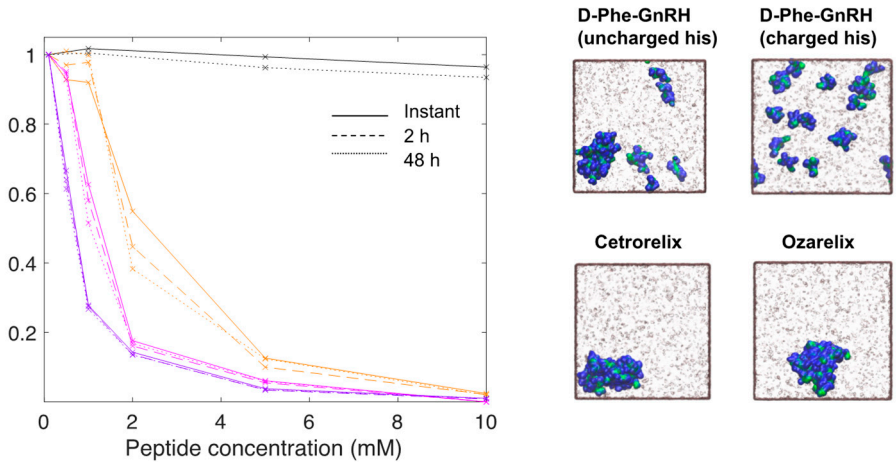


Figure 16. Left panel: plots of CNAI with increasing peptide concentration for D-Phe^6-GnRH (black), ozarelix (orange), cetrorelix (pink) and degarelix (purple). Solid lines represents data of sampes at the time of preparation, dashed lines two hours later and dotted lines two days later. Right panel: simulation snapshots (at 500 ns) of cetrorelix, ozarelix, and D-Phe^6-GnRH. Peptide backbone and side chain atoms are colored green and blue, respectively. Simulations were performed at 10 mM.

From Figure 16, a difference in concentration dependent aggregation propensity can clearly be discerned. No aggregation is observed for D-Phe^6-GnRH, and the aggregation propensity of the other peptides can be ranked. Degarelix shows the most dramatic loss of integral values due to line broadening, and no difference is observed at the different times, indicating that the aggregation is instant. For cetrorelix, aggregation is also instant, whereas for ozarelix, a time dependence is observed at the intermediate concentrations, 1 and 2 mM. Surprisingly there is an increase in absolute integral values over time at 1 mM, and after two days, no signal is lost. This effect was observed in replicate experiments. At 2 mM, about half of the expected integral value is lost, and the values decrease over time.

A slight decrease is observed after two days for D-Phe^6-GnRH. This is the only peptide in the series with a histidine residue. In an aqueous solution, both charge species of histidine would be expected to be present in equilibrium. To better understand the effects of this, D-Phe^6-GnRH was simulated twice, with charged and uncharged histidine. At the end of the simulated time frame, D-Phe^6-GnRH with charged histidine showed no aggregation propensity, whereas an inclination for aggregation was observed for D-Phe^6-GnRH with uncharged histidine. This inclination was not as dramatic as what was observed for the other peptides. Nonetheless, observed loss of NMR absolute integral values could be explained by the presence of this species. The simulation data also provides a ranking of aggregation propensity, by plotting the fraction of free peptides over simulation time. This ranking order agrees with the experimental NMR ranking, verifying the compatibility of these methods, for more details see Paper I.

Another interesting detail observed by NMR is the development of the counterion peak with peptide concentration. The counterion is acetate and when extracting the acetate *CNAI* values for D-Phe^6-GnRH, ozarelix and cetrorelix, values at unity are obtained. This is not the case for degarelix, instead the *CNAI* values of acetate decrease, in the same way as the peptide peak. This indicates that degarelix aggregates include the counterion while ozarelix and cetrorelix aggregates do not.

Generally, in formulation science, aggregation as a phenomenon is avoided. This is because that formation of aggregates is usually irreversible, depleting the available active protein in solution. Moreover, if present in an injected solution, aggregates can induce an adverse immunogenic response in patients (Evers et al. 2019) However, degarelix is an exception where aggregation part of the formulation design. Upon injection degarelix aggregates and forms a depo in the subcutaneous tissue, from which the active peptide is continuously released over three months (Ozono et al. 2017; Pharmaceuticals 2011).

In summary, the extraction of absolute values from 1D ^{1}H NMR spectra is relatively simple and the comparison of these values for a concentration series can lead to an increased understanding of the system in question, be that of the biomolecule in question or an excipient in the presence of a biomolecule.

Mapping Surfactant Behavior

Surfactants have been an important class of excipients for a long time. In recent formulation development there are new areas of surfactant application, for example, ionic surfactants play an essential role in the development of lipid nanoparticles. The solution behavior of a diverse set of surfactants has been characterized by NMR (Lundberg et al. 2007; Wiedmer et al. 1997; Wennerstrom and Ulmius 1976; Söderman and Guering 1987; Söderman and Stilbs 1994; Drakenberg and Lindman 1973; Tehrani-Bagha et al. 2013) and these techniques can easily be applied to formulation science.

For instance, NMR was used to understand previous observations in the ternary hGH-SDS-DDM system, that indicate of inclusion of DDM into the hGH-SDS complex. Thus, a study was designed to characterize surfactant behavior in the ternary hGH, SDS, and DDM system. Given the complexity of the system and the simultaneous entities (monomers, complex and micelles), no absolute values can be expected. Instead, an improved qualitative understanding of this system was achieved, and the formation of an hGH-SDS-DDM complex, or triplex, was verified.

Three NMR techniques were applied together to provide complementary information about the system, 1D ^{1}H NMR, 1D ^{13}C NMR, and NMR diffusometry. Generally, chemical shifts are sensitive to both the immediate surrounding and conformation of the site in question. However, for the alkyl chain of surfactant tails, it has been shown that changes in ^{13}C shifts, to good approximation, can be ascribed to changes chain conformation alone (Batchelor et al. 1972; Persson, Drakenberg, and Lindman 1976). Meanwhile, observed diffusion coefficients correspond to a population weighted average of the sites in which the molecules reside. Assuming a that only two sites are available to a molecule, for example monomer and micelle, the populations of these sites can be estimated (for more information see Paper III). In summary, these methods show great complementary promise, providing insight to evolution of the surfactant tail conformation and estimated population distributions.

Figure 17 shows observed diffusion coefficients of hGH, SDS and DDM for the ternary system. Two sample series are compared at constant hGH concentration with two different SDS concentrations, 3.8 and 6.25 mM SDS. Each series follows an increase in DDM concentration and the DDM/SDS ratio for samples in the two series are matched.

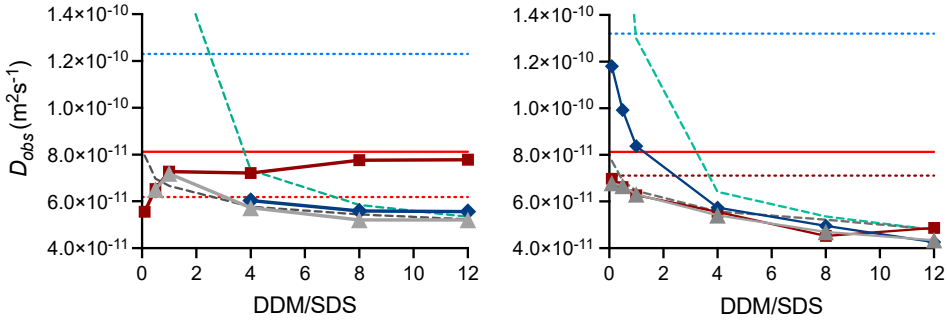


Figure 17. Observed self diffusion coefficients for ternary mixtures of SDS (blue diamonds), DDM (grey triangles) and hGH (red squares). The SDS concentration is 3.8 (left panel) or 6.25 mM (right panel), and the hGH concentration is 0.14 mM. Dashed green and grey lines represent D_{obs} for SDS and DDM in the binary SDS-DDM system. The red line represents D_{obs} of native hGH. The dotted blue and red lines represent D_{obs} for SDS and hGH at the corresponding SDS concentration in the binary SDS-hGH system.

In Figure 17, at the lower DDM/SDS ratios, the observed diffusion coefficients of DDM and hGH have the same values and follow the same trend. This is verification that DDM is complexed with the protein. At the highest DDM/SDS ratio, the observed diffusion coefficient of hGH, with 3.8 mM SDS, is very similar to that of the native protein, indicating that refolding has occurred. In contrast, at the higher SDS concentration, 6.25 mM, the protein diffusion coefficient is very similar to that of DDM in a binary SDS-DDM system, indicating that the protein has not refolded and is instead complexed with the surfactants. There is a separation of the observed diffusion coefficients for DDM and hGH, at 6.25 mM SDS and 12 DDM/SDS, but based on this data it is not possible to say whether this is the start of a refolding process or not.

These differences with SDS concentration can also be seen in the ^{1}H NMR spectrum, see Figure 18. The lower SDS concentration traces the native spectrum, while there are clear differences for the higher SDS concentration around 7.2 ppm, indicating that the there is a difference in protein conformation. In contrast, for the surfactant tails, also at 12 DDM/SDS, the presence of protein has no impact on the chain conformation. Figure 18 shows the chemical shift difference for a selected peak with and without hGH, for both SDS concentrations. Thus, when the values approach zero, the conformation of the carbon in question is the same regardless of the presence of the protein. Thus, at 6.25 mM SDS and 12 DDM/SDS, the conformation and diffusion coefficient of the protein is different from the native, while the conformation of hydrophobic surfactant tails is the same as in a pure surfactant system.

