Novel Approaches for Stabilization and Characterization of Therapeutic Proteins in Liquid Formulations

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Erklärung


Eidesstattliche Versicherung

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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD(s)</td>
<td>Cyclodextrin(s)</td>
</tr>
<tr>
<td>C$_{12}$DMPO</td>
<td>Dodecyldimethylphosphine oxide</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micellar concentration</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>EDC</td>
<td>N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>FNU</td>
<td>Formazine nephelometric units</td>
</tr>
<tr>
<td>FTIR-spectroscopy</td>
<td>Fourier-transformed infrared spectroscopy</td>
</tr>
<tr>
<td>GCSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>HES</td>
<td>Hydroxyethyl starch</td>
</tr>
<tr>
<td>HES-NTA</td>
<td>Coupling product of HES with NaNO$_2$-Bis(carboxymethyl)-L-lysine hydrate</td>
</tr>
<tr>
<td>hGH</td>
<td>Human growth hormone</td>
</tr>
<tr>
<td>HMDA</td>
<td>Hexamethylenediamine</td>
</tr>
<tr>
<td>HPβCD</td>
<td>Hydroxypropyl-beta-cyclodextrin</td>
</tr>
<tr>
<td>HP-SEC</td>
<td>High performance size exclusion chromatography</td>
</tr>
<tr>
<td>IFN-β-1b</td>
<td>Interferon-β-1b</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>KGF-2</td>
<td>Keratinocyte growth factor-2</td>
</tr>
<tr>
<td>LED</td>
<td>Light-emitting diode</td>
</tr>
<tr>
<td>mAbs</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>MβCD</td>
<td>Methyl-beta-cyclodextrin</td>
</tr>
<tr>
<td>MBPM</td>
<td>Maximum bubble pressure method</td>
</tr>
<tr>
<td>MS</td>
<td>Molar substitution</td>
</tr>
<tr>
<td>$M_w$</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
</tr>
<tr>
<td>NaBH$_3$CN</td>
<td>Sodium cyanoborohydride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NTA</td>
<td>N&lt;sub&gt;α&lt;/sub&gt;N&lt;sub&gt;α&lt;/sub&gt;-Bis(carboxymethyl)-L-lysine hydrate</td>
</tr>
<tr>
<td>PAT</td>
<td>Profile Analysis Tensiometer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PES</td>
<td>Polyethersulfone</td>
</tr>
<tr>
<td>Ph.Eur.</td>
<td>European Pharmacopoeia</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>Rh-GCSF</td>
<td>Recombinant human granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>SBEβCD</td>
<td>Sulfobutyhether-beta-cyclodextrin</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SLS</td>
<td>Static light scattering</td>
</tr>
<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Protein melting temperature</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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Chapter I

General Introduction

1. Introduction

Therapeutic proteins exhibit fascinating features and opportunities, and represent a promising substance-class for advanced pharmacotherapy. Research in the field of recombinant proteins goes back to the early 1970s, when the biotechnological techniques originated. [1] By now, biopharmaceuticals - which are mainly proteins [2] - get more and more important in the treatment of several severe diseases such as diabetes, cancer, haematological disorders, and others. [1] Beside the application for vaccination and diagnostic purposes, recombinant proteins are used to substitute lacking endogenous substances such as insulin and growth hormones. [3] Additionally, the group of monoclonal antibodies possesses highly selective and efficient targeting properties, which are beneficial in the treatment of various diseases. [3] Currently, about 30 monoclonal antibodies are already approved by the FDA, and 50 new antibodies were reported to enter clinical studies solely in 2008. [3, 4]

In addition to unique benefits and opportunities, protein pharmaceuticals implicate several challenges which have to be taken in consideration, and which lead to spiraling development costs. [3, 5, 6] Here, the route of administration represents one big challenge, since proteins are mainly delivered intravenously or subcutaneously due to their low bioavailability by other routes. [7, 8] Since both application routes exhibit some discomfort for the patients, novel administration techniques are currently being developed such as nasal or transdermal systems. [7, 9] A second issue upon therapy with biopharmaceutics is the induction of immune responses after administration of an exogenous protein [8, 10, 11] which could be reduced - but not eliminated - by the use of humanized proteins. [5] For the formulation scientist, the propensity of proteins for various instabilities represents the greatest challenge, since proteins undergo different chemical and physical degradation routes. [12, 13] Currently, several chemical instabilities are described for proteins such as deamidation as predominant chemical instability, oxidation at specific side chains, formation and breakage of disulfide bonds, isomerization, hydrolysis, and many others. [12, 14] The most predominant physical instability is the phenomenon of protein aggregation which will be discussed in detail in the next section. [12, 15]
2. Protein aggregation

