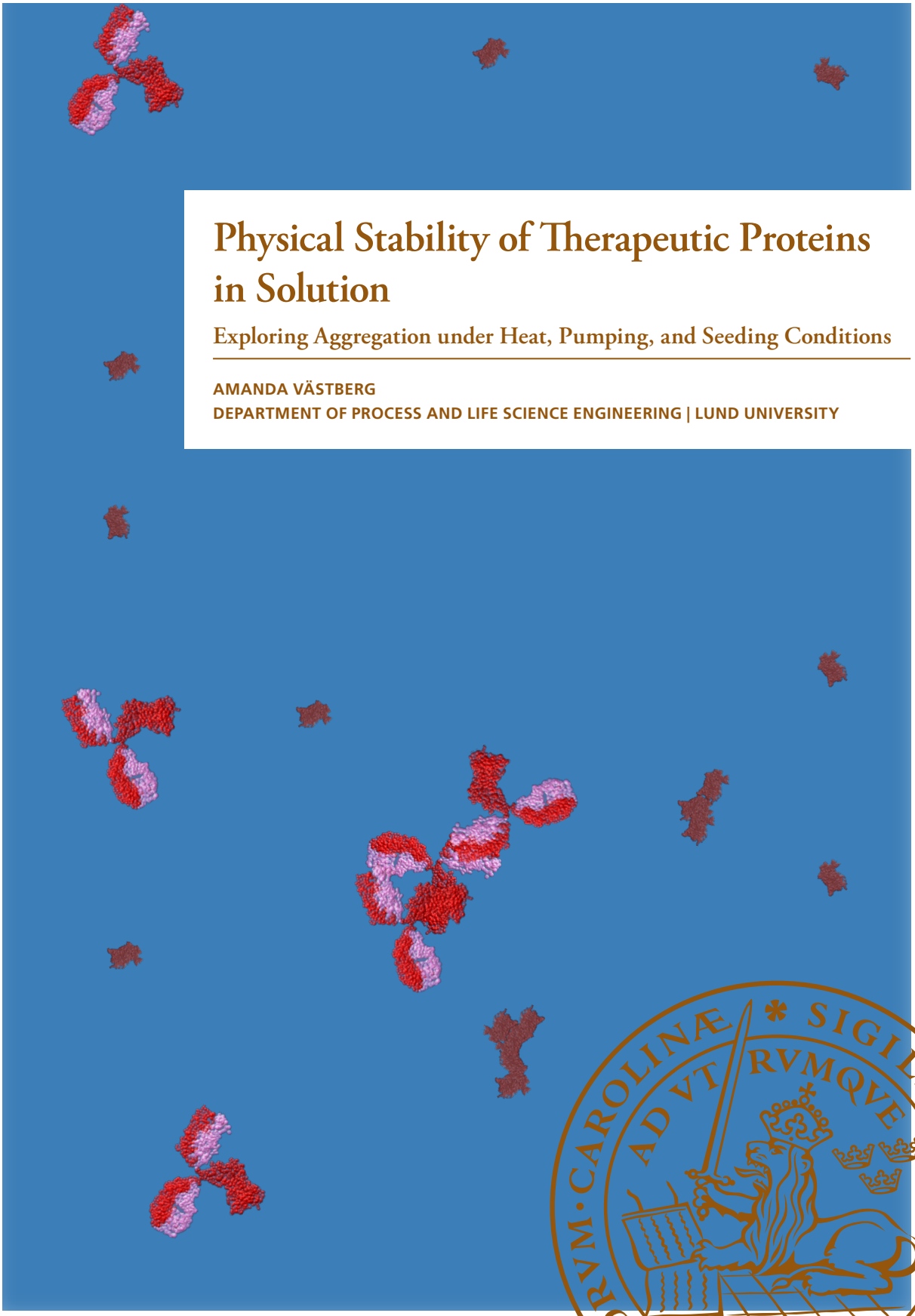

Physical Stability of Therapeutic Proteins in Solution

Exploring Aggregation under Heat, Pumping, and Seeding Conditions

AMANDA VÄSTBERG
DEPARTMENT OF PROCESS AND LIFE SCIENCE ENGINEERING | LUND UNIVERSITY

Physical Stability of Therapeutic Proteins in Solution

Physical Stability of Therapeutic Proteins in Solution:

Exploring Aggregation under Heat, Pumping, and Seeding Conditions

Amanda Västberg

LUND
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DOCTORAL DISSERTATION

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Title and subtitle: *Physical Stability of Therapeutic Proteins in Solution:* Exploring Aggregation under Heat, Pumping, and Seeding Conditions

Abstract:

This thesis aims to deepen the knowledge of physical stability and aggregation of proteins by studying two proteins under combinations of stress factors such as heat, pumping, and seeding. The work addressed questions such as how combinations of techniques can be used to study aggregation pathways, how the formulation might affect the formation of micron-sized particles during pumping, and finally, how different pre-formed aggregates can affect the stability of proteins in solution during storage.

Two different proteins, human growth hormone (hGH) and a monoclonal antibody, Antibody A, were investigated. These proteins represent different classes of therapeutic proteins, hormones, and antibodies; they differ in size and structure. The studies included investigating their physical stability and aggregation behaviour under a combination of stresses, which included heat, pumping, and storage at different temperatures. The storage studies investigated the effect of seeding and agitation on particle formation.

The aggregation pathway of human growth hormone upon heating at neutral pH could be elucidated utilising the powerful combination of MiniTEM for imaging, dynamic light scattering, and asymmetric flow field flow fractionation for size and concentration. In the second study, it was observed that the type of salt (Na2SO4, NaCl, and NaSCN) with anions ranking in the Hofmeister series dramatically influenced the particle formation propensity for Antibody A during peristaltic pumping. The combined effect of protein adsorption and protein-protein interaction can explain how the salts affect the number of particles formed. Interestingly, the ion-specific effects of NaCl, which is supposed to have the most neutral impact on the protein, created the “perfect storm” and decreased the stability during pumping. Finally, the evolution of small aggregates and micron-sized particles during different storage conditions (temperature, static, agitation) was examined. It turns out that small particles (<100 nm) created during heating follow a different process than large, micrometre-sized particles. Small aggregates might have a seeding effect, increasing size or number, while large particles undergo concurrent formation and dissolution processes during agitated storage. Elevated storage temperature (40 °C) mainly accelerates the degradation of the already formed particles rather than aggregation.

To summarise, studying aggregation by different combinations of stress factors and formulations of two therapeutic proteins has contributed to an improved understanding of physical stability and protein aggregation. These findings will hopefully give more insights into the continued research and development of therapeutic protein formulations.

Keywords: Therapeutic proteins, human growth hormone, antibody, protein aggergation, physical stability, protein particles, heat, pump, seeding
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In chess, the knight's strength sometimes lies in taking two steps back and one to the side, yet still finding its way forward.

- *Unknown*

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Popular Scientific Summary

The development and launch of new biological drugs has increased dramatically in the past decade. Between 2016 and 2023 alone, sales rose by just over 60%. Biological drugs are produced from or contain components derived from living organisms such as bacteria or yeast cells. Compared to traditional synthetic medicines, biological drugs are often much larger in size and have a more complex structure. Examples include proteins and enzymes. Protein-based drugs are used to treat a range of diseases, such as rheumatic disorders, cancer, and genetic conditions like growth hormone deficiency.

Proteins consist of building blocks called amino acids, linked together in a long chain. The amino acids have different side groups that give them various properties; they can be electrically charged, water-repellent, or water-attracting. These properties cause the long chain to fold in a specific way, forming the protein's unique (native) three-dimensional structure, which is crucial for the protein's therapeutic effect.

A protein's native structure is sensitive to changes in the surrounding environment. Factors such as heat, contact with different surfaces, mechanical stress, or the presence of various additives can affect the protein's structure. If the protein partially loses its unique structure, there is an increased risk of it clumping together to form small particles, which can range in size from a few nanometers to micrometres. Protein particles pose significant challenges in terms of quality and product safety. They reduce the therapeutic effect due to a decrease in the amount of active protein and increase the risk of allergic reactions, as the body no longer recognise the protein in its altered form. Regulatory authorities such as European Medicines Agency (EMA) or its U.S. counterpart, the Food and Drug Administration (FDA), have strict guidelines on acceptable particle levels.

Protein-based drugs are often administered in aqueous solutions, either intravenously or subcutaneously, to facilitate adsorption by the body and transport to the site of therapeutic action. Besides protein and water, these solutions also contain other substances, known as excipients, which enhance stability and compatibility. These can include buffers to stabilise pH or surfactants to protect the protein from interacting with surfaces. The complete mixture, comprising the active substance (protein or other drug) and excipients, is referred to as a formulation. Increasing our understanding of how protein particles form is of great importance. The risk of aggregation exists throughout the lifecycle of the drug— from production through transport, storage, and administration. A protein's tendency to aggregate strongly depends on various factors: external influences such as shaking or surfaces, solution conditions such as pH and salt content, and the inherent properties of the protein itself. Therefore, more knowledge about the effects of various system factors is needed. Greater knowledge of these factors will help

minimise and control the formation of protein particles, leading to more stable and reliable products.
This thesis investigates the particle formation of two different proteins under various stress conditions such as heat, pumping, and seeding. Key questions addressed include how combinations of analytical methods can be used to study protein aggregation, the impact of production handling through pumping, and the effect of prior mishandling that causes aggregation on stability during storage. These studies focus on two types of pharmaceutical proteins: growth hormone and a monoclonal antibody. These molecules differ in both size and structure.

To understand how particles arise, it is essential to have methods to study their formation. Since the size and shape can vary— from nanometres to micrometres and from round to elongated, thread-like structures, it is often necessary to employ multiple analytical techniques. During this work, the formation of growth hormone aggregates upon heating at neutral pH was found to follow a two-step process. First, elongated small aggregates form as protein molecules aggregate; these then transition into larger clusters with more complex structures. The formation could be explored using various techniques that examine the aggregates' size, concentration, and imaging.

The effect of production handling was also investigated. This study revealed that formulation composition significantly influences the extent of particle formation during peristaltic pumping. This effect is likely due to a combination of how strongly the protein interacts with the tubing surface and how strongly protein molecules attract one another.

Finally, the impact of both small and large aggregates on stability and behaviour during storage was examined. It was found that small aggregates (<100 nm) generated during heating followed a different progression than large, micrometre-sized particles. The small aggregates appear more reactive, increasing in number or size, while large particles underwent simultaneous formation and dissolution processes.

This thesis explores several aspects of the stability, aggregation, and therapeutic proteins, contributing valuable insight to the ongoing efforts to develop more stable and effective therapeutic protein formulations in the future.

Populärvetenskaplig Sammanfattning

Utvecklingen och lansering av nya biologiska läkemedel har ökat kraftigt det senaste årtiondet. Bara mellan 2016–2023 ökade försäljningen med drygt 60%. Biologiska läkemedel framställs av eller innehåller delar från levande organismer såsom bakterier eller jästceller. De skiljer sig i jämförelse med traditionella syntetiskt producerade läkemedel genom att de oftast är mycket större till storlek och har en mer komplex struktur. Några exempel är proteiner och enzymer. Denna typ av läkemedel används för att behandla ett flertal sjukdomar, såsom reumatiska sjukdomar, cancer och genetiska sjukdomar som tillväxthormonstörning.

Proteiner består utav byggstenar som kallas aminosyror som är ihopkopplade till en lång kedja. Aminosyrorna har olika sidogrupper vilket ger dem olika egenskaper, de kan vara elektrisk laddade sura eller basiska, vattenavvisande eller gilla att vara omgivna av vatten. Egenskaperna hos aminosyrorna gör att den långa kedjan veckar sig på ett visst sätt till proteinets unika (nativa) tredimensionella struktur. Strukturen är viktig för den behandlande effekten av proteinet.

Proteiners nativa struktur är känslig för förändringar i den omgivande miljön. Faktorer som värme, kontakt med olika ytor, mekanisk stress eller olika tillsatser kan därför påverka proteinets struktur. Om proteinet delvis förlorar sin unika struktur kan det öka risken för att proteinet klumpar ihop sig och bildar små partiklar. Dessa kan vara några få nanometer eller mikrometerstora. Partiklarna är problematiska då de ger problem med kvalité och produktsäkerhet. Det genom att den behandlande effekten minskar på grund av minskad mängd verksamt protein. De ökar även risken för allergiska reaktioner på grund av att kroppen inte känner igen proteinet utan sin rätta struktur. Det finns strikta riktlinjer för nivåer av partiklar från regleringsmyndigheter som European Medicines Agency (EMA) eller USAs motsvarighet Food and Drug Administration (FDA).

Proteinläkemedel ges oftast i vattenlösning intravenöst eller i underhudsfettet genom en spruta för att de lättare ska tas upp i kroppen och ta sig till det ställe där de har sin behandlande effekt. Lösningen innehåller förutom proteinet och vatten även andra ämnen, så kallade hjälpämnen, som är till för att öka stabiliteten och skonsamheten. Det kan till exempel vara salter för att stabilisera pH eller ytaktiva molekyler som skyddar proteinet mot ytor. Hela lösningen med aktiv substans (protein eller annat läkemedel) och hjälpämnen kallas för formulering. Det är av stor vikt att öka kunskaperna kring hur proteinpartiklar bildas. Risken för att proteinet klumpar ihop sig och bildar partiklar finns från produktion, transport, lagring tills när läkemedlet används. Benägenhet för proteinet att klumpa ihop sig är starkt beroende av systemet, det vill säga, yttre faktorer så som skakningar eller ytor, löningsförhållanden så som pH och salthalt, samt protein självt. Därför behövs mer kunskap kring effekterna av olika systemfaktorer. Det kommer i sin tur ge större

möjlighet att minimera och kontrollera formationen av dessa proteinpartiklar och därmed göra stabilare och bättre produkter.

Den här avhandlingen har undersökt partikelformation av två olika proteiner under olika stressfaktorer som värme, pumpning, och hur närvaro partiklar kan initiera bildningen av fler. De frågor som behandlas är hur kombinationer av metoder kan användas för att studera hur proteinet klumpar ihop sig, effekt av produktionshantering genom pumpning och hur tidigare felbehandling som orsakat partiklar kan påverka stabilitet under lagring. Två typer av läkemedelsproteiner har använts i studierna, tillväxthormon och en monoklonal antikropp. Dessa två molekyler har både olika storlek och struktur.

För att förstå hur partiklarna uppstår är det viktigt att ha metoder för att studera bildandet av dem. I och med att storleken och formen kan variera från nanometer till mikrometerstora och från runda till avlånga trådliknande strukturer så är det ofta nödvändigt att använda flera olika metoder. Under det här arbetet kunde bildning av partiklar av tillväxthormon vid värmning ses följa en två steg vid neutralt pH. Först formas avlånga små partiklar av att enkla proteinmolekyler adderas ihop som sen övergår till att dessa går ihop med varandra och formar större kluster med mer komplex struktur. Bildandet kunde utforskas genom att använda olika tekniker som undersöker olika både storlek, koncentration, och avbildning av aggregaten.