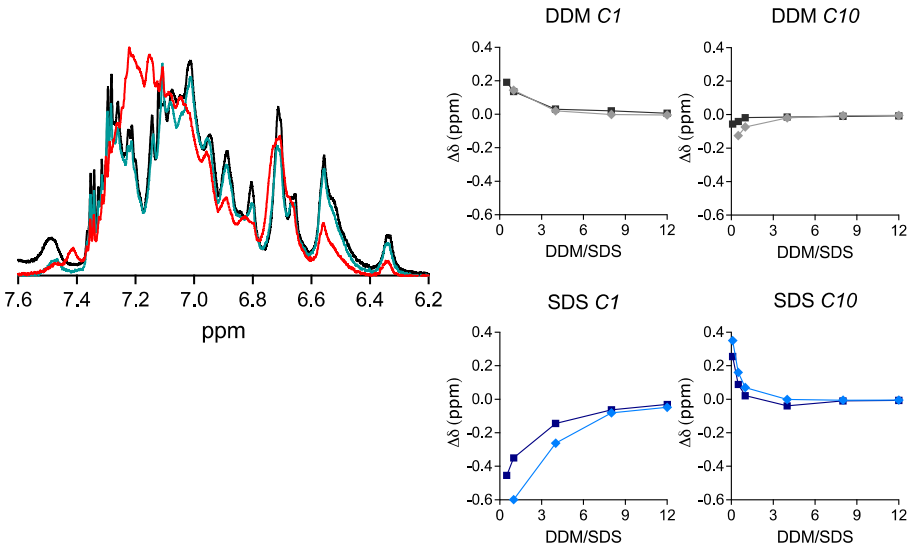


Figure 18. Left panel: partial ^{1}H NMR spectra of aromatic protein region at 3 mg/ml hGH. Comparison of 0:0:1 DDM:SDS:hGH, native protein spectra, (black) with 45:3.8:1 (purple) and 75:6.25:1 (red). Right panel: ^{13}C shift differences, for equivalent samples, with or without hGH, for selected peaks of SDS (blue) and DDM (grey). Series 2b (squares-lighter shade) consists of samples at 3.8 mM SDS with DDM concentrations corresponding to 0.1 – 12 DDM/SDS, Series 3a (circles-darker shade) consists of samples at 6.25 mM SDS with an equivalent DDM concentration increase.

The largest impact of protein presence is observed at low DDM/SDS for SDS *C1*, that is the first carbon on the alkyl tail, closest to the head group. This carbon would be expected to be impacted the most by changes in the mixed micelle composition, brought by an increase in DDM concentration. This is also the carbon for which the largest difference between the two SDS concentrations is observed.

At 0.1 DDM/SDS, with no protein present, observed diffusion coefficients indicate a higher concentration of free SDS monomers at 3.8 mM than at 6.25 mM. This is because, at a set DDM/SDS ratio, there will be less micelles that can incorporate SDS at a lower SDS concentration. 3.8 mM is also below the observed cmc of SDS in buffered D_2O. With hGH present, the estimated free SDS concentration is significantly reduced compared to without hGH, due to surfactant-protein interaction. Moreover, ^{13}C NMR measurements on the binary SDS-hGH system shows that electrostatic binding of SDS to the protein occur at 2.8 mM SDS.

All these puzzle pieces, provided by NMR, put together a picture, where the surfactant behavior at selected sample compositions comes into focus. Electrostatic adsorption of SDS occurs at lower SDS concentration, but above a critical DDM concentration both surfactants are incorporated to a micelle-like cluster complexed with the protein.

Chapter 5 – Excipient Interactions in Protein Formulations

Understanding the impact of a single excipient on a complex protein formulation is no simple task. Part of what makes an understanding of molecular mechanisms so challenging is the fact that different mechanisms lead to instability and small changes in formulation composition can completely alter the driving mechanism.

Protein Precipitation due to Clouding of Polysorbates

The incompatibility of polysorbates and preservatives like phenol has been documented for a long time, but not fully understood. Recently, it has been shown that the observed instability is due to cloud point depression (Paper IV). The presence of phenol causes a dramatic decrease in polysorbate cloud point. For example, at 0.1 w% PS80, addition 0.5 w% phenol causes clouding. This corresponds to about three polysorbate molecules per phenol.

This clouding phenomena and how it is impacted by the presence of different proteins was investigated in two master thesis projects I supervised (Börjesson 2022; Sjölund 2022). The phenol concentration at which precipitation occurred was determined at 0.1 w% PS80, with 0 or 0.15 M NaCl, and also without PS80. The results are detailed in Table 2 below, and the table combines results from Sjölund, Börjesson and myself (Börjesson 2022; Sjölund 2022). These studies were conducted on Probe Drum equipment. This allows automatic titration, with temperature control and mixing of the sample, whilst measuring laser scattering (637 nm) at a 90° angle to the sample.

If precipitation occurs at around 0.5 w% phenol, in the presence of PS80, it is assumed that precipitation is driven by the clouding phenomena. This is observed for lysozyme, BSA, and an albumin binding protein GA-Z. For this group of proteins, salt concentration did not have an impact on the clouding. In one of the projects, BSA precipitates were separated by centrifugation and both pellets and supernatant were studied with Fourier Transform Infrared Spectroscopy (FTIR) (Börjesson 2022). It could be demonstrated that BSA was found in both to an

equivalent extent, while the phenol concentration was a lot higher in the pellets than in the supernatant. GA-Z and BSA are found to precipitate at significantly higher phenol concentrations without PS80, whereas lysozyme precipitation occurs close to the PS80 cloud point.

Table 2. Phenol concentration where clouding is observed for a collection of portiens, with and without PS80, and also with and without 0.15 mM NaCl. Data was collected by Börjesson, Sjölund and myself (Börjesson 2022; Sjölund 2022).

Protein	PS80 (w%)	NaCl (M)	Phenol (w%)
Lysozyme	0.1	0.15	0.5
Lysozyme	0.1		0.6
Lysozyme			0.6
GA-Z	0.1	0.15	0.5
GA-Z	0.1		0.5
GA-Z			2.8
BSA	0.1	0.15	0.5
BSA	0.1		0.5
BSA			2.8
hGH	0.1	0.15	Instant
hGH	0.1		0.5
hGH			1.5
Trastuzumab	0.1	0.15	0.7
Trastuzumab	0.1		0.7
Trastuzumab			0.9
β-lactoglobulin	0.1	0.15	0.6
β-lactoglobulin	0.1		0.6
β-lactoglobulin			2.0
β-lactoglobulin	1	0.15	0.6
β-lactoglobulin	1		0.6

For hGH, two mechanisms are observed depending on the salt concentration. With no NaCl, precipitation is driven by the phenol induced cloud point depression, while at 0.15 M NaCl, the slightest addition of phenol results in instant turbidity. In this latter instance, a protein driven mechanism is assumed, as it was observed also in the absence of PS80 at 0.15 M NaCl (Börjesson 2022). This instant precipitation was also observed if phenol was replaced with metacresol, but not with benzyl alcohol (Börjesson 2022). This salt dependent protein precipitation behavior is further characterized in Paper V and is described in the section below.

For most proteins, titration with phenol leads to a sudden dramatic increase in scattering. For trastuzumab and β-lactoglobulin the increase was gradual, and the critical phenol concentration was more difficult to determine (Sjölund 2022). However, when the PS80 concentration was increased to 1 w%, in presence of β-

lactoglobulin, a dramatic precipitation event occurs at 0.6 w%. This is slightly above the cloud point for PS80, but β-lactoglobulin is known to interact with fatty acids (Spector and Fletcher 1970) so it is possible that an interaction between β-lactoglobulin and PS80 delays the onset of precipitation.

Protein Precipitation in the Presence of Salt and Phenol

The precipitation of hGH the presence of salt, preservative and surfactant was characterized by performing salt titrations on the Probe Drum equipment and measuring red laser scattering (637 nm) at a 90° angle to the sample. Photographs of selected samples are displayed in Figure 19.

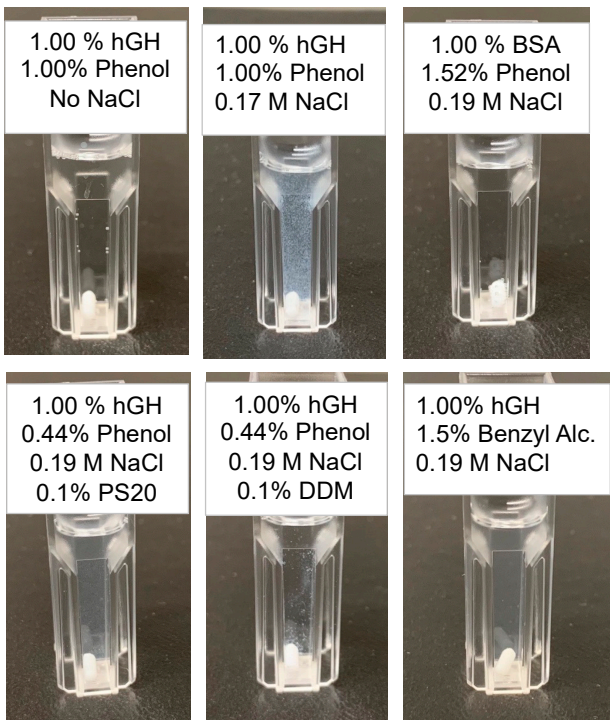


Figure 19. Photographs of cuvettes at the end of a salt titration on Probe Drum equipment. The content of each sample is listed in the figure.

In Figure 19, the impact of salt concentration on hGH and phenol is clear, without salt the sample is clear and at 0.17 M NaCl the sample is turbid with visible particles.

In comparison, the BSA sample, at a higher phenol and NaCl concentration, is clear, indicating that the effect is protein specific. This precipitation phenomenon could be shared with a wider class of proteins. In my group, similar precipitation with a positively charged therapeutic protein has been observed. The phenol-hGH interaction has been characterized by HMQC NMR, see Figure 12, the binding of phenol effects two leucine, one isoleucine and one valine. In the region where these residues are found, there are also positively charged arginine. It could be speculated that binding of phenol occurs due to cation-pi interaction and that this bound state is more vulnerable to aggregation, especially above a critical salt concentration.

When the preservative is exchanged to benzyl alcohol, see Figure 19, precipitation occurs but no visible particles are formed. The measured scattering intensity on Probe Drum increases suddenly, but the intensities reached are far lower with benzyl alcohol than with phenol. The choice of surfactant also effects precipitation. With PS20 at 0.44 w% phenol, the solution is cloudy with no visible particles, see Figure 19. The same is observed with PS20 or without surfactant at 0.44 w% phenol. However, with DDM (β-DDM), there are visible particles. In Figure 20, the effect of the type of non-ionic surfactant, comparing PS20 with PS80, as well as the anomers n-dodecyl-α-D-DDM and n-dodecyl-β-D-DDM (α-DDM with β-DDM), on precipitation of hGH in presence of phenol is shown.