The phrase “protein aggregate” is a collective term which stands for a highly heterogeneous group of protein assemblies starting from small, soluble oligomers in the nanometer-range right up to visible particles with a size of several hundreds of micrometers. [16, 17] Protein aggregates can be further categorized regarding their morphology which ranges from compact spherical shapes to elongated fibril-formations. Additionally, homogeneous and heterogeneous aggregates are described dependent on the contribution of extrinsic molecules to the protein assembly. While homogenous aggregates consist of similar extrinsic molecules, heterogeneous particles contain for instance silicone oil or stainless steel particles which act as precursor for further aggregation processes. [16, 18]

2.1 Relevance of protein aggregates

Prevention of protein aggregation represents an important task in the formulation of marketable protein pharmaceuticals, since aggregates show a reduced bioactivity which compromises their therapeutic success. [13] In the case of human calcitonin, aggregation-resistant mutants yielded in a higher physiological activity. [19] The reduced bioactivity of protein aggregates is ascribed to their loss of native protein structure which is known to reduce the affinity to the target-structure. [20]

Furthermore, the increased immunogenicity of protein aggregates represents a great challenge, since safety of the therapy may be endangered. [12, 13, 21] Up to now, a couple of studies were reported which demonstrate a linkage between the presence of aggregates and an increase in immune response. [22] Recently, aggregates of a monoclonal antibody with different size, structure, and morphology were compared regarding their immunogenic potential in wild-type mice. The results demonstrated that the application of aggregates lead to a significant higher immune response compared to the application of native protein, whereas the greatest reaction was observed for insoluble aggregates. [23] At present, there is a need for novel methods to elucidate the origin and immunogenic potential of therapeutic proteins, since counting and sizing of protein particles was shown as not sufficient to predict the general safety of biopharmaceutical products. [24, 25]
2.2 Mechanism of protein aggregation and influencing factors

The process of protein aggregation can proceed along different routes. Philo et al. reported five general mechanisms for protein aggregation which are (1) the reversible association between native monomers, (2) aggregation after conformational changes in the monomer conformation, (3) chemical protein alterations, which facilitate the attachment between proteins, (4) the presence of foreign particles such as silicone oil droplets or stainless steel particles, which act as nuclei for protein aggregation, and (5) surface-induced aggregation which implicates previous protein unfolding. [26] With the exception of the first mechanism, all other degradation routes comprise an initial change in the native protein conformation, where the “activation” of a monomer is often held as prerequisite for further aggregation processes. [15, 27]

Various stress factors are reported to induce conformational changes in the monomer structure - most notably partial unfolding - which subsequently triggers protein aggregation. [26, 28] Elevated temperature is one critical factor regarding protein stability, since increased temperatures provoke (partial) protein unfolding and the exposure of hydrophobic side chains on the protein surface. This increase in hydrophobicity leads to an increase in intermolecular protein-interactions which often results in the formation of aggregated species. [28, 29]

Exposure of the protein to different surfaces represents the second important stress-type due to the surface activity and unfolding-tendency of proteins upon contact to various interfaces. [30-32] These interfaces can be generated by the presence of undesired impurities such as silicone oil or stainless steel which was demonstrated to generate significant protein aggregation. [33, 34] Additionally, the air-water interface itself represents a dominant factor for aggregation-processes, and a constant compression and expansion of the surface area - which also occurs during shaking stress - was demonstrated as origin for the surface-induced degradation. [35] Also the propensity of proteins for aggregation during freezing is partially ascribed to the growth of ice-water interface. [36]

Besides physical reasons, also chemical alterations in the native monomer molecule are discussed as nucleating structure for further degradation and also aggregation processes. [37] Such chemical variations can be induced by a variety of triggers, such as the presence of oxygen or metal ions, which lead to protein oxidation [12]. Furthermore, light exposure is known to modify susceptible amino acid side chains [38], and the presence of additives, such as polysorbates for stabilization-purposes, can initiate peroxide formation and protein-oxidation processes. [39] These chemical alterations were considered as origin for protein aggregation since they can induce changes in hydrophobicity, three-dimensional structure, and a change in the barrier of protein unfolding. [15] A correlation between chemical alteration and protein aggregation was shown by Krishnan et al. where the oxidation-induced
formation of covalent dimers represented the rate-limiting step in fibril formation of α-synuclein. [40] Additionally, deamidation of a lens protein was identified as trigger for protein instability and aggregation. [41]