Fortsättningsvis undersöktes effekten av produktionshantering. I denna studie kunde det ses att formulering kraftigt påverkar effekten av partikelformation under peristaltisk pumpning. Det kan förklaras av en kombinerad effekt av hur mycket proteinet integrerar med ytan i pumpslangen samt hur mycket proteinet har en attraktiv interaktion med sig själv.

Sist undersöktes effekten av hur närvaro av små och stora partiklar påverkar stabilitet och beteendet under lagring. Det visar sig att små partiklar (<100 nm) som skapades under värmning har en annan process än de stora mikrometerstora partiklarna. De små aggregaten verkar vara mer reaktiva och ökar i mängd eller storlek medan stora partiklar verkar vara i samtida processer av bildning och upplösning.

Den här avhandlingen har behandlat ett flertal aspekter inom området för stabilitet, och partikelformation av läkemedelsproteiner. Med en förhoppning om att slutsatserna har tillfört kunskap för att kunna försätta arbetet med att tillverka stabilare och bättre läkemedelsproteiner i framtiden.

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I started this PhD journey almost five years ago; it feels like a lifetime ago, yet it has passed so quickly. It would not have been possible to finish without the guidance and support of so many people.

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To my daughter, **Rakel**: seeing you grow up brings me so much joy and gives me a beautiful perspective on what is truly important in life.

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List of Publications

Paper I: **Investigating Thermally Induced Aggregation of Somatropin- New Insights Using Orthogonal Techniques;** Amanda Västberg, Hans Bolinsson, Mats Leeman, Lars Nilsson, Tommy Nylander, Kushal Sejwal, Ida-Maria Sintorn, Kristina Lidayova, Helen Sjögren, Marie Wahlgren, Ulla Elofsson, In: International Journal of Pharmaceutics

Paper II: P **Particle Formation During Peristaltic Pumping of Therapeutic Proteins: Hofmeister Anions Effect**; Amanda Västberg, Natalia Markova, Lars Nilsson, Tommy Nylander, Balasubramanian Sivakumar, Marie Wahlgren, Ulla Elofsson: Manuscript-Submitted

Paper III: **Effect of Aggregate Seeding on Protein Stability in Solution** Amanda Västberg, Natalia Markova, Lars Nilsson, Patrick King, Jan Schaefer, Balasubramanian Sivakumar, Helen Sjögren, Marie Wahlgren, Ulla Elofsson. Manuscript- Submitted

Paper IV: **Protein Aggregation During Static and Agitated Storage in Different Solution Conditions,** Amanda Västberg, Hans Bolinsson, Ida Cederwall, Lars Nilsson, Tommy Nylander, Balasubramanian Sivakumar, Marie Wahlgren, Ulla Elofsson. Manuscript

Author's Contribution to the Papers

Paper I: I designed the study with my supervisors. I conducted most of the experimental work but with help from Kushal Sejwal and Kristina Lidoyova on MiniTEM and Mats Leeman and Hans Bolinsson on AF4 measurements. I analysed the 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 II: I designed the study 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 with my supervisors.

Paper III: I designed the study with some input from my supervisors. I conducted most of the experimental work and analyzed data with contributions from co-authors. I wrote the first draft of the manuscript and worked on the finished draft with my supervisors.

Paper IV: I designed the study with some input from my supervisors and conducted the majority of the experimental work with some help from Ida Cerderwall and Hans Bolinsson. Analyzed the data, wrote the first draft of the manuscript, and worked on the finished draft with my supervisors.

Abbreviations

mAb	Monoclonal antibody
hGH	Human growth hormone
DSC	Differential scanning calorimetry
FIM	Flow imaging microscopy
DLS	Dynamic light scattering
SEC	Size exclusion chromatography
UV	Ultra violette
RI	Refractive index
LS	Light scattering
TEM	Transmission electron spectroscopy

Introduction

Proteins are large molecules known as polypeptides, assembled by long chains of amino acids linked together by covalent peptide bonds. Insulin is one of the smallest proteins, comprising 51 amino acids [1]. The side chains of amino acids have different properties: they can be hydrophobic (non-polar), charged (positive or negative), polar, acidic, basic, or contain thiol groups with sulphur and aromatic rings [2]. These varying side chains interact with each other to form the three-dimensional structure of the protein. The structure of proteins is divided into four levels, as seen in Figure 1.

The primary structure describes the sequence of amino acids in the polypeptide chain.

The secondary structure describes the local folding patterns within the polypeptide chain, such as α-helices and β-sheets. These structures are stabilised by hydrogen bonds formed between the atoms in the polypeptide backbone.

The tertiary structure describes the overall three-dimensional shape of a single protein molecule. It results from folding the secondary structures into a thermodynamically favourable conformation. However, this energy minimum is often shallow, so the structure can easily be disrupted if conditions change around the protein. Interactions stabilising the tertiary structure include hydrogen bonds, hydrophobic interactions, covalent disulfide bridges, van der Waals forces, and dipole-dipole interactions. Most proteins exist in an aqueous environment. Therefore, the most hydrophobic regions are typically buried inside the protein [2].

The quaternary structure describes the assembly of multiple protein subunits into a larger complex. Two examples of proteins with a quaternary structure are hemoglobin and antibodies [3-5].

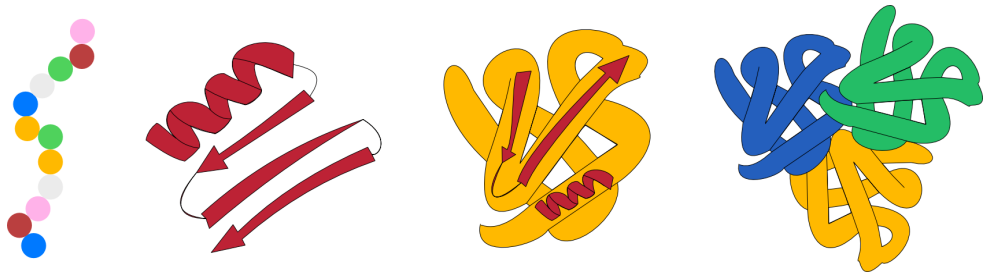

Figure 1 The four levels of protein structure from left to right: primary, secondary, tertiary, and quaternary structure.

Therapeutic Proteins

Therapeutic proteins have unlocked the possibility of treating various genetic conditions and diseases, such as autoimmune diseases or cancer [6]. As proteins have a specific function that aims to be replaced or targeted, this can be done with high specificity and less risk of interference with other biological processes, which decreases adverse effects [7]. Therapeutic proteins are mainly produced from living cells [7]. Compared to traditionally synthesised small-molecule drugs, proteins are large molecules with a complex structure. Therapeutic proteins are primarily administered intravenously or through subcutaneous or intramuscular injections [8, 9]. This is because, for example, if administrated orally, there is a high risk of protein degradation in the acidic environment and the presence of protease in the gastrointestinal tract. The uptake is also an issue due to the low cross-over through the epithelial barrier [10]. Furthermore, maintaining the structure is essential to ensure the efficacy and safety of therapeutic proteins. However, stability is a challenging aspect as proteins are sensitive to degradation, both chemically (deamidation, oxidation, etc.) and physically (unfolding, aggregation, adsorption) [11]. This thesis has focused on investigating the physical stability of proteins in solution, mainly aggregation, which will be further discussed in the following sections.

The aim of this thesis

This project aims to gain more profound knowledge about physical stability and aggregation behaviour of biological drugs in liquid formulations. The objectives are to investigate aggregation caused by combinations of stress factors like temperature, surface interactions, seeding and agitation in aspects of protein and formulation. Two therapeutic proteins, recombinant human growth hormone (hGH) and a monoclonal antibody, IgG1, Antibody A, have mainly been used as they have

different sizes and structures and represent two classes of proteins (hormones and antibodies). This will contribute to the improved understanding of triggering factors and mechanisms of protein aggregation, which will contribute to insight into the development strategies of therapeutic protein formulations.

The work has been divided into four parts: (1) the characterisation of heat-induced aggregation of hGH using orthogonal techniques, (2) studying the particle formation of Antibody A in different formulations during pumping, (3) the seeding effect of heat-induced aggregates on protein stability and aggregation in different storage temperatures for hGH and Antibody A, and lastly (4) aggregation behaviour of Antibody A in different formulations with and without pre-formed aggregation or agitation during storage.

Proteins used in this thesis

Human Growth Hormone

HGH, also known as Somatropin, is a small protein of a single polypeptide chain of 191 amino acids, 22.1 kDa, with an IP of about 5.3 [12-14]. Figure 2 shows the hGH main structure that comprises a four α-helices bundle. It has two disulfide bridges important for biological activity [15].

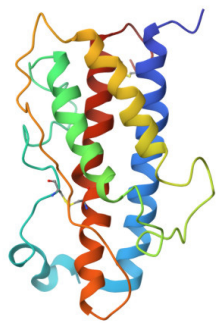

Figure 2 3d strucutre of hGH [16]. The structure was obtained from the RCSB Protein Data Bank (PDB ID: 1HGU).

Human growth hormone was successfully extracted from human pituitary glands in the 1950s [17]. This made treating children with severe growth hormone deficiency possible during the following decades. It was later found that the treatment could transfer a rare fatal neurodegenerative disease called Creutzfeldt-Jakobs disease [18]. However, in 1985, after discovering the biochemical structure in the 70s, the development of the recombinant DNA technique made it possible to produce

biosynthetic hGH [19]. Since then, the recombinant hGH has been used to treat various conditions successfully, such as growth hormone deficiency in children and adults, Turner syndrome, and Prader-Willi syndrome, but also for HIV-related conditions like wasting and fat accumulation caused by lipodystrophy [20].

Antibody A

Antibody A is a monoclonal Antibody of class IgG1. This protein is larger than hGH, with a molecular weight of 144 kDa and an IP of about 8.8. Figure 3 shows an antibody's Y-shaped structure comprising four polypeptide chains, with two identical heavy (H) chains and two light (L) chains. Covalent disulfide bridges stabilise the heavy and light chains to each other [21]. The heavy chain has three constant (C) domains (CH1, CH2 and CH3) and a variable domain (VH). The light chain has one constant (CL) and a variable (VL) domain. The two arms of the Y are the antigen-binding fragments known as the Fab region, which act as the antigen-binding sites [22]. The Fab has a highly variable region (Fv) that controls the binding to an antigen. This region steers the function of the antibody, such as inhibiting or neutralising the antigen. The base of the Y-shape is the constant region of CH2 and CH3 domains known as the fragment crystallised region (Fc). The Fc region and its functional properties decide the class of the antibody, which can be immunoglobulin G, M, D, E, and A (IgG, IgM, IgD, IgE, and IgA).

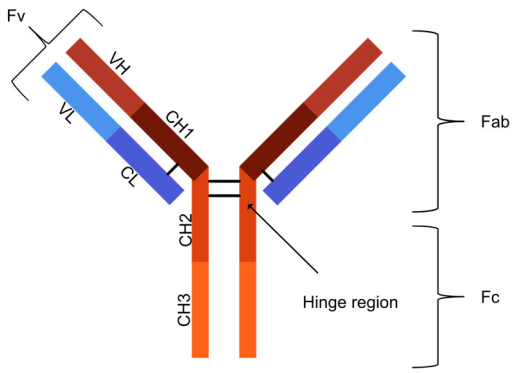

Figure 3 Illustration of antibody structure.

The first mAb was successfully produced in 1975. The first molecule was approved for clinical use in 1986 to prevent rejection after organ transplantation [23]. Since then, the field's development and possible treatments have advanced rapidly. Nowadays, they are used in various therapies such as hematologic conditions, cancer, immune diseases, etc [24].

β-lactoglobulin

β-lactoglobulin (β-lg) was also used in one part of this work, which is the major component of whey protein in milk. It is a small protein of 162 amino acids with a molecular weight of 18.3 KDa and an IP of about 5.2. The structure can be seen in Figure 4. β-lg consists of 9 anti-parallel β-strands; 8 of these strands form a β-barrel with a three-turn α-helix on the outside, and the ninth β-strands is placed along the first strand in the β-barrel [25, 26]. It has five cysteine residues. Four form two disulfide bridges, and the last one is buried in the core of the protein. This residue can be exposed upon unfolding, making it reactive, which gives β-lg a well-defined heat-induced aggregation behaviour. This and the well-characterised structure make it suitable a model protein [27].

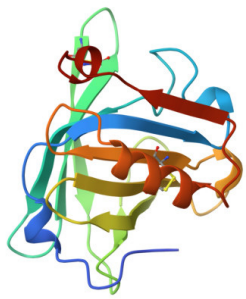

Figure 4 Crystal strucutre of β-lactoglobulin. The structure was obtained from the RCSB Protein Data Bank (PDB ID: 1BEB) [25].

Formulation of Therapeutic Proteins

The efficacy and safety of a therapeutic protein depend on keeping the protein structure intact. Therefore, the protein is combined into a formulation with ingredients that help stabilise the protein and decrease the risk of adverse immunogenic reactions. Table 1 shows different components commonly used in protein drug formulations and their effects [28-30].

Table 1 Lists examples of ingredients in protein drug formulations and their function.

Type of component	Reason
Buffer (phosphate, citrate, acetate, histidine, etc.)	Buffers are used to keep a stable pH that is optimal for protein stability.
Surfactants (polysorbate, poloxamer)	Proteins are surface-active molecules, and surfactants are added to prevent proteins from interacting with surfaces during production, primary packaging, or administration devices. They can also, in some cases, enhance the solubility of the protein.
Tonicity adjusters and Stabilisers (salt, sugars, polyols, amino acids)	Tonicity adjusters are used to match the solution osmolarity to that of body fluids, which helps reduce pain and irritation during administration. Stabiliser components help stabilise the protein's native conformation to prevent denaturation, aggregation, or degradation during manufacturing or storage.
Preservatives (benzyl alcohol, phenol, m-cresol)	Preservatives are added to prevent microbial growth if the protein drug product is produced for multi-dosage.
Antioxidants (amino acids, vitamins, other components like glutathione)	Antioxidants protect amino acid residues towards oxidational stress that can be caused by oxygen or light exposure.
Chelating agents (EDTA)	It is used to bind metal ions, which inhibit the risk that they will catalyse oxidative reactions in the formulation.
Cryo/lyoprotactants (sucrose, trehalose ect.)	Protects the protein during freezing and drying.