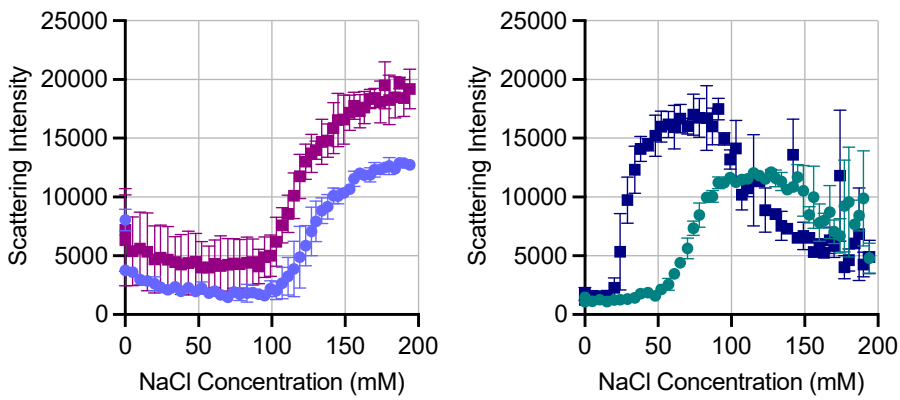


Figure 20. Evolution of scattering intensity during titration of 1 M NaCl to 10 mg/ml hGH, 0.44 w% phenol and different surfactants at a concentration of 1 mg/ml. All solutions are prepared with 10 mM sodium phosphate buffer, pH 7. The surfactants are, in the left panel: PS20 (violet circles), PS80 (plum squares) and in the right panel: β-DDM (blue squares), α-DDM (teal circles). Titration steps were 5 µl. The data show 90° scattering at 637 nm.

As can be seen in Figure 20, PS80 increases the measured scattering intensity compared to PS20. Both PS80 and PS20 increase the critical NaCl concentration slightly, compared to no surfactant, indicating a small stabilizing effect.

Surprisingly, the presence of sugar surfactant, α-DDM or β-DDM significantly decreases the critical NaCl concentration. The effect is more dramatic for β-DDM. DDM is known to incorporate phenol in the palisade layer. The presence of phenol in the micelles reduces curvature and changes the micelle shape (Lu and Somasundaran 2007). However, the exact mechanism of hGH-DDM-phenol aggregation is not yet understood. Moreover, the presence of sugar surfactant introduces a phenol concentration dependence that is not seen without surfactants, is demonstrated in Figure 21.

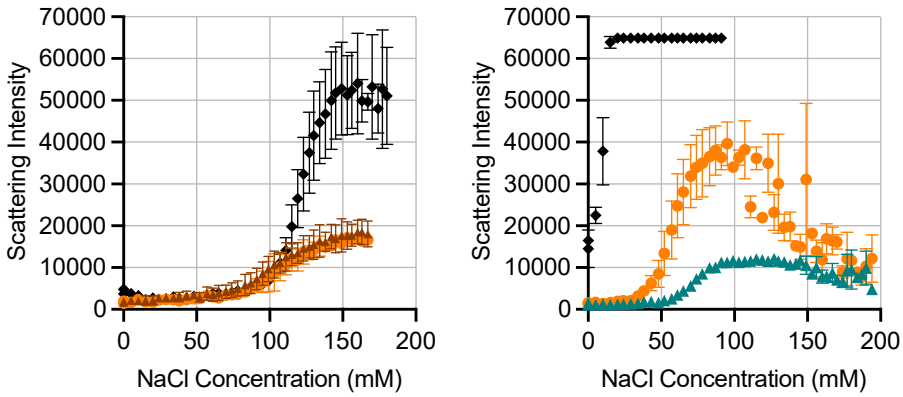


Figure 21. Left panel: evolution of scattering intensity during titration of 1 M NaCl to 10 mg/ml hGH, and varying phenol concentartion; 0.36 w%(brown), 0.68 w%(orange), and 1.00 w%(black). Right panel: evolution of scattering intensity during titration of 1 M NaCl to 10 mg/ml hGH, 1 mg/ml α-DDM, and varying phenol concentartion; 0.44 w%(teal), 0.68 w%(orange), and 1.00 w%(black). All solutions are prepared with 10 mM sodium phosphate buffer, pH 7. Titration steps were 5 µl. The data show 90° scattering at 637 nm.

Without surfactant, precipitation is observed at about the same NaCl concentration, regardless of phenol concentration, see Figure 21. The scattering intensity is the same at 0.36 and 0.68 w% phenol but increases between 0.68 w% and 1 w% phenol, with visible particles occurring in the latter case. In the presence of sugar surfactant, both α-DDM and β-DDM, the scattering intensity gradually increases with increasing phenol concentration and the onset of precipitation occurs at lower salt concentration with increasing phenol concentration. At 1 w% phenol and 0.1 w% β-DDM, precipitation occurs instantly with the addition of salt. Visible particles are observed at all phenol concentrations.

These results highlight the applicability of the Probe Drum equipment. The occurrence of different mechanisms in different sample compositions is indicated, which could be interesting to further investigate with more advanced techniques like SANS or NMR. In this study, the effects on precipitation by exchanging protein,

preservative and surfactant was investigated. Moreover, the effect of titrating with different salts was studied and the results are shown in Figure 22.

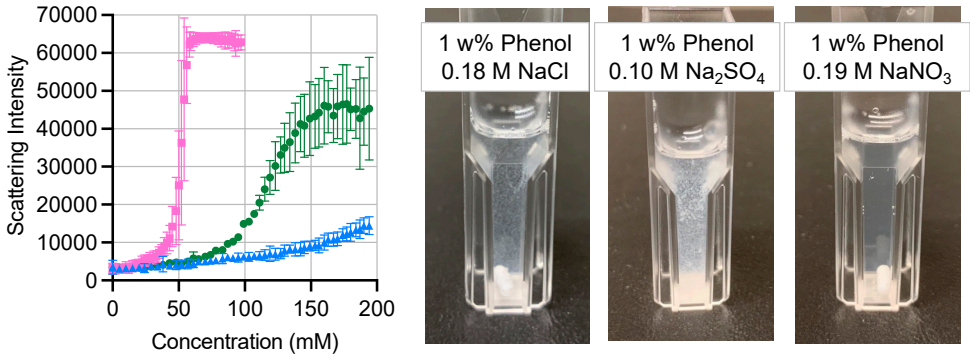


Figure 22. Left panel: evolution of scattering intensity during titration of different salts to 10 mg/ml hGH and 1 w% phenol titrated. Titrants are 1 M NaCl (green circles), 1 M $NaNO_3$ (blue triangles) and 0.5 M Na_2SO_4 (pink squares). Data in displayed as a function of salt concentration. The titration was done as steps of 5µl. The data show 90° scattering at 637 nm. Right panel: photographs of cuvettes at the end of a salt titration on Probe Drum equipment. The phenol and salt content of each sample is listed in the figure.

The investigated salts are NaCl, Na_2SO_4 and $NaNO_3$. According to the Hofmeister series, Na_2SO_4 is considered a salting-out salt, decreasing protein solubility, whereas $NaNO_3$ is considered a salting-in salt, increasing the protein solubility, and NaCl falls in the middle of the series. In Figure 22, visible particles can be seen for NaCl and Na_2SO_4. These salts also induce a dramatic increase of scattering intensity whereas the increase induced by $NaNO_3$ is gradual. The concentration where increased scattering occurs, and the amount/size of visible particles follow what is expected of Hofmeister series.

Refolding of SDS-unfolded hGH

As mentioned in the previous chapter, effects of SDS and DDM (β-DDM), individually and together, on the hGH structure has been studied. These studies were also conducted on Probe Drum equipment. In this case, the intrinsic fluorescence of hGH was tracked, by exciting the sample at 280 nm and collecting emission spectra. Other than hGH, surfactant titrations were performed on BSA and β -lactoglobulin. The evolution of the maximum position of the protein intrinsic fluorescence peak, for all three proteins, during titration of DDM and SDS is shown in Figure 23.

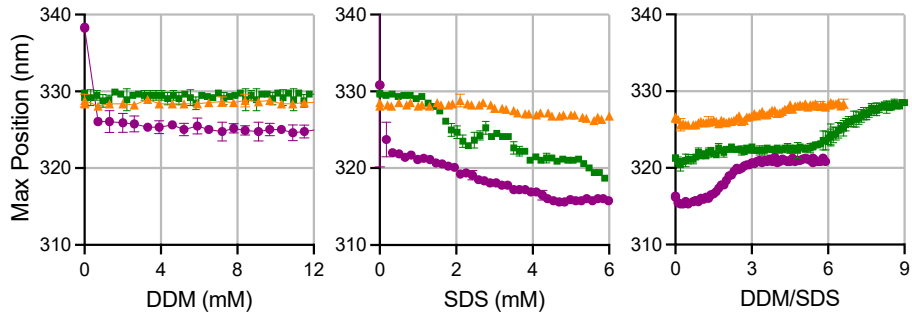


Figure 23. Evolution of intrinsic fluorescnce maximum peak position of 1.5 mg/ml protein during surfactant titration. (i) DDM titration to protein, (ii) SDS titration to protein, (iii) DDM titration to protein with 6 mM SDS. The proteins are hGH (green squares), BSA (purple circles) and bLG (orange triangles).

The titration of DDM to hGH and β-lactoglobulin has no effect on the fluorescence peak line shape or position, other than the expected dilution effect. In contrast, the intrinsic fluorescence for BSA is instantly blue shifted with the addition of DDM. First, there is a dramatic shift from 338 to 326 nm at the first titration point (0.7 mM DDM), then a gradual shift from 326 to 325 nm at 12 mM DDM. Generally, a blue shift is attributed to a more hydrophobic environment. Moreover, a biological function of BSA is to bind fatty acids (Spector, John, and Fletcher 1969), and BSA is also known to bind ten SDS molecules or thirteen alkyl glucosides without unfolding (Reynolds et al. 1967; Wasylewski and Kozik 1979). Thus, a binding of DDM is probable which affects the fluorophore environment without unfolding BSA.