2.3 Stabilization of liquid protein formulations

Stability of proteins in liquid formulations is affected by various parameters such as solution pH, buffer strength and composition, protein concentration, or structural protein-modifications, for instance by glycosylation and PEGylation. [12, 42] The addition of suitable additives plays a major role in protein stabilization during formulation development. In general, stabilization of proteins against aggregation can be achieved by several mechanisms, such as (1) the preferential hydration / preferential exclusion phenomenon, which leads to a compact native protein structure and which is achieved by the addition of amino acids and sugars, (2) preferential binding and electrostatic shielding of the protein by particular salts, (3) steric hindrance of protein-protein interactions in the presence of polymers, or (4) competitive adsorption of surfactants to surfaces inhibiting surface-induced aggregation processes. [12, 43, 44]

To this end, addition of sugars and surfactants represents the most commonly applied approach to prevent the protein from aggregation [42, 45]; however, both substance-classes exhibit some drawbacks which limit their applicability in protein formulations. Sugars such as sucrose or trehalose are known to stabilize proteins by the preferential exclusion mechanism which was explicitly described by Tiamsheff et al. [46, 47] Preferential exclusion of solutes lead to a compact, native protein conformation, which goes along with an increased protein stability during quiescent long time storage and under thermal stress. [48, 49] However, sugars are ineffective in stabilizing proteins against mechanical stress and surface-induced damage. [48, 50] By contrast, surfactants such as polysorbates are highly effective in displacing proteins from surfaces [51] which protects them from surface-induced degradation and mechanical stress. [48, 50] However, the polyethylene glycol structure in the polysorbate-molecule also leads to the formation of peroxides which triggers protein oxidation and further protein-aggregation processes. [39, 52]
3. Highly concentrated protein formulations

Therapy with monoclonal antibodies often requires high doses in frequent applications, which leads to some discomfort for the patients due to the high infusion-volume. For this reason, formulation of antibodies as highly concentrated solutions would enable a more convenient application together with lower costs for storage and shipping. [45, 53] Therefore, the development of antibody formulations in a concentration right up to more than 100 mg/mL is of great interest, but an increase in protein concentration also implicates several alterations and challenges compared to dilute formulations. [54] These phenomena and challenges are explained in the next subsections.

3.1 The phenomenon of macromolecular crowding

"Macromolecular crowding" or the "effect of excluded volume" describes the conditions in highly concentrated solutions of macromolecules, where a large fraction within the solution is occupied by the macromolecules itself, and the limited amount of available solvent notably affects the properties of the present macromolecules. [55, 56] By this, crowded media are distinguished from concentrated media as "crowded" means the presence of one solute in a concentrated solution of a different solute, and "concentrated" means the presence of one single solute in a high concentration. [57] Despite that difference, the well-studied properties of proteins in crowded solutions are very helpful to better understand the phenomena in highly concentrated protein formulations.

In crowded environments, proteins are affected regarding their kinetics in unfolding, self-association, as well as aggregation. [55] By this, crowding diminishes the rate of protein unfolding and the protein is held in a compact conformation since unfolding of proteins would occupy a larger, not available space. [55, 58] For instance, increased conformational protein stability – as observed by higher T_m-values – was reported for phosphoglycerate kinase in the presence of 50 to 200 mg/mL of the crowding agent Ficoll. [59] Similar to crowded media, an increase in the conformational protein stability was also seen in a concentrated antibody solution where the higher protein melting temperature was ascribed to the excluded volume effect. [60] However, this thoroughly desired effect is mitigated by the observation that if a protein is unfolded in a crowded environment, it might be rapidly forced to a compact non-native conformation, which could initiate further degradation routes. [55]