Hofmeister series

In paper II, three different anions, SO_4^-, Cl^-, and SCN^- ranked in the Hofmeister series, were used to vary the conformational, colloidal, and interfacial stability of Antibody A to investigate particle formation during peristaltic pumping. Franz Hofmeister first introduced the Hofmeister series in the late nineteenth century. It was an empirical ranking of ions and their ability to affect the stability and solubility of proteins and other macromolecules in aqueous solutions [31]. The order ranks the proteins in terms of their efficiency in stabilising the protein structure [32].

Anion series:

$$SO_4^{2-} > HPO_4^{2-} > F^- > CH_3COO^- > Cl^- > Br^- > NO_3^- > I^-ClO_4^- > SCN^-$$

Cation series:

$$(CH_3)_4N^+ > Rb^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^+$$

The left side in the series is often referred to as a Kosmotropic “structure maker”, while the right is a Chaotropics “structure breaker”. The left side tends to increase stability but decreases the solubility of the protein, which gives the “salting out” effect. The right side has the opposite effect, reducing protein stability while increasing protein solubility, giving a so-called “salting in” effect. The origin of the Hofmeister effect, or “specific ion effect”, has been debated over the years [15]. Two explanations are ion binding or exclusion of ions. Ion binding explains that ions directly interact with residues on the protein's surface. This can be described by “matching water affinities”, where similar-sized ions have higher affinity and differently sized ions are less likely to interact [33]. Instead, exclusion describes how small, well-hydrated ions are excluded from the protein's hydration shell and will not directly interact with the protein surface [34, 35]. The order of the Hofmeister series is not constant; it can reverse or change in order depending on solution conditions like pH, ionic strength or surface characteristics [36], highlighting the complexity of specific ion effects.

Formulations used in this thesis

Table 2 summarises the system used in the studies.

Table 2 Description of each system used in the papers and other studies in this thesis.

Experiment	Protein	Buffer	pH	Other
Paper I	hGH	10 mM phosphate buffer	5.8 and 7	n.a
Paper II	Antibody A	25 mM histidine-HCl	6	150 mM-Na_2SO_4, NaCl, NaSCN
Paper III	hGH, Antibody A	10 mM phosphate buffer, 25 mM histidine-HCl	7, 6	n.a
Paper IV	Antibody A	25 mM histidine-HCl	6	150 mM NaCl, 0.1% PS20
MiniTEM β-lactoglobulin	β-lactoglobulin	50 mM phosphate buffer	7	n.a
Hydrophilic and hydrophobic glass beads	hGH, Antibody A	25 mM histidine-HCl	6, 7	n.a

Aggregation Mechanisms

The structure of a protein is essential for the biological function. However, proteins are sensitive to changes in their surrounding environment, such as changes in temperature, solution pH, and ionic strength [37]. Aggregation can happen through different pathways, which will explained in this chapter. Understanding aggregation mechanisms for therapeutic proteins can help choose mitigation strategies during formulation and product development. However, although much knowledge about aggregation and aggregation mechanisms exists, the area is complex because aggregation highly depends on the system, such as the protein, solution conditions, and stress factors [38, 39]. Furthermore, it is also challenging to study the aggregates as they can have a wide range of sizes from nanometres up to hundreds of microns and different structures from spherical to tread-like [40, 41].

Aggregation pathways

Aggregation can occur through different pathways, as illustrated in Figure 5. Proteins can self-assemble in a native, partly unfolded, or denatured state, which can be the initiator of aggregation [42, 43]. The equilibrium of the native and unfolded/partly unfolded protein can be pushed to the right by different factors, such as increasing temperature, which exposes more reactive parts of the proteins. Chemical changes like oxidation or deamidation can also initiate structural changes in the protein. The same forces and interactions driving protein folding are involved in protein aggregation, which is why structural rearrangement can initiate protein-protein interaction. Depending on the solution condition, such as pH, ionic strength, or other additives, protein can also be self-assembled in the native state [44]. The small assemblies of protein molecules can be reversible or irreversible, depending on the pathway [45]. Reversibility can be tested by changing the solution condition, such as temperature, concentration or salt addition [46]. The smallest irreversible assembly can be seen as the nucleus for non-native aggregation [39]. Nucleus formation is often the rate-limiting step for protein aggregation and can be called the lag phase. After the initial lag phase, the aggregates grow more rapidly. This can occur through monomer addition followed by aggregate-aggregate interaction. Eventually, there can be phase separation.

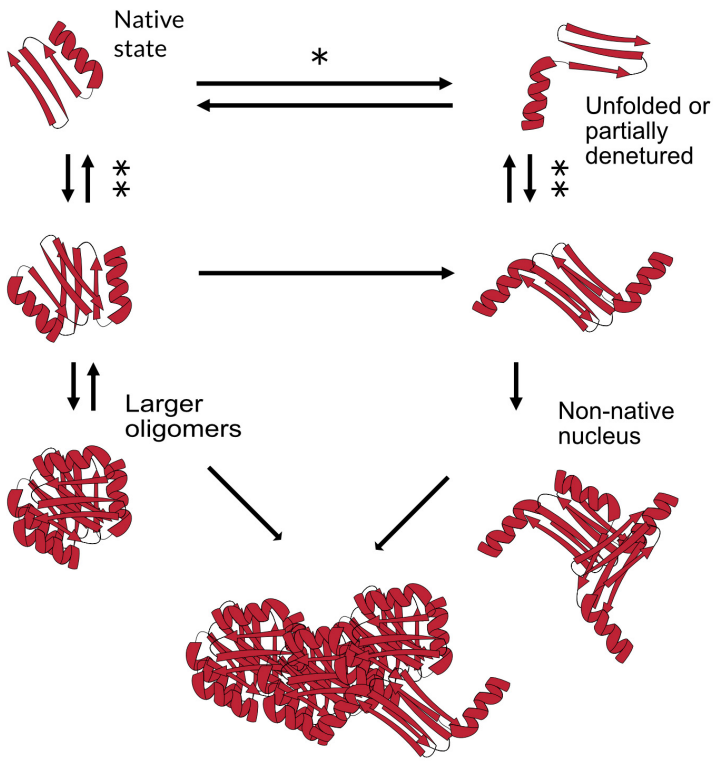

Figure 5 Schematic of different aggregation pathways. *Points to the structural changes caused by conformational instabilities. ** Points to the colloidal stability, thus the propensity of self-assembly. Adopted from [30].

Triggering Factors

Several factors can affect aggregation, and they can be intrinsic or extrinsic. Intrinsic factors include the protein structure from the amino acid sequence, which can consist of more aggregate-prone regions that can be mentioned as “hot spots” [38, 47]. Extrinsic factors include solution conditions like pH and ionic strength or the presence of reactive species [46, 48]. Extrinsic factors can also be interfacial, mechanical or temperature stress, accelerating protein aggregation [49-51]. Extrinsic stress factors present risks during the entire life cycle of a therapeutic protein drug. It can be during manufacturing, transport, storage, or administration [52].

Several steps during manufacturing involve different stresses [16]. These include homogenisation, sterile filtration, filling, drying, and primary packaging. Transport can cause low-frequency vibrations, damaging the protein [53]. Storage aspects are

also important; for example, the choice of primary packaging, storage temperature, and storage time can harm protein stability. Furthermore, preparation before administration can also be risky, such as dilution in IV bags, exposure to UV light, etc. [54-57].

Types of Aggregates

Aggregate is a broad term, and it is essential to distinguish between different types when studying aggregation mechanisms. Protein aggregates can range from dimers to large visible particles [58]. They can be categorised by size, reversibility, conformation, chemical modification, or morphology [59]. For example, aggregates can have a perturbed structure or be more native-like depending on the aggregation pathway, as seen in Figure 5. Aggregates, like amyloids, have a distinct structure, while other aggregates can also be amorphous with no particular structure [60].

Probably the most common way to categorise aggregates is based on their size. Oligomers refer to small assemblies of only a few protein molecules. Submicron aggregates often refer to the interval of 0.1-1 μm, while subvisible particles are usually mentioned in the 1-100 μm interval, and visible particles are > 100 μm. Some researchers instead distinguish the aggregates and use the terms soluble and insoluble aggregates [61, 62]. This can refer to whether they are visible by the eye or not or depending on which analytical technique is used to measure them. In this thesis, the aggregates were categorised by size. *Oligomers* have been used for the small aggregates seen by SEC, eluting just before the monomeric peak. *Small aggregates* have been used for the heat-induced aggregates seen by DLS and MinTEM. Lastly, *micron-sized particles* have been used for the aggregates seen by FIM, which is above 1 μm.

Analytical Techniques to Study Aggregates

One challenge in studying the aggregation of proteins is that no technique can simultaneously study aggregates in their full range of size and shape [63]. Therefore, it is essential to use orthogonal techniques, which were especially a focus in paper I. summarises the methods used for studying aggregates in this thesis.

Table 3 Summary of the techniques used to study aggregates in this thesis.

Technique	Principle	Information	Size	Comments
Dynamic light scattering (DLS).	Measures the fluctuation of scattered light due to the movement of particles by Brownian motion. The fluctuation rate is correlated to the size of the particles.	The hydrodynamic size distribution.	1 nm -10 µm.	Non-destructive. Relatively low resolution in multimodal samples due to the higher scattering intensity from larger-sized species.
Flow imaging microscopy (FIM).	A light microscopy technique that captures images of each particle in a flow cell.	Size distribution, shape, morphology, and number concentration.	1 µm up to millimeters.	Visual and quantitative. Particle characteristics such as transparency and settings can affect output.
Asymmetric flow field flow fractionation (AF4).	Aggregates are fractioned in a small channel under a cross-sectional flow. The particles are separated based on their diffusion coefficient in the flow. Larger particles will be closer to the bottom of the channel and elute slower.	Size distribution, molecular weight, concentration.	1 nm- 1 µm.	It can be coupled with UV, RI, MALS, DLS detectors, etc. It has a lower surface interaction than SEC.
Size exclusion chromatography (SEC).	Aggregates are separated with gel chromatography. The packed particles will elute depending on how they travel in the column. Small particles will enter the pores of the beads while larger ones will not and, therefore, elute faster.	Size distribution, molecular weight, concentration.	From about 1 kDa up to MDa.	It can be coupled with UV, RI, MALS, DLS detectors, etc. Risk of interaction with the stationary phase, dissociation of weakly bound aggregates.
Transmission electron microscopy (TEM).	Image the particles by an electron beam.	Size, structure, and morphology are at very high resolution.	~1 nanometer-100 nm.	Visual and at very high resolution. Not in solution; sample preparation can induce artefacts.

Several other techniques can be used to study aggregates. Some examples are light obscuration (LO), nano-tracking particle analysis (NTA), analytical ultracentrifugation (AUC), cryo-transmission electron microscopy, atomic force microscopy (AFM), and multi-angle light scattering/ static light scattering (MALS) [64].

It is crucial to consider that orthogonal techniques provide different size measures depending on the principle. For example, DLS provides the hydrodynamic radius based on their movement by Brownian motion, which can be affected by the ionic strength in the solution or shape of the particles. Microscopy methods can measure size based on different approaches. For example, the ferret diameter is the longest or shortest length of the particle. In contrast, the area-based diameter (ABD) assumes the diameter of a sphere with the same area as the particle.

Thermal stress

Temperature highly affects the stability of the protein in the solution. As seen in the previous section, an equilibrium exists between the folded and unfolded states of the protein. However, as Figure 5 shows, it is rarely an ideal reversible scenario, and proteins tend to self-associate and aggregate as it is forced to the unfolded state. From a thermodynamic perspective, $\Delta G=\Delta H-T\Delta S$, an increase in temperature can disrupt the non-covalent interaction, leading to a negative ΔG, favouring the unfolded state due to the increased entropy (disorder). When the temperature rises, more proteins will unfold until all are denatured at a high enough temperature. This process is also most often accompanied by aggregation. Thermal stability refers to the resistance of the protein to unfold at increasing temperatures. Different solution conditions, such as pH, ionic strength, and concentration, can change the thermal stability of the protein, as they affect conformational and colloidal stability.

Techniques

Several techniques can be used to study a protein's thermal stability. These techniques measure features that can be connected to changes in secondary or tertiary structure. Some examples are infrared spectroscopy (IR-spectroscopy), isothermal calorimetry (ICT), circular dichroism (CD), and differential scanning calorimetry (DSC). In this work, DSC was used. DSC provides insights into the thermodynamic properties of protein folding by measuring the heat flow as temperature increases [65]. As the protein unfolds, it absorbs heat, leading to endothermic changes in the thermogram. The peak maximum is the midpoint transition temperature (T_m), where 50% of the protein is unfolded, indicating the protein's thermal stability. Analysing these transitions helps understand protein folding mechanisms and stability under varying conditions. Except for T_m, parameters such as the onset temperature (T_{onset}) and the half-width temperature (T_{half}) can be helpful for assessment. T_{onset} describes the starting temperature where the structure starts to change. A higher T_{onset} gives higher resistance towards aggregation as most of the protein will be folded below T_{onset}. T_{half} describes the width of the peak at half of its height at T_m. A narrower peak describes a cooperative unfolding process, while a broader indicates a less cooperative unfolding process. The cooperativity describes the process of protein unfolding, a cooperative process where the folded native protein (N) unfolds (U) over a small temperature interval as

one unit in a two-state dependent manner, with no intermediate steps, an effect from the shallow energy minima of the protein structure [66]. The information that DSC can obtain makes it a valuable tool for evaluating therapeutic proteins' stability in different solution conditions [67].

Thermal unfolding

Figure 6 shows the non-two-state fits of hGH in 10 mM phosphate at pH 7 and Antibody A in 25 mM histidine-HCl at pH 6. A non-two-state fit of a thermogram indicates that the protein does not unfold through a simple, reversible cooperative transition from the folded to the unfolded state, as noted in the simplified schematic in Figure 5. Instead, the unfolding process involves intermediate states or multiple sequential transitions. This can occur due to distinct structural domains within the protein that unfold independently or through partially folded intermediates [68, 69]. Such unfolding behaviour suggests that multiple factors influence the protein's stability. Analysing these transitions can provide deeper insights into the folding mechanisms and the effects of external factors like pH, ionic strength or structural changes on the protein's thermal stability.

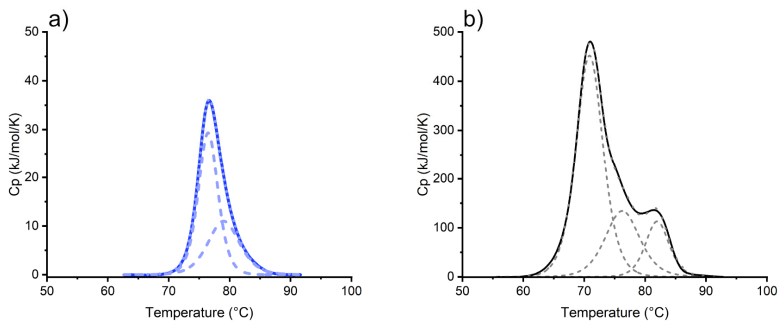

Figure 6 DSC thermogram and Non-two state fits for (a) hGH and (b) Antibody A

Concentration dependence during thermal unfolding

Measuring a concentration series can reveal specific protein features in that environment. An ideal two-state unfolding of a protein is reversible and not dependent on concentration [70]. If T_m decreases with increasing concentration, it tells that the formation of intermediates or aggregation most likely occurs during unfolding. For example, this is very noticeable for hGH (paper I); see Table 4. At the same time, Antibody A shows some concentration trends, with decreasing T_m with higher concentrations in most studied conditions see Table 4.