The titration of SDS results in a blue shift for all proteins, and this observed shift is generally gradual. However, for BSA there is again a dramatic shift at the first titration steps, from 338 to about 322 nm at 0.3 mM SDS (13 SDS/BSA), probably corresponding to initial binding, followed by unfolding as more SDS is added. For hGH, a red shift is observed at 2.4 – 2.7 mM SDS, this corresponds to about 35-40 SDS/hGH. It is known from NMR measurements (Sanchez-Fernandez et al. 2020) that the only tryptophan residue on hGH becomes solvent exposed at about 43 SDS/hGH. Perhaps there is a gradual conformation change with increasing SDS concentration resulting in solvent exposure of tryptophan followed by inclusion in a micelle-like cluster.

Lastly, DDM was titrated to the proteins in the presence of 6 mM SDS. For β-lactoglobulin, the blue shift induced by SDS is reversed. This protein has the smallest observed blue shift from 328 to 327, while the other proteins shift at least 10 nm. This could be connected to the fact that the secondary structure of β-

lactoglobulin is β-sheets, while hGH and BSA are clusters of α-helices that might be more surfactant accessible (Nielsen et al. 2007).

The maximum peak position of BSA plateaus at 321 nm, and this position is reached at about 3 DDM/SDS. This position is at a shorter wavelength than what was observed during the DDM titration, about 325 nm. It is also known that alkyl glucosides bind to BSA with lower affinity than anionic surfactants (Wasylewski and Kozik 1979). Nonetheless, CD measurements of these proteins in the presence of SDS and DDM (Paper II) show that the secondary structure is retrieved for all proteins. However, at least for hGH, the refolding is not complete, as previously shown with NMR. The refolding process of hGH has been further studied at different SDS concentrations, see Figure 24.

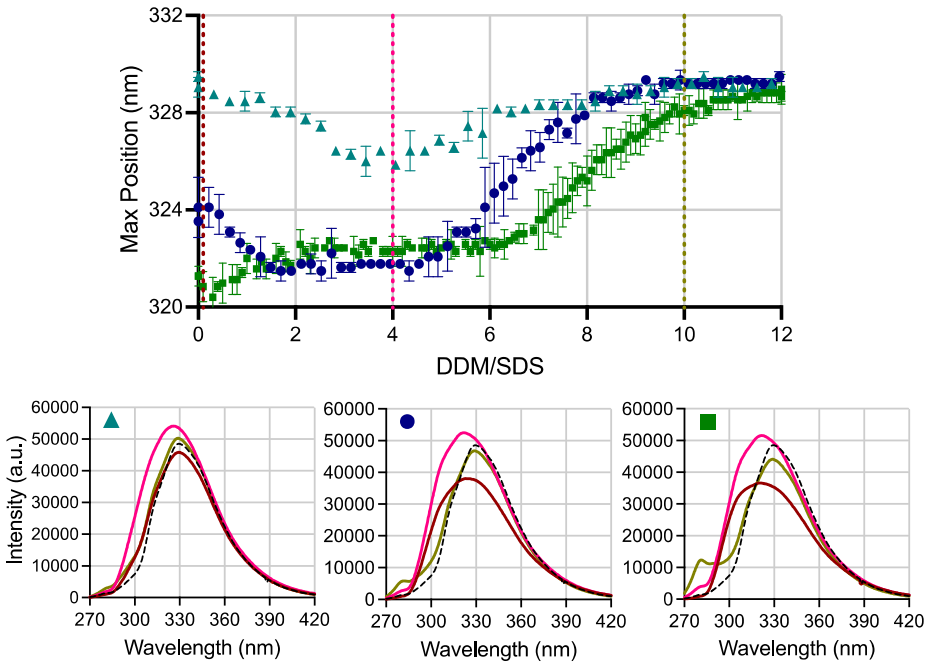


Figure 24. Evolution of intrinsic fluorescence maximum peak position of 1.5 mg/ml hGH with varying concentration of SDS during DDM titration; 1 mM SDS (teal triangles), 3 mM SDS (blue circles), and 6 mM SDS (green squares). Dotted lines represent selected DDM/SDS ratios for which fluorescence spectra are displayed in the bottom panels. Each bottom panel corresponds to an SDS concentration, from left to right; 1 mM, 3 mM, and 6 mM. The black dashed line represents the native protein spectrum.

DDM was titrated to hGH and SDS, the hGH concentration was constant and the SDS concentration was 1, 3 and 6 mM. Interestingly, at 1 and 3 mM SDS a blue shift is observed at 1- 4 DDM/SDS. This is an unexpected feature in the refolding

process and could indicate inclusion of DDM to the hGH-SDS complex. The verification of hGH-SDS-DDM complex by applying NMR techniques, is detailed in the previous chapter and in Papers II-III. Furthermore, far-UV CD used on hGH, BSA and βLG, see Paper II, provides calculated secondary structure contents that demonstrate a further loss of the native secondary structure with the presence of low amounts of DDM (DDM/SDS ≤ 4) for all three proteins. These results confirm the synergistic effect of SDS and DDM in protein unfolding.

Again, titration with the Probe Drum equipment provides a road map over key mechanisms in multicomponent formulations. At 3 mM, the blue shift plateaus at about 321 nm in between 2 and 4 DDM/SDS. The impact of a variety of variations of this titration is shown in Figure 25.

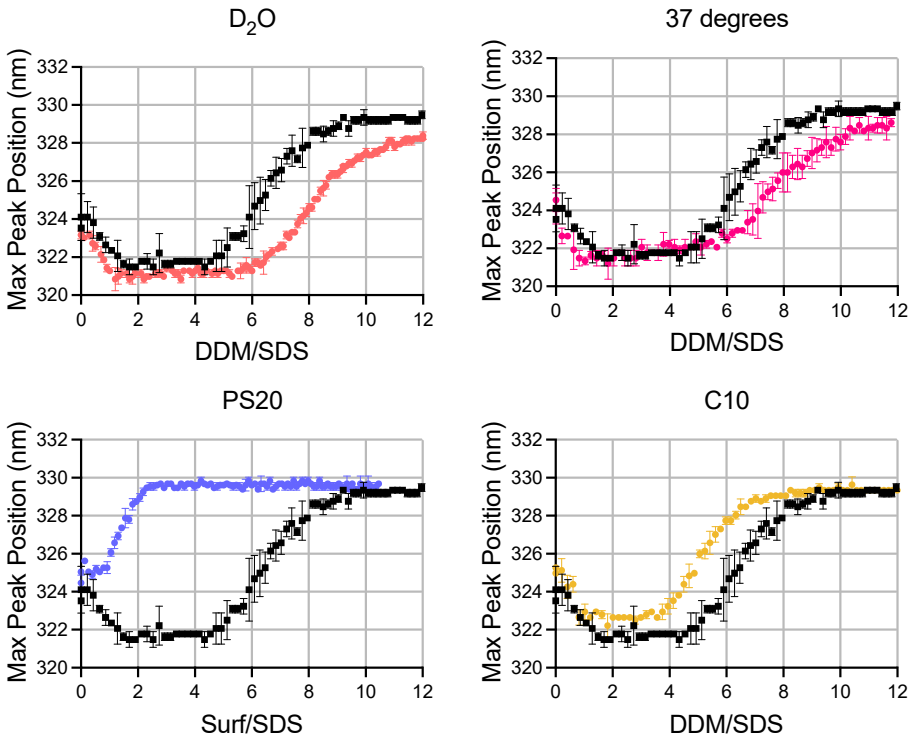


Figure 25. Evolution of intrinsic fluorescence maximum peak position of 1.5 mg/ml hGH with 3 mM SDS during non-ionic surfactant titration. All panels include DDM titration at 25°C in H_2O (black). Other variants are DDM titration at 25°C in D_2O (orange), DDM titration at 37°C in H_2O (pink), PS20 titration at 25°C in H_2O (purple), and DM (C10) titration at 25°C in H_2O (yellow).

As can be seen in Figure 25, titration with PS20 instead of DDM results in refolding already at about 2 PS20/SDS and no blue shift occurs. Thus, the hGH-SDS-DDM complexation is a phenomenon specific for this combination of components. From

NMR measurements we know that DDM does not directly interact with hGH, instead DDM seems to be included in a micelle-like cluster in the complex. Perhaps, the fact that DDM and SDS have the same alkyl tail length, can explain this joint complexation. The complexation occurs in a limited window of DDM to SDS ratio and this window is affected by solvent, temperature, and tail length. Changing the solvent to D_2O or increasing the temperature to 37°C widens the window. D_2O is known to decrease the cmc of alkyl glycosides (Ericsson et al. 2005). It is interesting that at 7 DDM/SDS an increased temperature or changing the solvent to D_2O, results in a preference toward the triplex state over a separation of mixed micelles and protein. Moreover, shortening the alkyl tail on the sugar surfactant has the opposite effect.

pH observations

pH effects on hGH have also been studied using Probe Drum equipment, by titrating a hGH solution with a 0.1 M phosphoric acid solution. This titration was performed in the presence of SDS and DDM, see Figure 26.

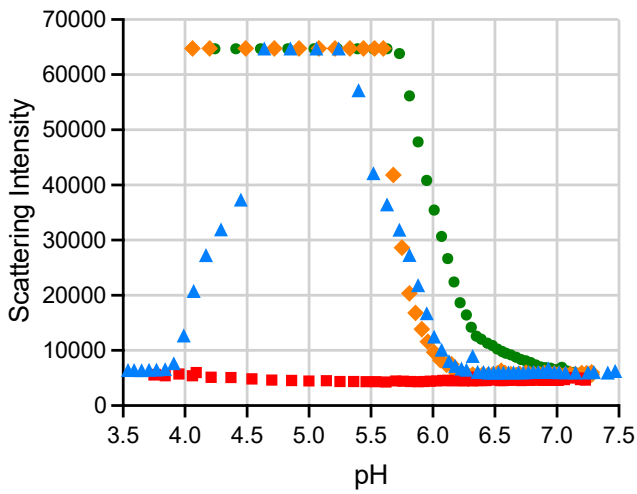


Figure 26. Evolution of scattering intensity of 1.5 mg/ml hGH and varying surfactant concentration during pH decrease; hGH (green circles), hGH and 1 mM SDS (orange diamonds), hGH and 12 mM DDM (blue triangles), hGH and 6 mM SDS (red squares).