Crowded media have a varied effect on intermolecular protein association, whereas rapid and slow association processes have to be considered separately. In general, rapid associations are based on intermolecular protein encounters which are diffusion-limited and
therefore reduced in highly concentrated media. By contrast, slow associations which require a transition-state are generally reaction-controlled and show an increase in crowded environment. [55, 56, 61] Rate of association is furthermore determined by the structure of the resulting di- and oligomers since only the formation of compact protein assemblies is favored by crowding. [56] Therefore, macromolecular crowding favors an increase in the formation of compact and well-structured aggregates such as fibrils due to the reduction in excluded volume [62] whereas unstructured, amorphous aggregates are not fostered. [55] Placing non-native proteins into crowded environments leads to significant protein aggregation which was observed for instance in the case of unfolded lysozyme, and the fast aggregation process prevents a correct refolding. [63]

3.2 Challenges in high concentration protein formulations

There are various differences between low and high concentrated protein solutions, which implicate several challenges and hurdles to overcome in the formulation of proteins at high concentrations. [54] In the next section, three main factors will be discussed in more detail, namely the influence of high concentration on protein stability, solution viscosity, and the lack of suitable, established analytical techniques.

3.2.1 Protein stability

The influence of high protein concentrations on conformational stability is controversially discussed. As already mentioned above, unfolding of the protein is decelerated in highly concentrated formulations due to the limited, available space. [60] This was also shown by thermal analysis of hemoglobin and fibrinogen in different concentrations where the conformational stability was significantly enhanced in the presence of high protein concentrations. [64] By contrast, lysozyme and BSA showed conformational destabilization with increasing concentrations, which demonstrates that various factors such as surface polarity and association kinetics contribute to the conformational protein stability, and protein stabilization by the effect of excluded volume cannot be generalized. [64]

Besides conformational stability aspects, association and aggregation plays an important obstacle in high protein concentrations since a higher rate of bi-molecular collisions is expected to increase intermolecular protein association and subsequent aggregation. [54, 65] The higher number of intermolecular encounters in concentrated solutions is in contrast to the situation in crowded media where the presence of crowders diminishes protein-protein interactions. [55] In concentrated protein solutions, a direct correlation between protein
concentration and rate of aggregation was observed after quiescent long-time storage of different protein formulations over several weeks where an increase in protein concentration led to a significant higher aggregation rate. [66] Interestingly, agitation induced aggregation was markedly reduced with higher protein concentrations which shows that here the ratio of air-water interface to the protein acts as determining factor for the protein aggregation rate. In general, there are several driving forces which foster protein-protein interactions, and the contribution of these forces is significantly affected by the respective protein concentration. In dilute formulations, intermolecular interactions are mainly caused by electrostatic and hydrophobic interactions, but with increasing protein concentration, hydrogen bonding, van der Waals forces, and steric effects due to excluded volume essentially arise. [65] A recently published study investigated the driving forces for protein-protein interactions and aggregation processes in dilute and concentrated protein solutions. Here, protein aggregation of a dual-variable domain immunoglobulin was ascribed to electrostatic interactions in the case of dilute protein solutions whereas predominant hydrophobic short-range forces caused the aggregation within a highly concentrated environment. [67]

3.2.2 Formulation viscosity

Besides the influence on protein stability, a higher rate of intermolecular protein association significantly contributes to a rise in solution viscosity. [65, 68] For a highly concentrated monoclonal antibody formulation, the origin of protein-protein interaction and viscosity-increase was ascribed to multiple associations between Fab-fragments. [69] Since the highest viscosities are obtained for buffer pH-values close to the isoelectric point of the protein, electrostatic attractive interactions were mainly hold responsible for the viscosity-increase. [68, 70]

An increased formulation viscosity drastically limits the syringeability and applicability for subcutaneous administration via thin syringe needles. [54, 71] Additionally, high viscosity can impose problems during processing, such as during diafiltration or filling. Therefore, the development of reduced-viscosity highly concentrated protein solutions represents an important task for the formulation scientist. Since the viscosity-increase is ascribed in many cases to electrostatic forces, reduction of viscosity can be achieved by the addition sodium chloride or chaotropic anions such as thiocyanate which leads to a disruption of the intermolecular protein network. [54, 68, 69]
3.2.3 Analytical characterization

A third big issue is the analytical investigation of highly concentrated protein formulations since a multitude of techniques, which are applied in dilute formulations, are not directly applicable at high concentrations without prior sample dilution. [37, 54] Reasons for inapplicability are a) overloading of the system such as for liquid chromatography, light obscuration, UV-spectroscopy, fluorimetry, or SDS-PAGE, b) unacceptable sample volume consumption such as for light obscuration, and c) the presence of intermolecular protein interactions, which prohibit correct data interpretation such as for light scattering techniques. Since sample dilution to lower concentrations does not resemble the “natural state” within the original highly concentrated solution, resulting data might contain some failures which impede correct interpretation. [60]