.

Table 4 Thermal parameters from DSC measurements for Antibody A and hGH in different solution conditions.

Name	Total Area (kJ/mol)	T_{Onset} (°C)	T_{m_1} (°C)	T_{m_2} (°C)	T_{m_3} (°C)	T_{mHalf_1} (°C)	T_{mHalf_2} (°C)	T_{mHalf_3} (°C)
Antibody A								
0.375 mg/ml 25 mM Hist-HCl	3475±21	62.8±0.1	71.4±0.0	81.7±0.1		5.9±0.0	18.7±0.0	
0.75 mg/ml 25 mM Hist-HCl	3985±7	62.6±0.1	71.2±0.0	81.6±0.0		5.9±0.0	18.5±0.0	
1.5 mg/ml 25 mM Hist-HCl	3965±7	62.8±0.1	71.1±0.0	81.6±0.0		5.9±0.0	18.3±0.0	
3 mg/mL 25 mM Hist-HCl	4085±21	63.0±0.1	70.9±0.0	81.4±0.0		5.9±0.0	18.2±0.0	
6 mg/mL 25 mM Hist-HCl	4085±21	63.0±0.0	70.7±0.0	81.3±0.0		6.0±0.0	18.1±0.0	
0.375 mg/ml 175 mM Hist-HCl	3415±64	61.6±0.1	69.9±0.0	80.7±0.0		6.5±0.0	19.3±0.1	
0.75 mg/ml 175 mM Hist-HCl	3585±21	61.3±0.2	69.7±0.0	80.5±0.0		6.7±0.1	19.2±0.0	
1.5 mg/ml 175 mM Hist-HCl	3615±7	61.2±0.0	69.5±0.0	79.8±0.2		7.0±0.0	19.1±0.0	
3 mg/ml 175 mM Hist-HCl	3805±49	61.0±0.0	69.3±0.0	79.8±0.0		7.2±0.0	19.1±0.0	
6 mg/ml 175 mM Hist-HCl	3495±7	61.0±0.0	68.8±0.0	79.9±0.0		7.7±0.0	19.2±0.0	
0.375 mg/ml NaSO4	3637±384	61.1±0.2	69.7±0.1	79.1±0.0		6.7±0.0	18.6±0.1	
0.75 mg/ml NaSO4	3810±251	61.0±0.2	69.6±0.1	78.7±0.0		6.9±0.1	18.5±0.1	
1.5 mg/ml NaSO4	3807±116	61.0±0.1	69.6±0.0	78.8±0.0		7.0±0.0	18.6±0.0	
3 mg/ml NaSO4	3810±17	61.0±0.1	69.4±0.0	79.0±0.1		7.2±0.1	18.6±0.0	
6 mg/ml NaSO4	3887±111	60.9±0.1	69.2±0.0	79.0±0.1		7.5±0.1	18.7±0.0	
0.375 mg/ml NaCl	3595±64	61.4±0.3	69.6±0.1	80.5±0.1		6.7±0.0	19.2±0.0	
0.75 mg/ml NaCl	3800±28	61.0±0.0	69.6±0.0	79.7±0.0		7.1±0.0	19.3±0.0	
1.5 mg/ml NaCl	3835±49	61.0±0.0	69.3±0.0	79.9±0.2		7.4±0.1	19.3±0.0	
3 mg/ml NaCl	3895±7	60.9±0.0	69.1±0.1	79.9±0.0		7.7±0.1	19.3±0.0	
6 mg/ml NaCl	3990±0	60.9±0.0	68.7±0.0	80.0±0.0		8.2±0.1	19.4±0.0	
0.375 mg/ml NaSCN	3435±7	55.6±0.1	63.7±0.0	67.0±0.2	75.9±0.0	13.9±0.1	9.0±0.0	20.1±0.1
0.75 mg/ml NaSCN	3415±21	56.50.3	63.6±0.0	67.0±0.0	76.1±0.1	12.9±0.2	8.9±0.1	20.2±0.0
1.5 mg/ml NaSCN	3615±35	56.2±0.0	63.7±0.1	67.0±0.0	76.1±0.0	13.2±0.1	9.1±0.0	20.3±0.0

3 mg/ml NaSCN	3580±14	56.3±0.0	63.8±0.1	66.9±0.0	76.2±0.1	13.3±0.0	9.4±0.0	20.4±0.0
6 mg/ml NaSCN	3705±35	56.2±0.0	63.8±0.1	66.8±0.0	76.4±0.0	13.4±0.0	9.8±0.0	20.5±0.0
0.75 mg/ml 0.1 % PS20	3805±35	63.1±0.0	70.8±0.0	81.4±0.0		6.4±0.0	18.4±0.0	
1.5 mg/ml 0.1 % PS20	3865±7	63.0±0.1	70.5±0.0	81.3±0.2		6.6±0.0	18.3±0.0	
3 mg/ml 0.1 % PS20	3865±7	63.4±0.0	70.4±0.0	81.2±0.0		6.5±0.0	18.0±0.0	
6 mg/ml 0.1 % PS20	3905±35	62.8±0.1	70.0±0.2	80.9±0.2		7.4±0.9	18.4±0.1	
hGH								
0.3 mg/ml hGH pH 5.8	97±11	68.7±0.3	80.5±0.0			4.2±0.1		
0.5 mg/ml hGH pH 5.8	128	71.15	78.4			4.0		
1 mg/ml hGH pH 5.8	160±1	71.8±0.0	76.8±0.1			4.0±0.0		
3 mg/ml hGH pH 5.8	173±1	69.5±0.0	74.6±0.0			4.1±0.0		
0.3 mg/ml hGH pH 6	108±6	75.0±0.7	80.9±0.3			4.0±0.1		
1mg/ml hGH pH 6	147±0.1	72.5±0.4	77.5±0.1			3.8±0.0		
3 mg/ml hGH pH 6	160±0	70.4±0.0	75.1±0.1			3.9±0.0		
0.3 mg/ml hGH pH 7	163±4	73.9±0.6	82.2±0.0			5.0±0.1		
0.5 mg/ml hGH pH 7	194±0	73.2±1	80.9±0.0			5.2±0.0		
1 mg/ml hGH pH 7	204±0	72.7±0.3	79.2±0.0			5.1±0.0		
3 mg/ml hGH pH 7	208±1	70.72±0	76.4±0.0			4.7±0.0		

Thermal Unfolding of Human Growth Hormone

Figure 7 (a and b) shows the thermal unfolding of hGH at two solution pHs. The pH highly affects the thermal stability of hGH; Tm was significantly lower at pH 5.8 (paper I). As seen by others, this acid destabilisation is suggested to be due to the protonation of carboxyl groups in hGH [71, 72]. Furthermore, hGH is a single-domain protein but shows a small shoulder in the thermograms, which is best accounted for by a fit with two peaks, as seen in Figure 6. As noted in paper I, Van't Hoff's and calorimetric enthalpy ratio is over one for all concentrations assessed at pH 5.8 and 7. This means that hGH unfolds less cooperatively, indicating intermediate states or aggregation during unfolding [68]. Several studies have reported different intermediate structures and aggregation during the unfolding of hGH [71, 73-75]. Gomez et al. could see two distinct transitions at pH 7-8. They presumed that the first transition involves self-association as the T_m decreases with higher concentration. The second peak increased with higher concentrations up to 1.5 mg/ml, which they associated with oligomer dissociation. They did not observe a second transition at higher concentrations, which they suggest is due to more extensive aggregation. However, our results differ as we see a concentration dependence at pH 5.8 and 7 up to a concentration of 3 mg/ml. Furthermore, our studies showed a decrease in both $T_m s$ with increasing concentration. Kasimova et al. saw two transitions at neutral pH, which could be connected to the formation of partly folded oligomers [75]. Our results suggest an intermediate form of hGH with slightly higher T_m, and both forms tend to aggregate upon unfolding [74, 76].

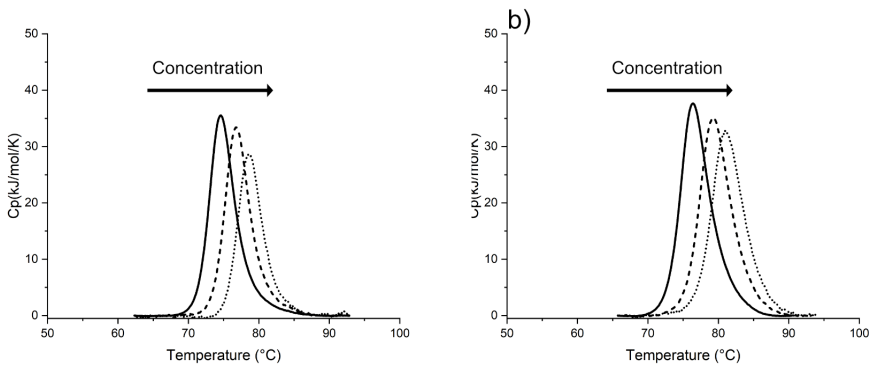

Figure 7 DSC thermograms of hGH concentration series, 0.5, 1, and 3 mg/ml in 10 mM phosphate at (a) pH 5.8 and (b) pH 7.

In paper III, the thermal stability of hGH was evaluated after heating, as seen in Figure 8. The calorimetric enthalpy had significantly decreased after hGH was heated at 75 °C for 60 minutes. However, it fits well with the second peak in the

thermogram of the untreated hGH, which strengthens the theory that a more stable form of hGH was present after the heat stress. It should also be noted that although hGH is heated at such a high temperature for 60 minutes, it was only associated with about a 5 % change in the secondary structure content (paper III), which tells that the secondary structure is relatively stable. That is consistent with the findings of Cauchy et al., where there was a drastic shift in the secondary structure of hGH only when reaching a temperature of 93 °C (FTIR at pH 7.4) [72].

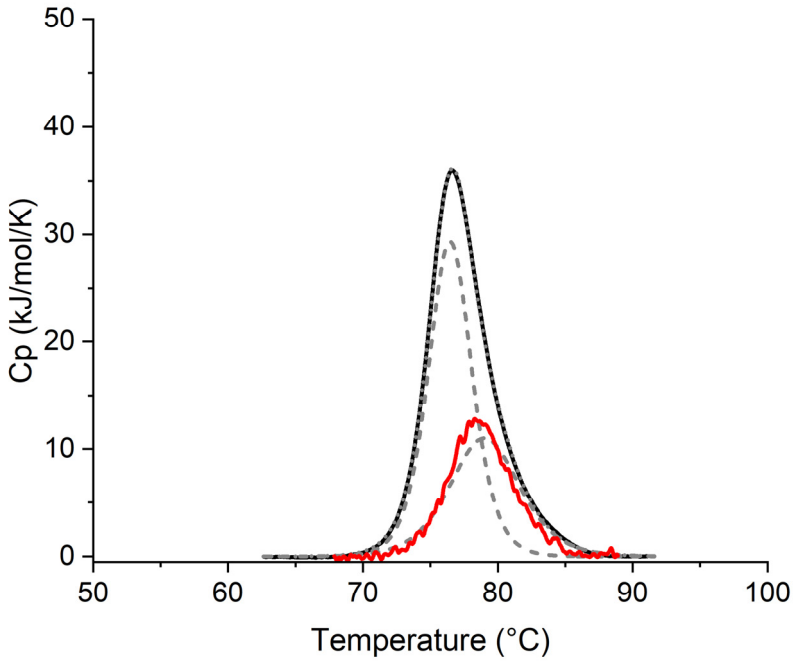

Figure 8 DSC thermogram of hGH untreated at pH 7 (red solid) with the non-two state fit (grey dotted and dashed) and the heated hGH (red solid).

Thermal Unfolding of Antibody A

Antibody A shows two distinct peaks and a shoulder between them, as seen in the DSC thermogram and the non-two-state fit in Figure 6 (b). The most prominent peak in a thermogram of a mAb is usually associated with the Fab region [77, 78]. In contrast, the two smaller peaks correspond to the CH2 and CH3 domains of the Fc region. For Antibody A, this means that the Fab region domains would be the least stable. Figure 9 (a) and Table 4 show that adding salt decreased the thermal stability of Antibody A, as seen by a lower T_{onset} and T_m (paper II). This is probably due to the higher ionic strength, as a histidine-HCl buffer at 175 mM shows similar

behaviour as adding 150 mM NaCl and Na_2SO_4. A higher ionic strength affects the inter- and intra- and intermolecular interaction, which can destabilise the structure of a protein [79]. Ions can also interact with the protein surface, which can induce specific ion effects [80, 81], as discussed in the previous chapter about the Hofmeister series. This can, for example, be noted especially by the addition of NaSCN, which significantly changes the thermal profile of Antibody A, with an apparent fourth transition before the assumed Fab peak.