As can be seen in Figure 2, there is generally an increase in scattering intensity when pH is lowered. Interestingly, with 12 mM DDM the scattering intensity decreases again once the isoelectric point at pH 5 is passed. This indicates a transient and pH dependent aggregation. At 6 mM SDS, no increase in scattering intensity is

observed. This indicates that the hGH-SDS complex formed at this SDS concentration protects the protein from aggregation.

From a chemical stability standpoint, a protein solution at a lower pH could be beneficial. The effects of storing hGH with 6 mM SDS at a low pH was studied by a master thesis student that I supervised (Viriyayudhakorn 2023). The project evaluated physical stability using FlowCAM and DLS, and chemical stability using HPLC. From the physical stability standpoint, it was observed that on the first day of storage, the solution stored at low pH with SDS had the fewest number of particles, compared to reference solutions. However, this was dramatically increased a week later. From the chemical stability standpoint, HPLC data measured the highest fraction of native hGH in the samples stored at low pH with SDS, and this was consistent over 40 days. However, this does not account for the protein that is lost due to aggregation (Viriyayudhakorn 2023).

Conclusion

The aim of this thesis was to improve understanding of aggregation of proteins and peptides, improve understanding of protein-excipient interactions, and explore integrative approaches to connect in-house methods to advanced methods like NMR and SANS.

The outcome of Papers I and V have contributed to an increased understanding of aggregation of proteins and peptides by differentiating aggregation behaviours. Aggregation behavior of structurally similar peptides was differentiated using NMR in Paper I, and the driving mechanisms of precipitation in Paper V were differentiated using Probe Drum. Meanwhile, the outcome of Papers II, III and V have contributed to an increased understanding protein-excipient interactions in liquid formulation. In Papers II-III, an in-depth understanding of protein-surfactant complexation has been achieved primarily for hGH, SDS and DDM. The selected combination of methods can also be applied to elucidate the behavior of other surfactant-protein systems.

The incompatibility between polysorbate and phenol has been tied to a depression in cloud point in Paper IV. This phenomenon also occurs in the presence of protein, and in Paper V the specific role excipients play in inducing precipitation is shown. Instant precipitation is observed when hGH, phenol, PS80 and NaCl are combined in solution. By exchanging one component at a time, the driving mechanism of the observed incompatibility is elucidated. Whether clouding or protein precipitation dominates depends on the ionic strength of the solution and increased ionic strength results in protein precipitation.

In all projects an integrative approach has been successfully applied. The effect of separate components was differentiated, mostly with the bench-top Probe Drum equipment, but also with NMR in Paper I. Selected phenomena were characterized in greater detail with all atom molecular dynamic simulations (AAMD) (Paper I), NMR (Papers II, III and V) and SANS (Paper II).

The applied NMR methodology in Paper I was proven successful in ranking aggregation propensity of a series of structurally similar peptides. Further, the integrative combination of NMR and AAMD provided new insights to the aggregation behaviour of the selected peptides. The ranking order determined by NMR was matched by that determined by AAMD. Moreover, the NMR

methodology gave insight to counterion incorporation and indications for further investigation of D-Phe^{6}-GnRH and ozarelix.

Protein-surfactant complexation was characterised in great detail with an approach combining Probe Drum, SANS and NMR in Papers II-III. This approach revealed an unexpected incorporation of DDM to preformed protein-SDS complexes, as indicated by Probe Drum, and verified for hGH by NMR. In a limited composition window, this hGH-DDM-SDS complex effects the conformation of protein, as seen by intrinsic fluorescence and NMR, through a cooperative unfolding event. When the DDM concentration is high enough, a refolding process is observed, but hGH does not completely regain the native fold. Moreover, the refolding capability of hGH seems to depend on the concentration of SDS it has been exposed to. As shown in this thesis, this cooperative unfolding behavior is not observed for SDS and PS20.

In summary, this works meets the aim of aiding development of liquid protein formulation by elucidating underlying phenomena linked to physical stability of biomolecules. This is beneficial in several steps of formulation development: in formulation design, for selection of excipients, in stability studies and in production. By aiding the industry, the insights and approaches laid out in this work is also to the benefit of people who need drug products based on biomolecules.

Future Studies

If this work were to continue, an in-depth investigation of the hGH/DDM/phenol system would be an intriguing avenue. For example, one could use diffusion NMR, in the same way as in Paper III, to investigate the distribution of DDM in the system, and compare observed diffusion coefficients for hGH/phenol, DDM/phenol and hGH/DDM/phenol systems, at a salt concentration below particle formation. With this information at hand, contrast variation SANS studies could be designed to further investigate the complex structures at varying component composition. However, characterization of this system would be significantly more difficult than the characterization of hGH-SDS-DDM.

Firstly, this system would introduce a fourth parameter, salt concentration. Also, because of the dramatic formation of visible particles, a wide distribution of aggregate sizes would be expected. Polydispersity makes analysis of both diffusion NMR and SANS significantly more complicated. If pre-aggregate structures, in a narrow size range, exist at a limited phenol and salt concentration range, these could be characterized, but careful consideration of sample preparation would be required.

Another avenue of further investigation, using Probe Drum equipment, would be to expand on the refolding study by investigating the impact of salt. Starting with titration of SDS to hGH at a couple selected sodium chloride concentrations, then moving on to PS20 driven refolding, where no non-ionic surfactant complexation is observed, and lastly investigating salt effect on the DDM induced refolding process. This could be further expanded using other salts from the Hofmeister series.

Yet another avenue could be to investigate the effect of phenol and polysorbates on a wide selection of proteins with 2D NMR, like HMQC or diffusion NMR. This kind of work could possibly tie observed differences in onset of precipitation to characteristics of the proteins and pave the way for general conclusions regarding different classes of proteins.

Lastly, these suggestions are provided from an academic research perspective. For research on protein-excipient interactions in the industry, my number one recommendation is to acquire Probe Drum equipment and find dedicated master thesis students.

References

Aguilar, Marie-Isabel. 2004. *HPLC of Peptides and Proteins* (Springer).

Alford, John R., Andrew C. Fowler, Deborah S. Wuttke, Bruce A. Kerwin, Ramil F. Latypov, John F. Carpenter, and Theodore W. Randolph. 2011. 'Effect Of Benzyl Alcohol on Recombinant Human Interleukin-1 Receptor Antagonist Structure and Hydrogen–Deuterium Exchange', *Journal of pharmaceutical sciences*, 100: 4215-24.

Anson, M. L. 1939. 'The Denaturation of Proteins by Detergents and Bile Salts', *Science*, 90: 256-57.

Arora, Jayant, Sangeeta B Joshi, C Russell Middaugh, David D Weis, and David B Volkin. 2017. 'Correlating the effects of antimicrobial preservatives on conformational stability, aggregation propensity, and backbone flexibility of an IgG1 mAb', *Journal of pharmaceutical sciences*, 106: 1508-18.

Aubin, Yves, Geneviève Gingras, and Simon Sauvé. 2008. 'Assessment of the Three-Dimensional Structure of Recombinant Protein Therapeutics by NMR Fingerprinting: Demonstration on Recombinant Human Granulocyte Macrophage-Colony Stimulation Factor', *Analytical Chemistry*, 80: 2623-27.

'Automate and combine proven biophysical techniques in a new way'. 2023 Probation Labs Sweden AB, Accessed Wed Mar 6.

Baird, Rosamund M, Norman A Hodges, and Stephen P Denyer. 2000. *Handbook of microbiological quality control in pharmaceuticals and medical devices* (CRC Press).

Bam, Narendra B, Jeffrey L Cleland, and Theodore W Randolph. 1996. 'Molten globule intermediate of recombinant human growth hormone: stabilization with surfactants', *Biotechnology progress*, 12: 801-09.

Bam, Narendra B, Jeffrey L Cleland, Janet Yang, Mark C Manning, John F Carpenter, Robert F Kelley, and Theodore W Randolph. 1998. 'Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions', *Journal of pharmaceutical sciences*, 87: 1554-59.

Batchelor, J. G., J. H. Prestegard, R. J. Cushley, and S. R. Lipsky. 1972. 'Conformational analysis of lecithin in vesicles by 13C NMR', *Biochemical and Biophysical Research Communications*, 48: 70-75.

Blommers, M. J. J., and N. Cerletti. 1997. 'High Resolution NMR, a Useful Tool to Control Batch-to-batch Consistency of Disulphide Bonds in Biopharmaceuticals: Application to Transforming Growth Factor-β3', *Pharmacy and Pharmacology Communications*, 3: 29-36.

Börjesson, Anna-Maria 2022. 'Cloud Point of Non-ionic Surfactant Mixed With Preservative for Biological Drug Formulation', Lund University.