Currently, there are several approaches to overcome the lack in analytical methods. By this, low volume techniques are required to keep sample consumption and development costs at an acceptable minimum. Several studies are reported which investigate the colloidal and conformational protein stability directly within the highly concentrated state, for instance by FTIR-spectroscopy, variable pathlength UV-spectroscopy, UV- and circular dichroism spectroscopy by using short path length cells, front surface fluorescence spectroscopy in triangular cuvettes, ultrasonic rheology, and viscosity measurements on a low volume viscometer. [60, 64, 72-75] However, there is still a lack of methods which are suitable to directly determine actual aggregates within the highly concentrated state.
4. Protein stabilization by cyclodextrins and polymers

As beforehand discussed, addition of suitable excipients represents the main-approach to achieve satisfying protein stabilization in liquid formulations. By this, different cyclodextrins and polymers were regarded as promising stabilizing agents [12, 28, 44, 76], and the properties of both substance classes as well as their influence on protein stability will be more precisely described in the next section.

4.1 Protein-cyclodextrin formulations

4.1.1 Structural characteristics of cyclodextrins

Cyclodextrins are circularly shaped oligosaccharides composed of six (α-cyclodextrin), seven (β-cyclodextrin), eight (γ-cyclodextrin), or rarely more α-(1,4) linked glucopyranose subunits which are obtained after degradation of starch by the cyclodextrin glycosyl transferase enzyme. [77] The ring-structure of the cyclodextrin-molecule implicates a three-dimensional shape of a truncated cone where skeletal carbons are present in the interior, and the hydroxyl groups are outwardly oriented. [77, 78] This special molecule assembly leads to a polarity-difference between the cyclodextrin cavity (which is hydrophobic) and shell (which is hydrophilic). [78] A schematic picture of natural β-cyclodextrin is illustrated in Figure I.1.

![Figure I.1: (A) Chemical structure and (B) three-dimensional configuration of native β-cyclodextrin as taken from [79].](image-url)
Non-substituted, natural cyclodextrins are crystalline and exhibit a remarkable low water solubility which is ascribed to the high crystal energy, and - especially in the case of β-cyclodextrin - the formation of strong intramolecular hydrogen bonds. [80] By contrast, substitution of hydroxyl-groups with variable substituents causes amorphous derivatives with notably increased solubility. [78, 80]

Regarding the toxicological profile of cyclodextrins, different properties are reported based on the respective substitution pattern and the route of application. In general, the size and polarity of hydrophilic cyclodextrins prevent them from crossing biological membranes which leads to a negligible systemic absorption after oral administration, and facilitates their use in oral dosage forms. For orally applied hydrophobic cyclodextrins, a very slow and almost negligible diffusion through the intestinal wall was shown. [80, 81] By contrast, native β-cyclodextrin shows a high affinity to cholesterol and phospholipids which causes extraction of these molecules from membranes, and explains the high hemolytic activity of β-cyclodextrin after parenteral application. [76] Furthermore, the formation of cyclodextrin-cholesterol complexes was observed for native and methylated β-cyclodextrin which leads to the accumulation of insoluble crystals in the kidney and consequently to nephrotoxicity. [81, 82] This negative effect of β-cyclodextrin can be effectively prevented by using hydroxypropyl- or sulfobutyl-residues, since both cyclodextrin-derivatives show significantly reduced toxicity after intravenous application which renders them as suitable excipients for parenteral formulations. [83, 84] For instance, hydroxypropyl-β-cyclodextrin is approved for parenteral use with doses up to 16 g per day. [85]

The hydrophobic cavity of the cyclodextrin molecule is capable of incorporating small lipophilic molecules and to give them a hydrophilic cover which offers various benefits such as increasing the solubility of the guest molecule, protection against microbial or chemical degradation, masking of bad taste or smell, and many others. [77] These desirable properties coupled with a good biocompatibility renders cyclodextrins as useful excipients in many pharmaceutical formulations of small molecules. [80, 82, 85]