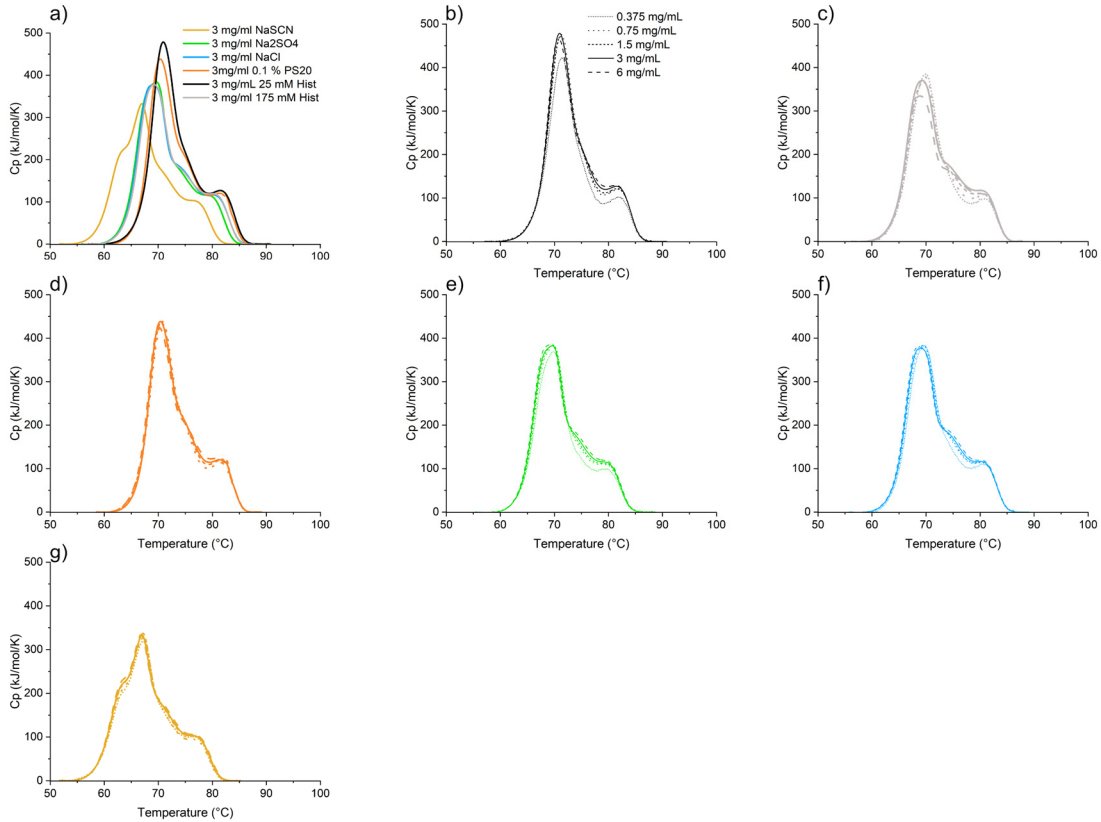

Figure 9 DSC thermograms of Antibody A for different solution conditions. a) 3 mg/mL in 25 mM histidine-HCl buffer (black), 175 mM histidine-HCl (grey),150 mM Na_2SO_4 (green), 150 mM NaCl (blue), 150 mM NaSCN (yellow), 0.1 % PS20 (orange), (b)-(f) concentration series 0.375, 0.75, 1.5, 3, and 6 mg/ml of Antibody A in the same solution conditions as seen in (a).

Furthermore, the thermal profile after heat stress of Antibody A showed a complete unfolding of the first domain as seen in (paper III), which could be expected because the heating temperature was over T_m of the Fab domain. Furthermore, it seemed like parts of Antibody A unfolding and aggregation might be reversible, as a regain in the thermogram peaks (DSC) and chromatogram (SEC) monomer fraction was observable after two weeks of storage (paper II). This contradicts the common conception that thermal aggregation is irreversible.

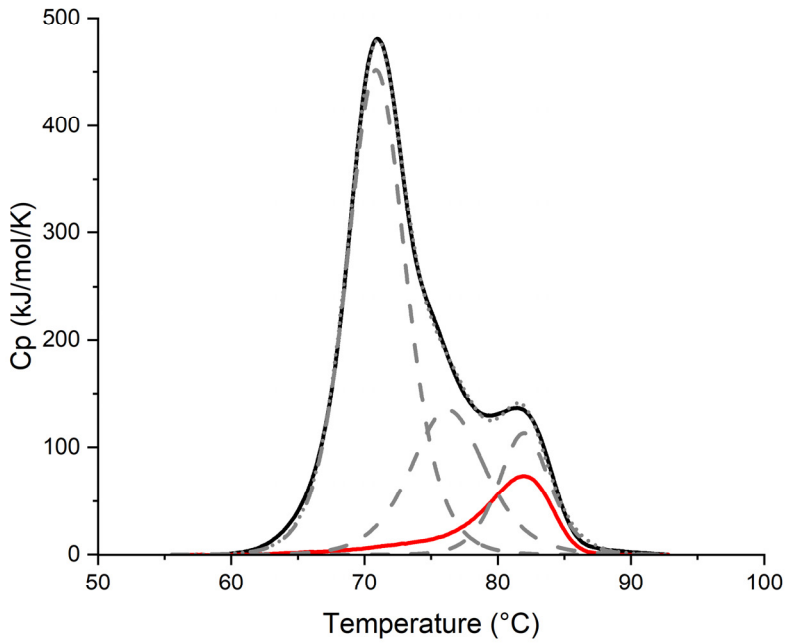

Figure 10 DSC thermogram of Antibody A untreated (black) with the non-two-state fitting (grey dashed) and heated (red).

Heat-induced aggregation

As mentioned, aggregation is most often inevitable when proteins are subjected to higher temperatures. In this thesis different aspects of heat-induced aggregation are investigated by varying time (hGH paper I, b-lg, unpublished data), pH (hGH, paper I), and heating temperature (hGH, paper I).

Heat-induced aggregation of human Growth hormone

In paper I, we investigated the thermal aggregation of hGH in pH 5.8, close to IP and pH 7, where hGH has a net negative charge. pH significantly affected the aggregation behaviour. The electrostatic repulsion of hGH in pH 7 reduced the growth of the aggregates. The heating temperature in relation to T_m did not change the rapid formation of large aggregates in pH 5.8. MiniTEM and AF4 could demonstrate that the aggregates most likely grow by monomer addition at pH 7 up to a specific size [42, 43]. Then, during prolonged heating, as the monomeric concentration decreases, the growth is dominated by aggregate-aggregate growth, forming clusters of the initial elongated aggregates. This shows that electrostatic repulsion and colloidal stability are vital for minimising aggregation propensity [82, 83]. Others have studied the aggregation of hGH and found that the heat-formed aggregates are most likely formed through hydrophobic interactions. This is because the aggregation is significantly reduced in organic solvents or the presence of non-ionic surfactants [71, 76]. Surfactants have been shown to form complexes with hGH [73]. This could lead to improved colloidal stability due to the steric hindrance when electrostatic repulsion might not be sufficient. Hence, adding a non-ionic surfactant might reduce the fast formation of large aggregates at pH 5.8.

MiniTEM and image analysis of small aggregates of β-lactoglobulin

MiniTEM was used in paper I, which helped to interpret the heat-induced aggregation of hGH at pH 7. The MiniTEM technique was further evaluated compared to DLS for heat-induced aggregation of β-lactoglobulin (unpublished data). β-lactoglobulin was heated in the same manner as hGH. MiniTEM images in Figure 11 show that β-lactoglobulin showed a different aggregate formation than hGH. It initially formed small, rounded aggregates. After 60 minutes of heating, some elongated aggregates had formed. The thermal aggregation of β-lactoglobulin did not show a tendency of aggregate-aggregate growth like hGH during this heating time and solution condition. DLS peak size were in quite good agreement with MiniTEM, seen in Figure 12. However, that there is no aggregate-aggregates interaction cannot be seen in DLS measurements (Figure 12(a)) but becomes evident in the analysis of the MiniTEM images. As seen in Figure 12 (b) for β-lactoglobulin, both mean and mode Ferret max diameter continued to increase during the 60 minutes of heating. In contrast, for hGH, the Feret Max mode size is almost constant between 15-60 minutes of heating while the mean continued to grow. Using the MiniTEM technology to study the same type of aggregation for another protein shows that a semi-automated bench-top TEM technique and a more straightforward technique like DLS can be a powerful combination to study aggregation mechanisms.

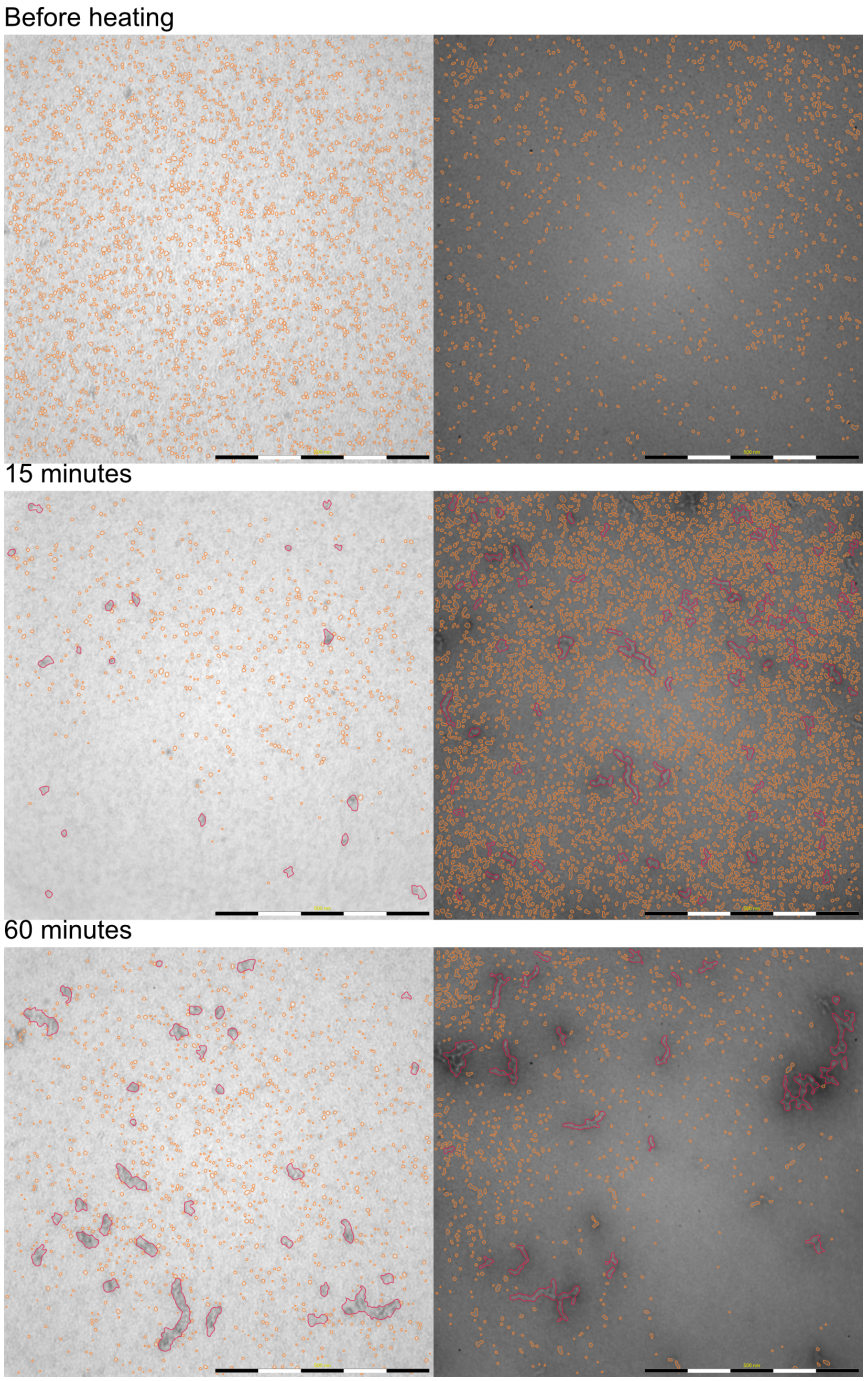

Figure 11 MiniTEM images with detection of monomers (yellow) and aggregates (red) for β-lactoglobulin (left) and hGH (right).

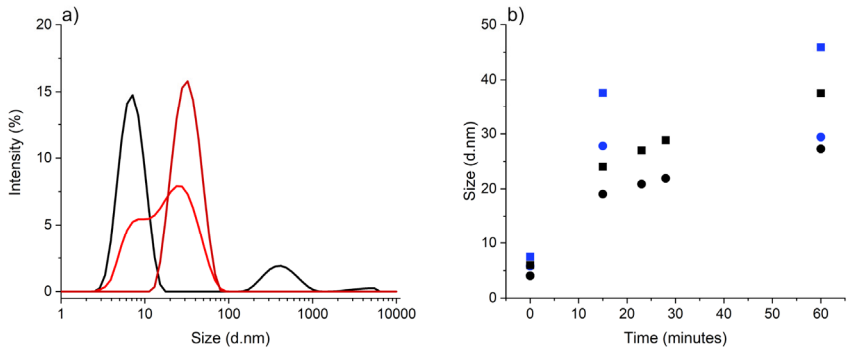

Figure 12 The (a) DLS intensity size distribution untreated (black), heated (light red-15 and dark red-60 minutes) and (b) comparison of the change with time of the aggregate ferret diameter max mean (●) and mode (■) size by analysis from MiniTEM images for β-lactoglobulin (black) and hGH (blue).

Protein Adsorption

A therapeutic protein will encounter many types of interfaces from production to administration, such as solid surfaces: stainless steel (pumping, vessels), aluminium (pistol pumps, vessels), different types of polymeric surfaces (filters, syringe plunger-head, IV-bags, pump tubing), glass (syringes, vials) or dynamic air-water (head-space) or water-oil interfaces (lubricant in syringes, coating in glass vials) [84, 85]. Depending on the protein, solution conditions and interface, they adsorb to various degrees [86]. Interfacial interactions can often be accompanied by conformational changes in the protein, especially on hydrophobic surfaces [87]. By rearranging the protein structure, entropy will be gained when the hydrophobic parts of the protein adsorb, dehydrating the surface and the protein from highly ordered water and releasing counterions into the bulk solution [88]. These structural changes can also lead to aggregation as aggregate-prone regions of the protein may be exposed. Figure 13 shows two proposed mechanisms of surface-induced aggregation. The left side shows how the surface introduces structural rearrangement of the protein, which, can cause aggregation via a non-native aggregation mechanism [84]. The second pathway shows that the protein forms a film on the surface that can be shredded from the surface by mechanical force or shear, leading to particles in the bulk solution. The second mechanism has, for example, been described for protein particle formation during pumping [89, 90]. Protein adsorption is critical to control and minimise when developing therapeutic proteins to ensure efficacy and safety.

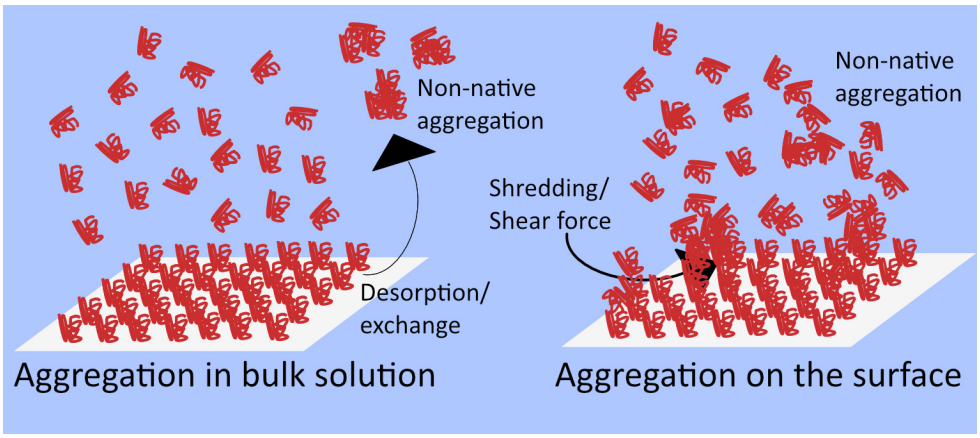

Figure 13 Illustration of pathways of surface-induced aggregation.