Bramham, J. E., A. Podmore, S. A. Davies, and A. P. Golovanov. 2021. 'Comprehensive Assessment of Protein and Excipient Stability in Biopharmaceutical Formulations Using (1)H NMR Spectroscopy', *ACS Pharmacol Transl Sci*, 4: 288-95.
Byler, D. Michael, and Heino Susi. 1986. 'Examination of the secondary structure of proteins by deconvolved FTIR spectra', *Biopolymers*, 25: 469-87.
Cai, Y., M. Xu, M. Yuan, Z. Liu, and W. Yuan. 2014. 'Developments in human growth hormone preparations: sustained-release, prolonged half-life, novel injection devices, and alternative delivery routes', *Int J Nanomedicine*, 9: 3527-38.
Castaneda Ruiz, A. J., M. A. Shetab Boushehri, T. Phan, S. Carle, P. Garidel, J. Buske, and A. Lamprecht. 2022. 'Alternative Excipients for Protein Stabilization in Protein Therapeutics: Overcoming the Limitations of Polysorbates', *Pharmaceutics*, 14.
Chantalat, L, ND Jones, F Korber, J Navaza, and AG Pavlovsky. 1995. 'The crystalstructure of wild-type growth hormone at 2.5 Aresolution', *Protein Pept Lett*, 2: 333-40.
Chawla, Jyoti, and R. K. Mahajan. 2011. 'Cloud Point Studies of Tween and Glycol in the Presence of Salts', *Journal of Dispersion Science and Technology*, 32: 822-27.
Claridge, Timothy D. W. 2009. 'Chapter 9 - Diffusion NMR spectroscopy.' in Timothy D. W. Claridge (ed.), *Tetrahedron Organic Chemistry Series* (Elsevier).
Cohen, Laurie E. 2016. *Growth Hormone Deficiency* (Springer).
Cueto, Madaisy, M. Jesús Dorta, Munguı, x, Obdulia a, Matı Llabrés, x, and as. 2003. 'New approach to stability assessment of protein solution formulations by differential scanning calorimetry', *International journal of pharmaceutics*, 252: 159-66.
Dave, Nikunj, and Tejas Joshi. 2018. 'Cloud point analysis: Influence of additives on polysorbate', *Journal of Dispersion Science and Technology*, 39: 548-51.
de la Torre, B. G., and F. Albericio. 2023. 'The Pharmaceutical Industry in 2022: An Analysis of FDA Drug Approvals from the Perspective of Molecules', *Molecules*, 28.
Doñate, Fernando, Antonio Artigues, ANA Iriarte, and Marino Martinez-Carrion. 1998. 'Opposite behavior of two isozymes when refolding in the presence of non-ionic detergents', *Protein science*, 7: 1811-20.
Drakenberg, Torbjörn, and Björn Lindman. 1973. '13C NMR of micellar solutions', *Journal of Colloid and Interface Science*, 44: 184-86.
Eftink, Maurice R. 2002. 'Intrinsic fluorescence of proteins.' in, *Topics in fluorescence spectroscopy: volume 6: protein fluorescence* (Springer).
Ericsson, Caroline A, Olle Söderman, Vasil M Garamus, Magnus Bergström, and Stefan Ulvenlund. 2004. 'Effects of temperature, salt, and deuterium oxide on the self-aggregation of alkylglycosides in dilute solution. 1. n-nonyl-β-D-glucoside', *Langmuir*, 20: 1401-08.
———. 2005. 'Effects of temperature, salt, and deuterium oxide on the self-aggregation of alkylglycosides in dilute solution. 2. n-tetradecyl-β-D-maltoside', *Langmuir*, 21: 1507-15.
Evers, A., S. Pfeiffer-Marek, M. Bossart, C. Heubel, U. Stock, G. Tiwari, B. Gebauer, B. Elshorst, A. Pfenninger, U. Lukasczyk, G. Hessler, W. Kamm, and M. Wagner. 2019. 'Peptide Optimization at the Drug Discovery-Development Interface: Tailoring of Physicochemical Properties Toward Specific Formulation Requirements', *J Pharm Sci*, 108: 1404-14.

Fisher, Benjamin G, and Carlo L Acerini. 2013. 'Understanding the growth hormone therapy adherence paradigm: a systematic review', *Hormone Research in Paediatrics*, 79: 189-96.
Ford, R. R., P. H. Gilbert, R. Gillilan, Q. Huang, R. Donnelly, K. K. Qian, D. P. Allen, N. J. Wagner, and Y. Liu. 2023. 'Micelle Formation and Phase Separation of Poloxamer 188 and Preservative Molecules in Aqueous Solutions Studied by Small Angle X-ray Scattering', *J Pharm Sci*, 112: 731-39.
Foster, M. P., C. A. McElroy, and C. D. Amero. 2007. 'Solution NMR of large molecules and assemblies', *Biochemistry*, 46: 331-40.
Frahm, J., K. D. Merboldt, W. Hänicke, and A. Haase. 1985. 'Stimulated echo imaging', *Journal of Magnetic Resonance (1969)*, 64: 81-93.
Franklin, Sherry L, and Mitchell E Geffner. 2011. 'Growth hormone: the expansion of available products and indications', *Pediatric Clinics*, 58: 1141-65.
Geller, Markham J. 2010. *Ancient Babylonian medicine: theory and practice* (John Wiley & Sons).
Gellerfors, P., G. Eketorp, K. Fhölenhag, B. Pavlu, S. Johansson, and L. Fryklund. 1989. 'Characterisation of a secreted form of recombinant derived human growth hormone, expressed in Escherichia coli cells', *J Pharm Biomed Anal*, 7: 173-83.
Gerber, Sven , Vasil M. Garamus, Götz Milkereit, and Volkmar Vill. 2005. 'Mixed Micelles Formed by SDS and a Bolaamphiphile with Carbohydrate Headgroups', *Langmuir*, 21: 6707-11.
Gervasi, V., R. Dall Agnol, S. Cullen, T. McCoy, S. Vucen, and A. Crean. 2018. 'Parenteral protein formulations: An overview of approved products within the European Union', *Eur J Pharm Biopharm*, 131: 8-24.
Ghosh, Soumen, and Asok Banerjee. 2002. 'A Multitechnique Approach in Protein/Surfactant Interaction Study: Physicochemical Aspects of Sodium Dodecyl Sulfate in the Presence of Trypsin in Aqueous Medium', *Biomacromolecules*, 3: 9-16.
Gilbert, P. H., Z. Zhang, K. K. Qian, D. P. Allen, R. Ford, N. J. Wagner, and Y. Liu. 2022. 'Aggregation Kinetics of Polysorbate 80/m-Cresol Solutions: A Small-Angle Neutron Scattering Study', *Mol Pharm*, 19: 862-75.
Giraudeau, Patrick, Virginie Silvestre, and Serge Akoka. 2015. 'Optimizing water suppression for quantitative NMR-based metabolomics: a tutorial review', *Metabolomics*, 11: 1041-55.
Glatter, Otto. 1977. 'A new method for the evaluation of small-angle scattering data', *Journal of Applied Crystallography*, 10: 415-21.
Greenfield, Norma J. 2006. 'Using circular dichroism spectra to estimate protein secondary structure', *Nature Protocols*, 1: 2876-90.
Hassan, Puthusserickal A., Suman Rana, and Gunjan Verma. 2015. 'Making Sense of Brownian Motion: Colloid Characterization by Dynamic Light Scattering', *Langmuir*, 31: 3-12.
Hindmarsh, P. C., and M. T. Dattani. 2006. 'Use of growth hormone in children', *Nat Clin Pract Endocrinol Metab*, 2: 260-8.
Hines, J. D., R. K. Thomas, P. R. Garrett, G. K. Rennie, and J. Penfold. 1997. 'Investigation of Mixing in Binary Surfactant Solutions by Surface Tension and Neutron Reflection: Anionic/Nonionic and Zwitterionic/Nonionic Mixtures', *The Journal of Physical Chemistry B*, 101: 9215-23.

Hofmeister, Franz. 1888. 'Zur Lehre von der Wirkung der Salze', *Archiv für experimentelle Pathologie und Pharmakologie*, 24: 247-60.
Holmberg, K., B. Jönsson, B. Kronberg, and B. Lindman. 2002. *Surfactants and Polymers in Aqueous Solution* (Wiley).
Holzgrabe, Ulrike. 2010. 'Quantitative NMR spectroscopy in pharmaceutical applications', *Progress in nuclear magnetic resonance spectroscopy*, 57: 229-40.
Hong, Paula, Stephan Koza, and Edouard S. P. Bouvier. 2012. 'A review size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates ', *Journal of Liquid Chromatography & Related Technologies*, 35: 2923-50.
Hovgaard, Lars, Sven Frokjaer, and Marco van de Weert. 1999. *Pharmaceutical formulation development of peptides and proteins* (CRC Press).
Hrabe, J., G. Kaur, and D. N. Guilfoyle. 2007. 'Principles and limitations of NMR diffusion measurements', *J Med Phys*, 32: 34-42.
Hutchings, R. L., S. M. Singh, J. Cabello-Villegas, and K. M. Mallela. 2013. 'Effect of antimicrobial preservatives on partial protein unfolding and aggregation', *J Pharm Sci*, 102: 365-76.
Huus, K., S. Havelund, H. B. Olsen, M. van de Weert, and S. Frokjaer. 2006. 'Chemical and thermal stability of insulin: effects of zinc and ligand binding to the insulin zinc-hexamer', *Pharm Res*, 23: 2611-20.
Huus, Kasper, Svend Havelund, Helle B. Olsen, Bent W. Sigurskjold, Marco van de Weert, and Sven Frokjaer. 2006. 'Ligand Binding and Thermostability of Different Allosteric States of the Insulin Zinc−Hexamer', *Biochemistry*, 45: 4014-24.
Israelachvili, Jacob N., D. John Mitchell, and Barry W. Ninham. 1976. 'Theory of self-assembly of hydrocarbon amphiphiles into micelles and bilayers', *Journal of the Chemical Society, Faraday Transactions 2: Molecular and Chemical Physics*, 72: 1525-68.
Jacobs, JJ, RA Anderson, and TR Watson. 1971. 'Nuclear magnetic resonance studies of interactions between cetomacrogol and phenol', *Journal of Pharmacy and Pharmacology*, 23: 786-93.
Jiskoot, Wim , and Michael Bloemendal. 2005. 'Circular Dichroism Spectroscopy.' in, *Methods for Structural Analysis of Protein Pharmaceuticals* (American Association of Pharmaceutical Scientists).
Kang, Beibei, Huicheng Tang, Zengdian Zhao, and Shasha Song. 2020. 'Hofmeister Series: Insights of Ion Specificity from Amphiphilic Assembly and Interface Property', *ACS Omega*, 5: 6229-39.
Kasimova, Marina R, Søren M Kristensen, Peter WA Howe, Thorkild Christensen, Finn Matthiesen, Jørgen Petersen, Hans H Sørensen, and Jens J Led. 2002. 'NMR studies of the backbone flexibility and structure of human growth hormone: a comparison of high and low pH conformations', *Journal of Molecular Biology*, 318: 679-95.
Kaspersen, Jørn Døvling, Anne Søndergaard, Daniel Jhaf Madsen, Daniel E. Otzen, and Jan Skov Pedersen. 2017. 'Refolding of SDS-Unfolded Proteins by Nonionic Surfactants', *Biophysical Journal*, 112: 1609-20.
Keeler, J. 2010a. *Understanding NMR spectroscopy* (John Wiley and Sons, Ltd).
Keeler, James. 2010b. 'The basic NMR experiment.' in, *Understanding NMR spectrocopy* (John Wiley & Sons Ltd: Chichester, West Sussex, United Kingdom).