4.1.2 Influence of cyclodextrins on protein stability

Besides various positive effects on low molecular weight drugs, cyclodextrins show significant impact on protein stability which was extensively analyzed in a recent review by Serno et al. [76] In general, the influence of cyclodextrins on protein stability cannot be absolutely predicted but has rather to be considered dependent on the respective protein, formulation medium, stress factor, and cyclodextrin-derivative. [76, 86] By this, differences were seen in colloidal as well as conformational protein stability. Concerning colloidal protein stability, HPβCD was reported as effective in reducing hGH-aggregation during vortex-stress
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[87], HPβCD and MβCD stabilized an IgG antibody against agitation induced aggregation to a comparable degree as by polysorbate 80, whereas no stabilization was observed after addition of SBEβCD [88], a variety of cyclodextrin-derivatives showed stabilizing properties against thermally induced aggregation which was significantly dependent on the pH-value of the buffer media [89]; however, SBEβCD leads to a markedly increased aggregation of GCSF during agitation and freeze-thaw stress [50], and HPβCD accelerated the aggregation of glycogen phosphorylase b during incubation at 48°C. [90]

There are also several reports about the influence of different cyclodextrins on the conformational protein stability as measured by the respective melting temperature. Here, different substituted α-, β-, and γ-cyclodextrins lead to a significant increase in the protein melting temperature of human growth hormone [87], and α-cyclodextrin was shown to increase the melting temperature of alcohol dehydrogenase which was also mirrored in a lower propensity for aggregation during storage at elevated temperature. [91] By contrast, a reduced thermal stability was observed after addition of α-cyclodextrin to four globular proteins [92], and for GCSF, a great decrease in melting temperature was observed in the presence of 10 mM SBEβCD whereas the same concentration of HPβCD had no effect on the thermal resistance of the protein. [50]

These observations demonstrate that cyclodextrins can potentially be useful excipients in protein formulation, since they are capable of overcoming the disadvantages of polysorbates (peroxide formation) or commonly applied sugars such as sucrose or trehalose (reduced stabilization against mechanical stress). However, the effect of cyclodextrins has to be accurately and separately considered for each single protein formulation.

4.1.3 Protein-cyclodextrin interactions

The capability of cyclodextrins to prevent protein aggregation during refolding processes is frequently reported. Here, cyclodextrins are on the one side used to incorporate hydrophobic amino acid residues on the surface of the unfolded protein in order to shield them from hydrophobic interactions and subsequent protein association and aggregation processes. [87, 93] On the other side, cyclodextrins are used in the so called “artificial chaperon technique” which describes a two-step method for protein refolding [94]. In a first step, the presence of detergent prevents the protein from aggregation, and in a second step, the addition of cyclodextrins leads to the stripping of the detergent molecules. [95, 96]

Considering the first case - the direct interaction between cyclodextrin and hydrophobic sites on the protein - there are many additional reports in literature which substantiate the hypothesis of a direct protein-cyclodextrin interaction [87, 90, 91, 97, 98], and the respective extent of binding was dependent on factors such as ionic strength [91], pH-value [89], size of
the cyclodextrin cavity [99], and solvent accessibility of surface exposed hydrophobic amino acids. [86] Furthermore, a binding between cyclodextrin and protein explains the reduction in conformational protein stability since cyclodextrins preferentially interact with solvent-exposed hydrophobic amino acids which are predominantly present after (partial) unfolding of the native state.

A further hypothesis for the influence of cyclodextrins on protein stability is based on the observation that distinct cyclodextrin-derivatives such as HPβCD are capable of preventing proteins from surface-stress induced aggregation. This effect was demonstrated during agitation of various proteins such as a monoclonal antibody [88], human growth hormone [87], porcine growth hormone [100], insulin [101], or the recombinant glycoprotein Antigen 18A. [102] Moreover, HPβCD was effective in preventing ovalbumin and lysozyme from surface-induced denaturation during methylene chloride/water emulsification. [103] The stabilizing mechanism of HPβCD on interfacially-driven protein aggregation was explained by (1) a shielding of hydrophobic sites on the protein which leads to a reduced protein-adsorption to the interface [103], or (2) the amphiphilic nature and surface activity of HPβCD which leads to a competition between protein and cyclodextrin on the air-water interface. [50, 102]