Solid surfaces

Adsorption occurs rapidly when a protein encounters a solid surface. The absorption mechanism can be divided into the following stages: how the protein reaches the surface via diffusion, convection, or flow; the initial attachment, which is by hydrophobic or electrostatic interactions or weak van der Waal forces. The initial attachment is followed by reorganisation on the surface, causing conformational changes and, lastly, desorption or exchange of the protein at the surface into the bulk solution [91].

Several factors can affect the extent of protein adsorption and its impact on protein stability, which is connected to substrate surface properties like surface hydrophobicity, charge, or roughness. Environmental conditions such as pH, temperature, and ionic strength also impact adsorption, affecting protein structure and surface affinities [92, 93]. Proteins often do not follow the conventional adsorption isotherms like Langmuir or Clausius-Clapeyron because they assume reversible adsorption [94]. Protein adsorption is usually irreversible or only partially reversible upon dilution. However, there are examples of reversible adsorption. For instance, if the protein is adsorbed through attractive electrostatic interactions, it can be desorbed by changing the pH or increasing the ionic strength [86]. Hydrophobic surfaces can have a more significant effect as the protein may undergo extensive conformational changes by exposing more hydrophobic internal parts towards the surface [87]. Electrostatically driven adsorption could also result in a significantly perturbed structure. If the protein has an attractive interaction with the surface but repulsive interactions with itself, it could make the protein less packed on the surface. This would give the protein more space to interact with the surface when

attractive patches on the protein are driven to interact with the surface, thereby initiating a significant change in structure [95, 96].

Hydrophilic and hydrophobic glass beads

Surface-induced aggregation was investigated by letting Antibody A and hGH interact with glass beads. Glass beads were chosen in the experiment to maximize the surface area. Figure 14 shows the AF4-UV chromatogram of Antibody A and hGH after interaction with hydrophilic (paper IV) or hydrophobic silanised glass beads on a tilting table with no air head space.

Salinisation of hydrophobic glass beads

Glass beads were salinised to achieve a hydrophobic surface [97]. The beads were first washed in a piranha solution (90% H_2SO_4 and 30% H_2O_2 mixed at a 3:1 ratio) during stirring for 30 minutes. Subsequently, the beads were rinsed in MilliQ water and dried at 110 °C. When the beads to be hydrophobised were dried entirely, they were mixed with 1 % silane solution (trichloro[octyl]silane) in toluene for 30 minutes while stirring. The beads were washed three times in ethanol (99%), followed by rinsing in MilliQ water. The hydrophobic beads were stored in ethanol until usage.

Experimental setup

The hydrophobic glass beads had a diameter of 1 µm while the hydrophilic had a diameter of about 0.5 µm. Both beads were washed extensively in miliQ-water and dried before the experiment. The beads were packed in clean glass vials of the same size, and the estimated surface area for the hydrophilic beads was about twice that of the hydrophobic beads. Protein solutions at 3 mg/ml of Antibody A (pH6) and hGH (pH 7) were added to glass vials with the glass beads. The filled glass vials were then put on a tilting table for 24 hours before sampling the bulk protein solution for analysis with AF4, DLS, and small angle x-ray scattering (SAXS).

AF4-UV and DLS after interaction

Antibody A was formulated at pH 6, giving a net positive charge as its IP is at pH 8.8. hGH was formulated at pH 7, which gives hGH a net negative charge as IP is about pH 5.3. Based on the decrease in concentration, Antibody A seemed to adsorb to the hydrophilic and hydrophobic beads after 24 hours of interaction, as seen in Figure 14. In contrast, hGH only showed a reduction in concentration after interaction with the hydrophobic beads, even though the surface area was larger for the hydrophilic beads. This shows the impact of solution conditions on adsorption behaviour and the effect on electrostatic interaction. Antibody A has an electrostatic attraction to the hydrophilic surface, while hGH does not, which highly affects the

adsorption of these two proteins. At the same time, for the hydrophobic beads, both proteins tend to adsorb, which is instead driven by hydrophobic interactions.

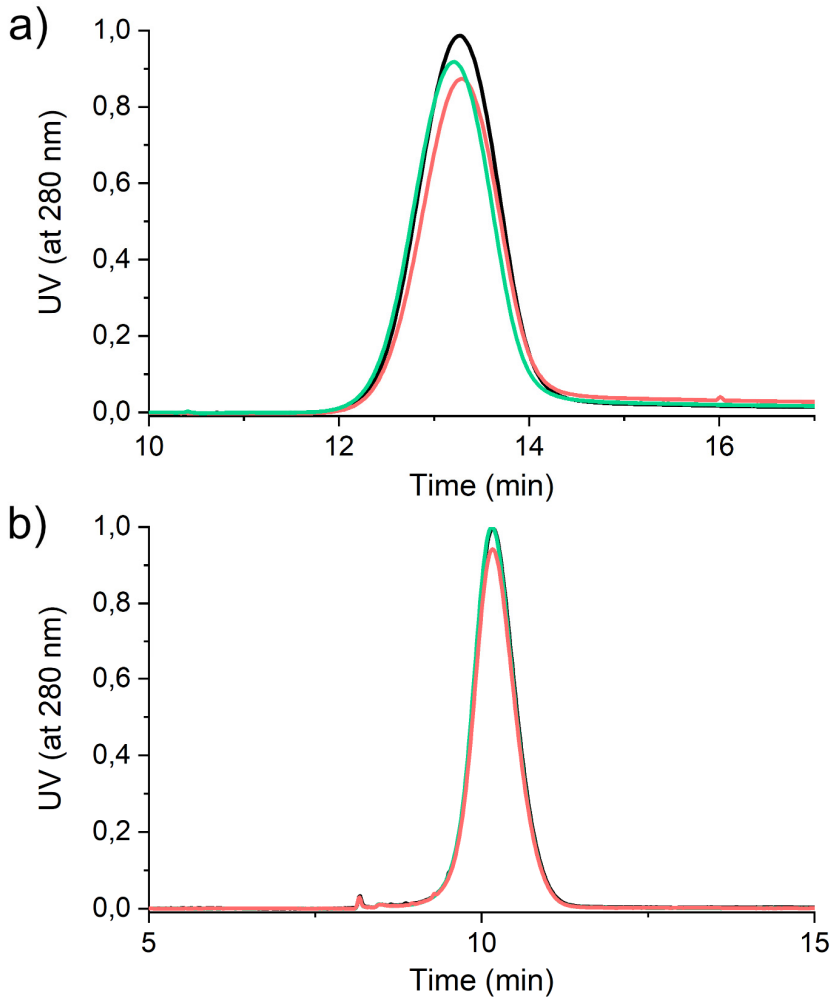

Figure 14 AF4-UV of (a) Antibody A and (b) hGH before (black) and after interaction with hydrophilic (green) and hydrophobic (red) glass beads.

As mentioned in the introduction of this chapter, surface interaction can lead to aggregation. In the glass beads experiment of Antibody A, DLS showed that aggregates were formed after the interaction, as shown in Figure 15. Hydrophilic beads seem to have a more significant effect on Antibody A aggregation, which could be connected to the protein being less prone to desorbing from the hydrophobic surface or the higher surface area of the hydrophilic beads [96].

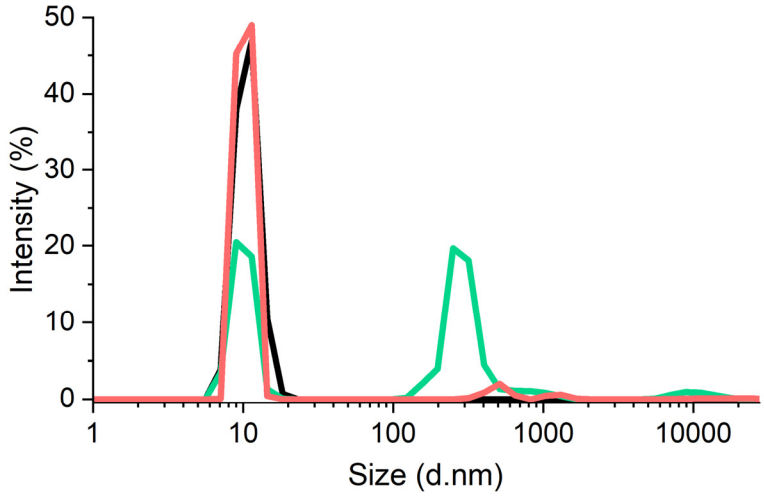

Figure 15 DLS intensity size distribution of Antibody A before (black) and after interaction with hydrophilic (green) and hydrophobic (red) glass beads.

One aspect of surface-induced aggregation is whether the protein desorbs or is exchanged from the surface and, if so, whether it has a native-like structure or is conformationally changed after interaction with the surface. Earlier experiments had shown a broadening of the peak in the AF4-LS chromatogram, indicating some structural changes after interaction with hydrophobic beads. A conformationally changed protein could trigger a non-native aggregation mechanism, as seen in Figure 13. Therefore, an attempt was made to investigate whether the interaction with the two different glass beads had triggered any significant conformational changes in the bulk solution. This was attempted using a novel method, where AF4 was coupled with SAXS [98]. SAXS can give information about the size and shape of a protein. Hence, conformational changes in a monoclonal antibody such as Antibody A could be investigated. However, monodisperse samples are essential to obtain high-quality SAXS data due to the scattering effect of larger particles [99]. This makes it necessary to have a fractionation step before measuring the Antibody A solution after the glass bead interaction, as DLS showed that aggregates had formed. The benefit of using AF4 instead of the more conventional SEC is less risk of surface interaction effects in an AF4 channel than in an SEC column [100]. The results from the AF4-SAXS are seen in Figure 16. Structural changes can be assessed by analysing the dimensionless Kratky plot [101]. However, as seen at higher q-values, the signal-to-noise ratio was high during the experiments, probably due to the dilution of the samples in the AF4, which made it difficult to evaluate the subtle changes found previously by AF4. The high noise in the scattering curve makes it difficult to tell if the molecules have an affected structure. It is also

important to mention that SAXS is a bulk method, and the result is an average of the whole fraction. So, if the concentration of conformational changed protein is low, it might be cancelled in the sample average. Therefore, based on these results, it is impossible to rule out structural changes in Antibody A after interaction with hydrophilic and hydrophobic glass beads. However, with the development of the experimental setup, AF4-SAXS could be a potential way of studying surface-induced conformational changes.

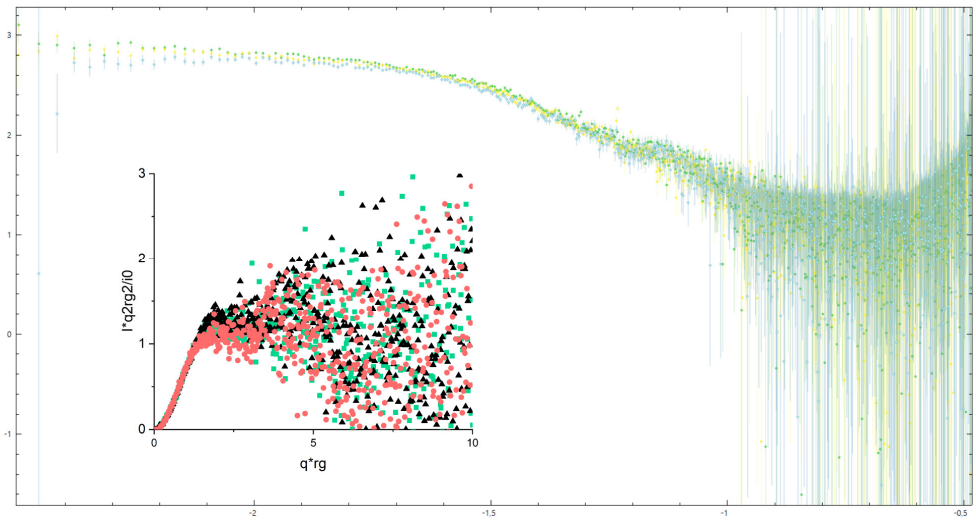

Figure 16 The SAXS curve q vs I on a double logarithmic scale and the dimensionless Kratky plot as an insert before (black dotts), hydrophilic (green), hydrophobic (red).

Antibody A adsorption on a hydrophobic surface in the presence of salts

The previous section evaluated Antibody A adsorption and aggregation with AF4 and DLS after interaction with hydrophilic and hydrophobic glass beads. Measuring the decrease in concentration is one way of assessing the degree of protein adsorption, especially when the surface area is large [102]. However, several techniques can be used to study protein adsorption behaviour in situ and the characteristics of the layer, such as thickness and structure—for example, quartz crystal microbalance dissipation (QCM-D), ellipsometry, and neutron reflectometry. QCM-D gives information about the wet-adsorbed mass through changes in frequency and information about the structure of the adsorbed layer by changes in dissipation. The decrease in frequency shows an increase in mass on the sensor when the protein is adsorbed. The degree of dissipation shift shows if the layer is ridged or flexible [103].

Figure 17 shows Antibody A adsorption to a PDMS-coated Au-sensor (paper II) as measured by QCM-D. The experiment was done with three different salts: Na_2SO_4, NaCl, and NaSCN. The anions had different rankings in the Hofmeister series. However, it was not the specific ion effects that were investigated; it was mainly a way to change the adsorption behaviour of Antibody A. Firstly, Antibody A adsorbed instantaneously after insertion in the presence of all salts. The adsorption was affected by the type of salt. The adsorption amount was lower, and the adsorption seemed slightly slower in the presence of NaSCN. At the same time, the adsorption behaviour was similar in solutions with Na_2SO_4 and NaCl. Furthermore, the layer was only partially desorbed upon buffer rinse for 30 minutes in the presence of all salts. The lower adsorption in NaSCN is probably due to the specific ion effect of SCN^-. The effect of NaSCN could be due to the interaction of SNC^- with the apolar regions of Antibody A [80]. This will reduce the net charge and, by the preferential binding, the hydrophobic interaction with the surface. The intrinsic stability is also lower for Antibody A, as seen in Figure 9 (Paper II), which could reduce the amount of adsorbed protein if it takes a more extended shape on the surface [104].