———. 2010c. 'Relaxation and the NOE.' in, *Understanding NMR spectroscopy* (John Wiley & Sons Ltd: Chichester, West Sussex, United Kingdom).
———. 2010d. 'Two-dimensional NMR.' in, *Understanding NMR spectrocopy* (John Wiley & Sons Ltd: Chichester, West Sussex, United Kingdom).
Kerwin, B. A. 2008. 'Polysorbates 20 and 80 used in the formulation of protein biotherapeutics: structure and degradation pathways', *J Pharm Sci*, 97: 2924-35.
Khatory, A, F Lequeux, F Kern, and SJ Candau. 1993. 'Linear and nonlinear viscoelasticity of semidilute solutions of wormlike micelles at high salt content', *Langmuir*, 9: 1456-64.
Kiss, Róbert, Ádám Fizil, and Csaba Szántay. 2018. 'What NMR can do in the biopharmaceutical industry', *Journal of Pharmaceutical and Biomedical Analysis*, 147: 367-77.
Ko, Suk Kyu, Gabriella Björkengren, Carolin Berner, Gerhard Winter, Pernille Harris, and Günther H. J. Peters. 2023. 'Combining Molecular Dynamics Simulations and Biophysical Characterization to Investigate Protein-Specific Excipient Effects on Reteplase during Freeze Drying', *Pharmaceutics*, 15: 1854.
Kunz, Werner, JWNB Henle, and Barry W Ninham. 2004. '‘Zur Lehre von der Wirkung der Salze’(about the science of the effect of salts): Franz Hofmeister's historical papers', *Current Opinion in Colloid & Interface Science*, 9: 19-37.
Lagassé, H. A., A. Alexaki, V. L. Simhadri, N. H. Katagiri, W. Jankowski, Z. E. Sauna, and C. Kimchi-Sarfaty. 2017. 'Recent advances in (therapeutic protein) drug development', *F1000Res*, 6: 113.
Lakowicz, JR. 2006. "In Principles of Fluorescence Spectroscopy." In.: Springer, US: Boston, MA.
Leader, Benjamin, Quentin J. Baca, and David E. Golan. 2008. 'Protein therapeutics: a summary and pharmacological classification', *Nature Reviews Drug Discovery*, 7: 21-39.
Lee, H. J., A. McAuley, K. F. Schilke, and J. McGuire. 2011. 'Molecular origins of surfactant-mediated stabilization of protein drugs', *Adv Drug Deliv Rev*, 63: 1160-71.
Liu, H., H. Zhang, and B. Jin. 2013. 'Fluorescence of tryptophan in aqueous solution', *Spectrochim Acta A Mol Biomol Spectrosc*, 106: 54-9.
Lo Nostro, Pierandrea, and Barry W Ninham. 2012. 'Hofmeister phenomena: an update on ion specificity in biology', *Chemical Reviews*, 112: 2286-322.
Lu, Shaohua, and Ponisseril Somasundaran. 2007. 'Intermolecular packing of sugar-based surfactant and phenol in a micellar phase', *Langmuir*, 23: 9960-66.
Lundahl, M. L. E., S. Fogli, P. E. Colavita, and E. M. Scanlan. 2021. 'Aggregation of protein therapeutics enhances their immunogenicity: causes and mitigation strategies', *RSC Chem Biol*, 2: 1004-20.
Lundberg, Dan, Johan Unga, Ashley L Galloway, and Fredric M Menger. 2007. 'Studies on an ester-modified cationic amphiphile in aqueous systems: Behavior of binary solutions and ternary mixtures with conventional surfactants', *Langmuir*, 23: 11434-42.
Maa, Yuh-Fun, and Chung C Hsu. 1996. 'Aggregation of recombinant human growth hormone induced by phenolic compounds', *International journal of pharmaceutics*, 140: 155-68.

Mach, Henryk, David B. Volkin, Carl J. Burke, and C. Russell Middaugh. 1995. 'Ultraviolet Absorption Spectroscopy.' in Bret A. Shirley (ed.), *Protein Stability and Folding: Theory and Practice* (Humana Press: Totowa, NJ).
Malmendal, A., J. Underhaug, D. E. Otzen, and N. C. Nielsen. 2010. 'Fast mapping of global protein folding states by multivariate NMR: a GPS for proteins', *PLoS One*, 5: e10262.
Manning, Mark C, Jun Liu, Tiansheng Li, and Ryan E Holcomb. 2018. 'Rational design of liquid formulations of proteins', *Advances in protein chemistry and structural biology*, 112: 1-59.
Manning, Mark Cornell, Danny K. Chou, Brian M. Murphy, Robert W. Payne, and Derrick S. Katayama. 2010. 'Stability of Protein Pharmaceuticals: An Update', *Pharmaceutical Research*, 27: 544-75.
Marion, Dominique. 2013. 'An Introduction to Biological NMR Spectroscopy*', *Molecular & Cellular Proteomics*, 12: 3006-25.
Mehan, Sumit, Vinod K Aswal, and Joachim Kohlbrecher. 2015. 'Tuning of protein-surfactant interaction to modify the resultant structure', *Physical Review E*, 92: 032713.
Merboldt, K. D., W. Hänicke, and J. Frahm. 1991. 'Diffusion imaging using stimulated echoes', *Magn Reson Med*, 19: 233-9.
Merrifield, RB. 1967. 'New approaches to the chemical synthesis of peptides', *Recent progress in hormone research*, 23: 451-82.
Meyer, B. K., A. Ni, B. Hu, and L. Shi. 2007. 'Antimicrobial preservative use in parenteral products: past and present', *J Pharm Sci*, 96: 3155-67.
Mukerjee, Pasupati. 1967. 'The nature of the association equilibria and hydrophobic bonding in aqueous solutions of association colloids', *Advances in Colloid and Interface Science*, 1: 242-75.
Mukherjee, Partha, Susanta K Padhan, Sukalyan Dash, Sabita Patel, and Bijay K Mishra. 2011. 'Clouding behaviour in surfactant systems', *Advances in Colloid and Interface Science*, 162: 59-79.
Muralidhara, Bilikallahalli K., and Marcus Wong. 2020. 'Critical considerations in the formulation development of parenteral biologic drugs', *Drug Discovery Today*, 25: 574-81.
Nielsen, Mette M, Kell K Andersen, Peter Westh, and Daniel E Otzen. 2007. 'Unfolding of β-sheet proteins in SDS', *Biophysical Journal*, 92: 3674-85.
'The Nobel Prize in Chemistry 1984'. Nobel Prize Outreach AB 2024, Accessed Tue. 5 Mar.
Olsson, Ulf, Olle Söderman, and Paul Guering. 1986. 'Characterization of micellar aggregates in viscoelastic surfactant solutions. A nuclear magnetic resonance and light scattering study', *The Journal of Physical Chemistry*, 90: 5223-32.
Otzen, Daniel E., Jannik Nedergaard Pedersen, Helena Østergaard Rasmussen, and Jan Skov Pedersen. 2022. 'How do surfactants unfold and refold proteins?', *Advances in Colloid and Interface Science*, 308: 102754.
Ozono, S., T. Tsukamoto, S. Naito, Y. Ohashi, T. Ueda, T. Nishiyama, H. Maeda, H. Kusuoka, R. Akazawa, M. Ito, and H. Akaza. 2017. 'Efficacy and safety of a 3-month dosing regimen of degarelix in Japanese patients with prostate cancer: a phase II maintenance-dose-finding study', *Jpn J Clin Oncol*, 47: 438-46.

Pai, Sheetal S., Boualem Hammouda, Kunlun Hong, Danilo C. Pozzo, Todd M. Przybycien, and Robert D. Tilton. 2011. 'The Conformation of the Poly(ethylene glycol) Chain in Mono-PEGylated Lysozyme and Mono-PEGylated Human Growth Hormone', *Bioconjugate Chemistry*, 22: 2317-23.

Patil, S. M., D. A. Keire, and K. Chen. 2017. 'Comparison of NMR and Dynamic Light Scattering for Measuring Diffusion Coefficients of Formulated Insulin: Implications for Particle Size Distribution Measurements in Drug Products', *AAPS J*, 19: 1760-66.

Pedersen, Jannik Nedergaard, Jeppe Lyngsø, Thomas Zinn, Daniel E. Otzen, and Jan Skov Pedersen. 2020. 'A complete picture of protein unfolding and refolding in surfactants', *Chemical Science*, 11: 699-712.

Perozzo, Remo, Gerd Folkers, and Leonardo Scapozza. 2004. 'Thermodynamics of protein–ligand interactions: history, presence, and future aspects', *Journal of Receptors and Signal Transduction*, 24: 1-52.

Persson, Bert O, Torbjorn Drakenberg, and Bjorn Lindman. 1976. 'Amphiphile aggregation number and conformation from carbon-13 nuclear magnetic resonance chemical shifts', *The Journal of Physical Chemistry*, 80: 2124-25.

Petkovic, Vibor, Maria Consolata Miletta, Andrée Eblé, Daniel I. Iliev, Gerhard Binder, Christa E. Flück, and Primus E. Mullis. 2013. 'Effect of Zinc Binding Residues in Growth Hormone (GH) and Altered Intracellular Zinc Content on Regulated GH Secretion', *Endocrinology*, 154: 4215-25.

Pharmaceuticals, Ferring. 2011. 'Investigating Efficacy and Safety of Two Degarelix Three-Month Dosing Regimens in Patients With Prostate Cancer Requiring Androgen Ablation Therapy', National Library of Medicine (US), Accessed March 12.

Pokorski, Jonathan K., and Michael J. A. Hore. 2019. 'Structural characterization of protein–polymer conjugates for biomedical applications with small-angle scattering', *Current Opinion in Colloid & Interface Science*, 42: 157-68.

Pramanick, Sougata , Deepak Singodia, and Vikas Chandel. 2013. 'Excipient Selection In Parenteral Formulation

Development', *Pharma Times*, 45.

Rahman Khan, Muhammad Akhlaqur, Md Ruhul Amin, Marzia Rahman, Malik Abdul Rub, Md Anamul Hoque, Mohammed Abdullah Khan, and Abdullah M. Asiri. 2019. 'Influence of Different Additives on the Clouding Nature and Thermodynamic Behavior of Tween 80 Solution in the Absence and Presence of the Amikacin Sulfate Drug', *Journal of Chemical & Engineering Data*, 64: 668-75.

Reynolds, Jacqueline A, Sarah Herbert, Helga Polet, and Jacinto Steinhardt. 1967. 'The binding of divers detergent anions to bovine serum albumin', *Biochemistry*, 6: 937-47.

Saha, D., D. Ray, J. Kohlbrecher, and V. K. Aswal. 2018. 'Unfolding and Refolding of Protein by a Combination of Ionic and Nonionic Surfactants', *ACS Omega*, 3: 8260-70.

Sanchez-Fernandez, Adrian, Carl Diehl, Judith E Houston, Anna E Leung, James P Tellam, Sarah E Rogers, Sylvain Prevost, Stefan Ulvenlund, Helen Sjögren, and Marie Wahlgren. 2020. 'An integrative toolbox to unlock the structure and dynamics of protein–surfactant complexes', *Nanoscale Advances*, 2: 4011-23.

Schellekens, H. 2009. 'Biosimilar therapeutics-what do we need to consider?', *NDT Plus*, 2: i27-i36.
Schott, Hans. 1984. 'Lyotropic numbers of anions from cloud point changes of nonionic surfactants', *Colloids and Surfaces*, 11: 51-54.
Seifert, Ivonne, and Wolfgang Friess. 2019. 'Established and Novel Excipients for Freeze-Drying of Proteins', *Freeze Drying of Pharmaceutical Products*: 33-52.
Shapiro, Arnold L., Eladio Viñuela, and Jacob V. Maizel. 1967. 'Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels', *Biochemical and Biophysical Research Communications*, 28: 815-20.
Sharfstein, Susan T. 2018. 'Non-protein biologic therapeutics', *Current Opinion in Biotechnology*, 53: 65-75.
Sjögren, Helen, Caroline A. Ericsson, Johan Evenäs, and Stefan Ulvenlund. 2005. 'Interactions between Charged Polypeptides and Nonionic Surfactants', *Biophysical Journal*, 89: 4219-33.
Sjölund, Johan. 2022. 'Phenol-Protein Solution Stability', Lund University.
Söderman, Olle, and Paul Guering. 1987. 'On the determination of micellar aggregation numbers from the concentration dependence of 13 C NMR chemical shifts', *Colloid and Polymer Science*, 265: 76-82.
Söderman, Olle, and Peter Stilbs. 1994. 'NMR studies of complex surfactant systems ', *Progress in NMR spectroscopy*, 26: 445-82.
Söderman, Olle, Peter Stilbs, and William S Price. 2004. 'NMR studies of surfactants', *Concepts in Magnetic Resonance Part A: An Educational Journal*, 23: 121-35.
Spector, A. A., K. John, and J. E. Fletcher. 1969. 'Binding of long-chain fatty acids to bovine serum albumin', *J Lipid Res*, 10: 56-67.
Spector, Arthur A. , and John E. Fletcher. 1970. 'Binding of Long Chain Fatty Acids to beta-Lactoglobulin', *Lipids*, 5: 403-11.
Standing, Kenneth G. 2003. 'Peptide and protein de novo sequencing by mass spectrometry', *Current Opinion in Structural Biology*, 13: 595-601.
Stejskal, E. O., and J. E. Tanner. 1965. 'Spin Diffusion Measurements: Spin Echoes in the Presence of a Time-Dependent Field Gradient', *The Journal of Chemical Physics*, 42: 288-92.
Stenstam, Anna, Daniel Topgaard, and Håkan Wennerström. 2003. 'Aggregation in a protein− surfactant system. The interplay between hydrophobic and electrostatic interactions', *The Journal of Physical Chemistry B*, 107: 7987-92.
Stilbs, Peter. 1987. 'Fourier transform pulsed-gradient spin-echo studies of molecular diffusion', *Progress in nuclear magnetic resonance spectroscopy*, 19: 1-45.
Stroppel, L., T. Schultz-Fademrecht, M. Cebulla, M. Blech, R. J. Marhofer, P. M. Selzer, and P. Garidel. 2023. 'Antimicrobial Preservatives for Protein and Peptide Formulations: An Overview', *Pharmaceutics*, 15.
Sun, Yang, LO Pedro Filho, José C Bozelli, Juliana Carvalho, Shirley Schreier, and Cristiano LP Oliveira. 2015. 'Unfolding and folding pathway of lysozyme induced by sodium dodecyl sulfate', *Soft Matter*, 11: 7769-77.
Takeda, Andrea, Keith Cooper, Alex Bird, Louise Baxter, Elena Gospodarevskaya, Geoffrey K Frampton, Karen Welch, and Jackie Bryant. 2010. 'Recombinant human growth hormone for the treatment of growth disorders in children: a systematic review and economic evaluation', *Health Technology Assessment*, 14.

Tanford, Charles. 1997. 'How protein chemists learned about the hydrophobic factor', *Protein science*, 6: 1358-66.
Tehrani-Bagha, Ali Reza, Krister Holmberg, Magnus Nydén, and Lars Nordstierna. 2013. 'Micelle growth of cationic gemini surfactants studied by NMR and by time-resolved fluorescence quenching', *Journal of Colloid and Interface Science*, 405: 145-49.
Viriyayudhakorn, Karnsinee 2023. 'Effects of mixed surfactant system on chemical and physical stability of human growth hormone', Lund University.
Wahlgren, Marie, Jenny Kedström, and Thomas Arnebrant. 1997. 'The Interactions in Solution between Nonionic Surfactants and Globular Proteins: Effects on Cloud Point', *Journal of Dispersion Science and Technology*, 18: 449-58.
Wahlund, Karl-Gustav, and Lars Nilsson. 2012. 'Flow FFF – Basics and Key Applications.' in S. Kim R. Williams and Karin D. Caldwell (eds.), *Field-Flow Fractionation in Biopolymer Analysis* (Springer Vienna: Vienna).
Walsh, Gary, and Roy Jefferis. 2006. 'Post-translational modifications in the context of therapeutic proteins', *Nature biotechnology*, 24: 1241-52.
Wang, Lei, Nanxi Wang, Wenping Zhang, Xurui Cheng, Zhibin Yan, Gang Shao, Xi Wang, Rui Wang, and Caiyun Fu. 2022. 'Therapeutic peptides: current applications and future directions', *Signal Transduction and Targeted Therapy*, 7: 48.
Wang, Wei. 2000. 'Lyophilization and development of solid protein pharmaceuticals', *International journal of pharmaceutics*, 203: 1-60.
Wasylewski, Zygmunt, and Andrzej Kozik. 1979. 'Protein—Non-ionic Detergent Interaction', *European Journal of Biochemistry*, 95: 121-26.
Wennerström, Håkan, and Björn Lindman. 1979. 'Micelles. Physical chemistry of surfactant association', *Physics Reports*, 52: 1-86.
Wennerstrom, Håkan, and Jan Ulmius. 1976. 'Proton NMR bandshapes in phospholipid bilayer vesicles', *Journal of Magnetic Resonance (1969)*, 23: 431-35.
Wiedmer, Susanne K., Marja-Liisa Riekkola, Magnus Nydén, and Olle Söderman. 1997. 'Mixed Micelles of Sodium Dodecyl Sulfate and Sodium Cholate: Micellar Electrokinetic Capillary Chromatography and Nuclear Magnetic Resonance Spectroscopy', *Analytical Chemistry*, 69: 1577-84.
Wishart, David. 2005. 'NMR spectroscopy and protein structure determination: applications to drug discovery and development', *Current Pharmaceutical Biotechnology*, 6: 105-20.
Yuan, Hua Xi, and Rosen Milton J. 1982. 'Synergism in Binary Mixtures of Surfactants', *Journal of Colloid and Interface Science*, 90.
Zapadka, K. L., F. J. Becher, A. L. Gomes Dos Santos, and S. E. Jackson. 2017. 'Factors affecting the physical stability (aggregation) of peptide therapeutics', *Interface Focus*, 7: 20170030.
Zardeneta, G., and P. M. Horowitz. 1994. 'Detergent, Liposome, and Micelle-Assisted Protein Refolding', *Analytical Biochemistry*, 223: 1-6.
Zhang, Lei, P Somasundaran, and C Maltesh. 1996. 'Electrolyte effects on the surface tension and micellization of n-dodecyl β-d-maltoside solutions', *Langmuir*, 12: 2371-73.
Zhang, Rui, Lei Zhang, and P Somasundaran. 2004. 'Study of mixtures of n-dodecyl-β-d-maltoside with anionic, cationic, and nonionic surfactant in aqueous solutions

using surface tension and fluorescence techniques', *Journal of Colloid and Interface Science*, 278: 453-60.
Zhang, Yanjie, and Paul S Cremer. 2006. 'Interactions between macromolecules and ions: the Hofmeister series', *Current opinion in chemical biology*, 10: 658-63.
Zhang, Ye, Shouvik Roy, Latoya S Jones, Sampathkumar Krishnan, Bruce A Kerwin, Byeong S Chang, Mark C Manning, Theodore W Randolph, and John F Carpenter. 2004. 'Mechanism for benzyl alcohol-induced aggregation of recombinant human interleukin-1 receptor antagonist in aqueous solution', *Journal of pharmaceutical sciences*, 93: 3076-89.
Zhi, Chen , Shuai Shi, Joseph M Rizzo, Andrew Semple, Sarita Mittal, Jason K Cheung, Daisy Richardson, Antochshuk Valentyn, and Mohammed Shameem. 2015. 'A Highly Sensitive Method for the Quantitation of Polysorbate 20 and 80 to Study the Compatibility between Polysorbates and m-Cresol in the Peptide Formulation', *Journal of Analytical & Bioanalytical Techniques*, 06.
Zubkov, Mikhail, Gary R. Dennis, Tim Stait-Gardner, Allan M. Torres, Scott A. Willis, Gang Zheng, and William S. Price. 2017. 'Physical characterization using diffusion NMR spectroscopy', *Magnetic Resonance in Chemistry*, 55: 414-24.

Printed by Media-Tryck, Lund 2024 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

Division of Food and Pharma
Department of Process and Life Science Engineering
Faculty of Engineering

ISBN 978-91-8096-038-0