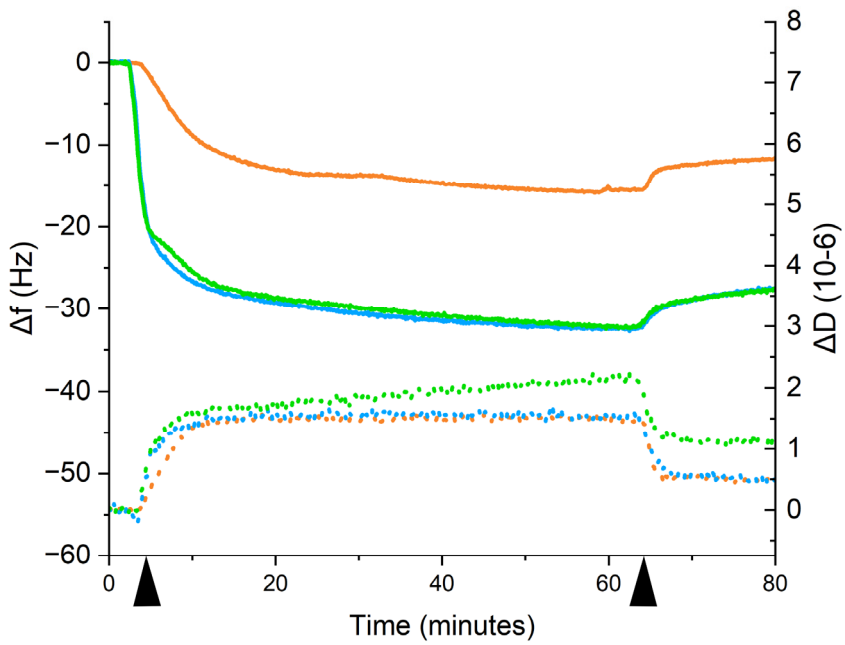

Figure 17 QCM-D adsorption experiment of Antibody A on a hydrophobic PDMS sensor in the presence of different salts, Na_2SO_4 (green), NaCl (blue), and NaSCN (orange).

Particle formation during pumping

Pumping therapeutic protein solutions has been highlighted as a critical step due to the risk of particle formation [52]. Pumping is standard when transferring liquids and can be used during manufacturing or pumps for continuous administration, such as insulin or human growth hormone. Different types of pumps, such as rotary piston pumps, rolling diaphragm pump system, and peristaltic pumps, are common. The literature describes particle formation during pumping as a surface-driven mechanism [89, 105], where the protein is adsorbed and forms a film on the surface, which then shreds off through mechanical and shear forces, showing up as particles in the bulk solution, as illustrated for peristaltic pumping in Figure 18.

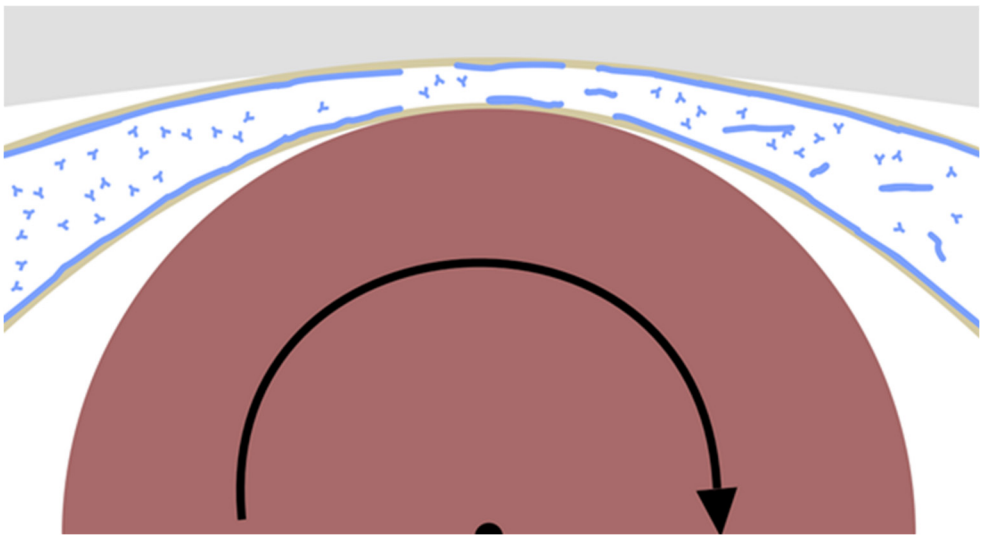

Figure 18 Schematic illustration of particle formation of a protein during peristaltic pumping.

Tubing, formulation, and protein have been shown to affect particle formation during pumping [89, 106-108]. In this thesis, the effect of pumping on hGH stability was investigated in two pHs, 6 and 7, and the impact of tubing and flow rate at pH 6 (unpublished data). This showed that hGH stability during pumping was affected by pH, as seen in Figure 19. According to the DLS measurements, larger aggregates are formed at pH 6 compared to pH 7. This is similar to the results of hGH during thermal stress in paper I. A lower pH decreases the stability of hGH (see Figure 7), and there will be less electrostatic repulsion between protein molecules as the net charge is lower at pH 6 than at pH 7 and thus affects the propensity to form large aggregates also during pumping [109].

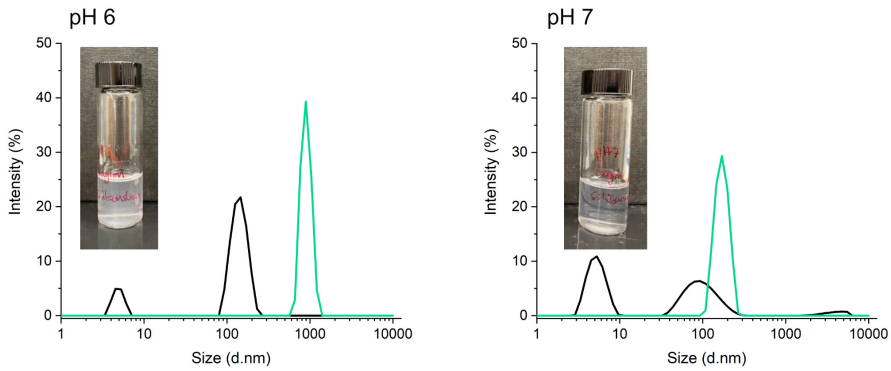

Figure 19 DLS intensity distribution before (black) and after (green) pumping hGH for 30 minutes at 2.5 ml/min.

Furthermore, hGH was pumped at two flows, 2.5 and 5 ml/min, in two different tubes: Tygon LMT-55 (PVC non-DEHP and phthalate-free) and PharMED-BPT (polypropylene-based thermoplastic elastomer). A test was also performed in another peristaltic pump with a slightly higher flow rate (6 ml/min) with the same tubings. The different combinations can be seen in Table 5. An aggregation index (A340/A280-A340) was calculated from UV-Vis measurements, as seen in Figure 20. Tubing seems to influence aggregation. This could be because of the differences in material characteristics or compression on the tubes between the pumps. The second pump was tighter over the tube, which has been shown to affect the aggregation [110]. Others have found the choice of tube material to be critical when addressing particle formation during peristaltic pumping [18, 106-108]. Furthermore, studies report differences in whether a higher or lower flow rate has more adverse effects [89, 107]. An increased flow rate might only have a small or no contribution to the particle formation, and other factors like protein, solution condition, or tube compression might have a more significant effect.

Table 5 The set-up for the hGH pumping experiment.

Tube	Flow rate (ml/min)	Pump
Tygon LMT-55	2.5	X
Tygon LMT-55	5	X
Tygon LMT-55	6	Y
PharMED-BPT	2.5	X
PharMED-BPT	5	X
PharMED-BPT	6	Y

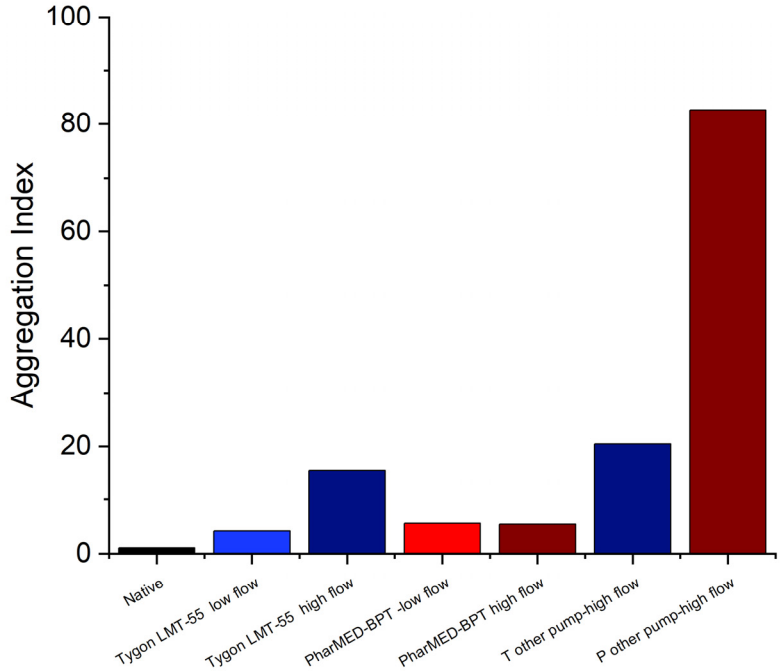

Figure 20 Aggregation index as measured by UV-vis (A340/A280-A340) for hGH before and after pumping in two different tubings Tyggon LMT-55 and PharMED-BPT at flow rates of about 2.5 and 5 ml/min. The two last bars are done using another peristaltic pump.

Furthermore, the propensity of particle formation during peristaltic pumping was further elucidated by investigating Antibody A in the presence of three different salts with anions ranking in the Hofmeister series (paper II). The reason for investigating the effect of different anions was to assess the impact of interfacial behaviour and conformational stability on particle formation during pumping. The highest number of particles was hypothesised to form in Na_2SO_4 because it was presumed to adsorb to a higher degree. However, Na_2SO_4 and NaCl were shown to have very similar adsorption behaviour, as seen in Figure 17. It should be noted that the ionic strength is not the same in these solutions, as the Na^+ concentration is double in Na_2SO_4. Some electrostatic effects could, therefore, still be present, especially for NaCl, and total screening might not be achieved until 275 mM [111]. An alternative hypothesis was that most particles would form in NaSCN due to lower conformational stability [112], as seen in Figure 9 (a). However, most particles were formed in the presence of NaCl, as seen in Figure 21 (a). Even though the particle concentration increased about 20 and 3.5 times after pumping for Na_2SO_4 and NaSCN, respectively, it did not affect particle size distribution, as seen

in Figure 21 (c). Particle formation propensity during pumping correlated with higher adsorption and attractive protein-protein interactions. Deiringer et al. highlighted the importance of protein interactions when they compared different mAbs' characteristics and evaluated their propensity to form particles during pumping [109]. The results in paper II, comparing the effect of the three salts Na_2SO_4, NaCl, and NaSCN, highlight that particle formation for a specific protein can be minimised by choice of the solution conditions, minimising adsorption and attractive protein-protein interactions. This is especially important as NaCl is a common additive to adjust isotonicity or solubility.

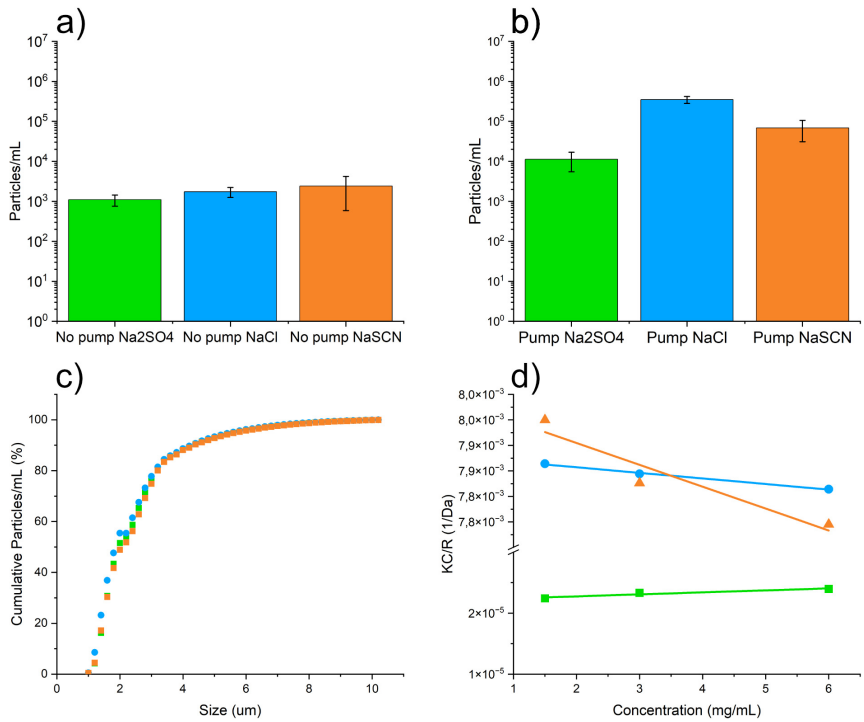

Figure 21 The total particle concentration as measured by FIM before (a) and after (b) pumping (OBS logarithmic scale y-axis), the cumulative particle concentration (%) 1-10 µm, and d) the estimation of the direction of the second virial coefficient B22 from the slope.

Storage Stability

Pharmaceutics should be stable over several years and in a broad temperature span. Traditional small molecule drugs are more commonly produced as dry formulations, typically more stable than liquid formulations. However, as mentioned before, most therapeutic proteins need to be administrated parenterally or intravenously, making liquid formulations necessary at some point. Stability screening of drug candidates and formulations is typically done during development by assessing the structural, colloidal, and interfacial stability [113]. Forced degradation can be relevant to evaluating stability, which is done by inducing harsh conditions like high temperatures or by shaking, stirring, or changing pH [114]. Accelerated stability tests assess short-term effects and are usually performed at sub-optimal temperatures like 25-45 °C. Long-term stability studies are typically done at the end of development to ensure the stability and quality in the intended storage condition.

Different protein characteristics, such as thermal stability or protein interactions, can sometimes be used to predict long-term stability [115].Table 6 shows some characteristics of Antibody A in various formulations. When assessing these parameters, the most stable option for Antibody A seems to be the 25 mM histidine-HCl buffer or with the addition of PS20, while presence of NaSCN lowers the stability.

Table 6 Characteristics that can be linked to degradation of protein.

Condition	Relative thermal stability 1-5 (Tonset×Tm1)	Self-association propensity during heating	Self-association propensity at 25 °C	Absolute heat capacity at 25 °C
25 mM hist-HCl	1	Yes	No	0.24
+ 150 mM Na_2SO_4	3	Yes	No	0.31
+ 150 mM NaCl	3	Yes	Yes	0.32
+ 150 mM NaSCN	4	No	Yes	0.33
+ 0.1 % PS20	2	Yes	No	0.24

Seeding

Previous sections have discussed stability and aggregation during short-term forced degradation conditions by means of heat or pumping stress of hGH and Antibody A. The potential that a protein solution could be mishandled to trigger aggregation, and then stored raises the question of whether pre-formed aggregates could accelerate aggregation. In paper III, the effect on stability and aggregation for these two proteins was assessed in the presence of pre-formed aggregates induced by heat at different storage temperatures: 4 °C, RT, and 40°C for two weeks. The investigation continued for Antibody A in paper IV where it was expanded to other formulations and seeding with different types of pre-formed aggregates (heat or pump) during static or agitated storage for up to twelve weeks.

Aggregates and particles should be minimised in production and handling due to the higher risk of immunogenic reactions and potential decrease in efficacy. However, producing small amounts of reactive species during production, transportation, storage, or administration preparation could accelerate protein degradation. This mechanism can be described as seeding. Seeding is often divided into heterogeneous or homogeneous seeding. Sheds from process equipment like tubing or metal surfaces could induce heterogeneous seeding [116]. Heterogeneous seeding has been shown, for example, for a pharmaceutical IgG in the presence of steel nanoparticles and recombinant human platelet-activating factor acetylhydrolase with hydrophilic silica nanoparticles [116, 117]. Homogeneous seeding could be unfolded protein species or aggregates acting as nuclei. Homogeneous protein seeding is an established mechanism for several neurodegenerative diseases like Alzheimer's and Parkinson's, where initial misfolded proteins form a seed that triggers amyloid formation [118-121]. However, the potential of small reactive protein aggregates in solutions may also be of concern. One study found that dimers of mAb formed at different temperatures, 37 or 50 °C induced a higher degree of aggregation when mixed with untreated mAb during storage at 37 °C for one year [48]. They also saw a difference in reactiveness depending on the temperature

during the formation of the dimers. The dimers had different structures depending on whether they were present in the native solution or formed at 37 or 50 °C, as determined using SEC-SAXS. Furthermore, the structure seemed to affect how reactive they were towards inducing aggregation. However, another study found that aggregates induced by heat or shaking stress did not seem to accelerate the degradation of a mAb during storage [62]. Another study found that there might be a threshold for heat-induced aggregate seed formation in high molecular weight species for one of the mAbs they investigated [122]. In this thesis, seeding effect on stability of hGH and Antibody A was investigated in terms of different formulations, storage temperature and aggregates in paper III and IV.

Seeding by Heat or Pump-induced Aggregates

In papers III and IV, hGH and Antibody A were heated in water baths to induce aggregation. After heating, small aggregates in the hundred-nanometre range and micron-sized particles formed in both protein solutions. In paper IV, in addition to the heat-induced aggregation, particles were induced by pumping in different formulations (histidine-HCl buffer, + 150 mM NaCl, or + 0.1 % PS20). 5 vol% of the heated solutions (paper III and IV) and 10 vol% of the pumped solution (paper IV) were added to the untreated solution. Both types of seeded samples contained micron-sized particles, and the heat-seeded ones also contained small aggregates seen by DLS. The seeded samples with the pump-induced particles contained significantly fewer particles than the heat-seeded samples but slightly more than the untreated sample.

In papers III and IV, we could see a potential seeding effect for the small heat-induced aggregates of hGH and Antibody A after storage for 2-12 weeks. The aggregates seemed to either increase in size or amount based on the peak size or area increase measured by DLS. As a two-step mechanism with small elongated aggregates growing by entanglement into larger aggregates was found for hGH during heating in paper I, it could be believed that the small aggregates continue to interact but at a lower rate when diluted and stored at lower temperatures. The seeding effect of Antibody A heat-induced aggregates was shown to be affected by formulation in paper IV. For Antibody A, PS20 reduced the impact, while NaCl seemed to accelerate the aggregation compared to no additive. Higher ionic strength can significantly affect the aggregation propensity by screening electrostatic interactions [46, 123, 124]. Meanwhile, surfactants can hinder protein interactions through complex formation with the protein, stabilising the protein structure or creating steric hindrance for protein-protein interaction [125].

In contrast to the small heat-induced aggregates, the micron-sized particles formed by heating (hGH and Antibody A) or pumping (Antibody A) did not show the same seeding tendency during the studied conditions. Instead, the micron-sized particle concentration did decrease or not significantly change during more extended storage

of 12 weeks (paper IV). The formulation did not seem to make a difference in the behaviour of the micron-sized particles during static storage, considering that the initial particle concentration differed. The decrease in total particle concentration could be caused by aggregate-aggregate interaction (growth in size). However, there was no apparent correlation with a shift in size distribution, so this is not the case for most conditions. This indicates that a decrease in micron-sized particles might partly be due to the dissolution of particles, which could be driven by chemical degradation, changing the electrostatics or hydrophobicity characteristics of the proteins.

Others have investigated the effect of seeding on mAb after pumping [90, 126]. The heterogeneous seeding is interesting because tubing or inside of other pump types have been demonstrated to shed particles during pumping. However, these studies focused on heterogeneous seeding, where they incubated protein solutions with polystyrene micro-particles or pumped buffer, which showed no seeding effect on protein aggregation.

Our studies show that heat-induced small aggregates and micron-sized particles behave differently during storage. Due to their perturbed structure, the small heat-induced aggregates may be more prone to act as seeds for continued aggregation. At the same time, the micron-sized particles formed by pumping might be more native-like, hence less reactive seeds.

Storage temperatures

Therapeutic protein often needs to be stored at lower temperatures, frozen or refrigerated (4-8 °C), to reduce the rate of chemical and physical degradation. Accelerated stability studies can often be performed at sub-optimal temperatures or thermal stress conditions to investigate protein stability [127]. However, aggregation behaviour at higher temperatures does not always extrapolate to the behaviour at lower temperatures [128]. For the two proteins hGH and Antibody A studied in this thesis, a higher temperature of 40 °C did not promote a higher degree of micron-sized particle formation as detected by FIM (paper III); see Figure 22. We could not see a significant decrease in monomeric content for either of the two proteins at a higher storage temperature using SEC. 40 °C did promote the formation of oligomers and a higher amount of undefined small aggregates of hGH. However, the presence of oligomers did not change for Antibody A (paper III). This highlights the protein and solution-specific characteristics affecting stability. The higher storage temperature was also connected to a higher degree of structural degradation for both proteins (paper III). Chemical degradation was not evaluated in this study but could play a part in forming aggregates. Chemical degradation routes like deamidation or oxidation could cause changes in electrostatics or fragmentation,

affecting the interactions of proteins. Chemical degradation is often accelerated at higher temperatures, which might explain the changes in stability and behaviour of aggregates and particles at 40 °C [129].

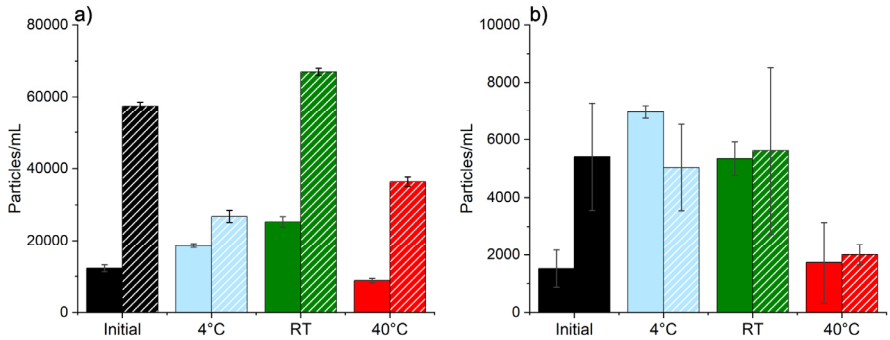

Figure 22 Change in micron-sized particles as measured by FIM during two weeks of storage at 4 C, RT, or 40 C for (a) hGH and (b) Antibody A. Solid bars are the untreated protein solution, while striped bars are sample seeded with 5% heated solution. The error bars are the standard deviation of 3 replicates x 1 sample (hGh) and 3 replicates x 2 samples of two samples (Antibody A).

Agitation

Agitation adds another complex factor to storage stability, which generates air-water disruption. This was investigated in paper IV for Antibody A in different formulations, with or without pre-formed aggregates. The air-water interface is critical for protein aggregation. The effect of the air-water interface has been studied extensively as it is unavoidable but considerably damaging to protein stability. Studies have investigated the influence of temperature, shear, time, protein concentration, and air-water interface area on protein aggregation [130]. There are different ways to study the air-water interface, such as bubbling, shaking, vibrating, etc. Interference of an air-water interface introduces renewal or compression and dilation of the interface, which generally leads to a higher amount of aggregation and a higher effect on protein stability due to the increased surface area [49, 131, 132]. However, most studies seem to have a shorter time frame during agitation in combination with air-water interface than the twelve weeks followed in paper IV.

In paper IV, the micron-sized particles increased during twelve weeks of agitated storage of Antibody A in 25 mM histidine-HCl. In the presence of NaCl or PS20, particle concentration reached a maximum, after which it decreased. The micron-sized particles were also significantly lower throughout the storage time in NaCl and PS20 compared to pure 25 mM histidine-HCl buffer. In addition to the air-water

interface, this system has a glass-water interface and a three-phase line between the glass-water and air. The interchanging of the three face-lines seems specifically damaging for Antibody A in the pure histidine-HCl compared to when NaCl or PS20 is present. Glass is typically negatively charged in pH 6. In contrast, Antibody A has a net positive charge at pH 6 (IP 8.8). This means that Antibody A has an electrostatic attraction to the glass surface. DLS showed that aggregates had formed after interaction with hydrophilic glass beads (removing the air-water interface) detected by DLS, as seen in Figure 14. The use of beads maximised the surface area, which allowed for an accelerated effect, reducing the interaction time to 24 hours. However, it indicates that the glass surface in the vial might be an additional stress factor in this system. This makes sense when considering the behaviour in NaCl. The addition of NaCl screens the attractive electrostatic range between Antibody A and the glass surface and decreases the particle formation during agitation in this system. PS20 is known to compete competently adsorb to surfaces, giving protection for surface-induced aggregation.

Seeding with pre-formed micron-sized particles generated a maximum or a plateau of micron-sized particles in all formulations during agitation. In general, this indicates that the micron-sized particles are formed and dissolved simultaneously [62]. The heat-seeded samples had small aggregates, as visible by DLS, which were unaffected by the additional agitation. This difference indicates that the small heat-induced aggregates follow a bulk-mediated pathway, while a surface-mediated pathway might drive the formation of micron-sized particles [133]. Figure 23 shows the suggested passage of events of the small heat-induced aggregates and the surface-induced micron-sized particles.

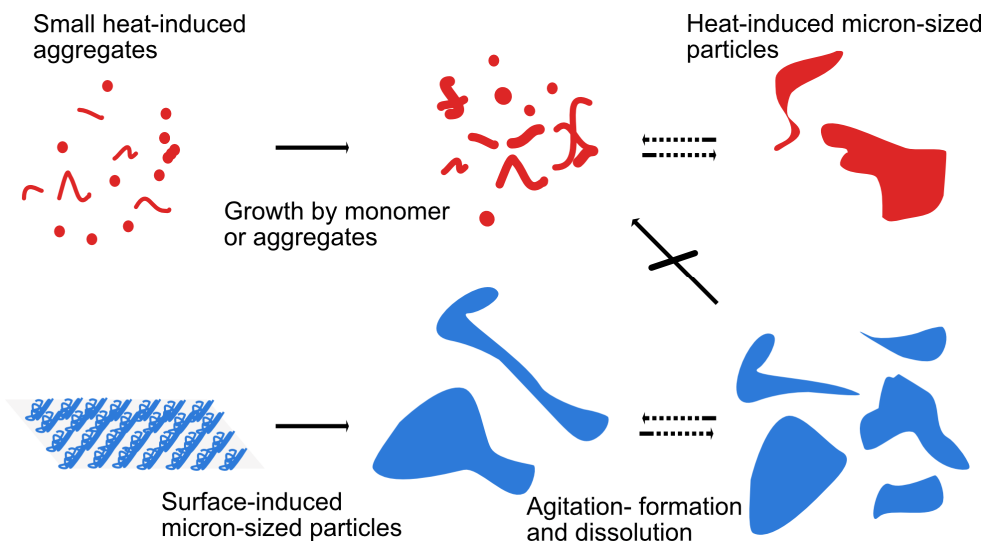

Figure 23 A schematic illustration of what could be the passage of events for small aggregates and micron-sized particles during agitated storage.

Conclusions

This thesis aimed to improve knowledge about the physical and aggregation behaviour of therapeutic proteins in liquid formulations. Some of the primary conclusions from this work are highlighted here.

Using MiniTEM, a bench-top nanometer-resolution image technique, and image analysis of the size and shapes of the monomer and aggregates made it possible to interpret the measurements of size and concentration (DLS, AF4) to elucidate the heat-induced aggregation of hGH. This highlights the power of visualisation in studying protein aggregation mechanisms.

Using different salts in the Hofmeister series, it was found that particle formation propensity during peristaltic pumping was connected to both the interfacial and bulk solution stability of Antibody A. Furthermore, due to the specific ion effects, Antibody A was found to be least stable during pumping in the presence of NaCl. This is an interesting fact as it is a common additive in therapeutic protein formulations.

The evolution of small heat-induced aggregates appeared disconnected from micron-sized particles from the same protein. Agitation did not affect the small aggregates as it did for micron-sized particles. The small aggregates seemed to depend on colloidal or conformational stability of the protein. At the same time, the micron-sized particles seem to form and dissociate/degrade simultaneously during agitation, and the formation is correlated to protein interfacial interactions. This highlights the importance of different formulation approaches to mitigate the formation of varying aggregate populations.

To summarise, studying aggregation by different combinations of stress factors and formulations of two therapeutic proteins has contributed to an improved understanding of physical stability and protein aggregation. These findings will hopefully give more insights into the continued research and development of therapeutic protein formulations.

Future aspects

This thesis investigated different aspects of protein aggregation.

The pumping results show that the particle formation propensity is linked to interfacial and colloidal stability. As sodium chloride seemed to initiate the perfect storm for Antibody A, it would be interesting to expand this matrix to include other proteins to see if the interfacial and colloidal stability affects particle formation similarly. This would give more knowledge about the interplay between bulk and interfacial behaviour concerning aggregation propensity during pumping and specific ion effects.

It would be interesting to confirm the dissolution of the micron-sized particles and investigate the protein function and structure more carefully to assess the possible effect particle formation and dissolution would have in long-term storage. This would give more knowledge about the impact of a protein drug product that had accidentally been mishandled. Furthermore, verifying the potential seeding effect of small heat-induced aggregates would be interesting, revealing whether it includes growth by monomers or is only aggregate-aggregate growth.

Studying aggregate and particle formation in combination with chemical degradation, like deamidation, would be beneficial in further elucidating the mechanistic understanding of aggregate dissolution or seeding effect. Including image analysis of DLS ranges with TEM would help clarify the mechanical aspect of the aggregation indicated by the DLS results. However, adjustments to the experimental set-up and sample preparation would be needed to achieve high enough concentrations of aggregates.

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