In the literature, one can find evidence for the surface activity of HPβCD which is furthermore significantly influenced by the number of hydroxypropyl-residues on the cyclodextrin-ring. [87, 88, 104] Since surfactants such as polysorbates are well known to stabilize proteins due to competitive displacement from interfaces [12], it was often speculated that the surface activity of HPβCD leads to a comparable mechanism in stabilizing proteins against interfacial aggregation [87, 100, 102, 103], and a direct correlation between surface activity of the respective HPβCD-derivative and reduction in protein aggregation was demonstrated by Tavornvips et al. [87] However, first investigations with a monoclonal antibody indicate that - despite the surface activity of HPβCD - a behavior analogous to the interfacial competition between protein and polysorbate seems unlikely for the cyclodextrin but the exact effect of HPβCD at the air-water interface of protein solutions is still not clarified. [50]

4.2 Polymers in protein formulations

According to Wang, the stabilization-mechanism of polymers on proteins can be either based on surface activity or preferential exclusion phenomena which are already known for classical surfactant and sugar-based excipients, or polymers generate a reduction in protein mobility due to steric effects or due to the increase in solution viscosity which leads to a reduced protein aggregation. [12] An overview about various applications of polymers in protein formulations was recently published by Ohtake et al. who estimated the
macromolecular crowding effect as dominating factor for protein stabilization by hydrophilic polymers. [105] The effect of polymers on protein aggregation was shown as dependent on the polymer concentration, molecular weight, and hydrophobicity. [106] By this, polyethylene glycol (PEG) exhibits a special status since PEG - which is in general of hydrophilic nature - shows some hydrophobic properties which counterbalance the positive effect of steric exclusion by hydrophobic interactions with the protein. [105, 107]

4.2.1 Hydroxyethyl starch - properties and capabilities

The fundamental structure of hydroxyethyl starch (HES) is illustrated in Figure I.2 and shows an amylopectin-based configuration consisting of α-(1,4)- and α-(1,6)-glycosidically linked anhydroglucose units. [108]

![Figure I.2: Structure of hydroxyethyl starch as taken from [109].](image)

HES is obtained from degraded waxy maize starch by etherification with ethylene oxide in alkaline medium, where hydroxyethyl moieties are mainly attached to the hydroxyl-group at C₂, less at C₆, and only minor at C₃ position. [108] Hydroxyethyl starch derivatives are generally classified by their absolute molecular weight, the molar substitution, and the so-called C₂/C₆-ratio. These parameters show a significant influence on the pharmacokinetic properties of HES and are crucial for its applicability as plasma volume expander. [110] By this, the molar substitution refers to the medium number of hydroxyethyl-substituents per glucose subunit, and the higher the degree of molar substitution, the lower the degradation of HES by serum α-amylase is. [110] Dependent on the molar substitution (MS), HES-derivatives are classified as tetrastarch (MS 0.4), pentastarch (MS 0.5), hexastarch (MS 0.6),
or hetastarch (MS 0.7). [110] The C₂/C₆-ratio gives information about the substitution pattern of HES and indicates the predominant position of the hydroxyethyl moiety. Since hydroxyethylation at position C₂ hinders the access for α-amylase, higher C₂/C₆-ratios are associated with reduced HES-degradation and higher plasma concentration rates. [110] Besides the differences in pharmacokinetic properties, the variety of HES-derivatives also shows significant differences in side-effects after parenteral application. In general, first generation HES is characterized by high molecular weights and high molar substitution rates which leads on the one side to adverse effects on the renal function, and on the other side to accumulation in the tissue with associated pruritus. However, third generation HES - which is characterized by lower molecular weights and lower molar substitution - exhibits a reduction in circulation half-life, significantly reduced side effects, and offers a desirable good biocompatibility. [111] HES-pharmaceuticals are used as plasma volume expanders, where an iso-oncotic pressure is obtained with 6%-solutions. [110, 111] The good tolerance and low adverse reactions after parenteral application renders low molecular weight HES with low molar substitution as promising excipient in protein formulation.

4.2.2 PEGylation versus HESylation

Covalent linkage between polymers and therapeutic proteins offers several benefits and is predominantly performed by using polyethylene glycol (PEG). [112, 113] The main advantages in the PEGylation of proteins are (1) the extension of plasma half life due to reduced renal clearance and lower access for proteolytic enzymes and (2) the increase in protein solubility. [113] In 2011, eight therapeutic proteins and peptides were available in the market as approved PEGylated therapeutics [114], and a number of further protein-candidates is currently in the pipeline. [112] Besides the favorable effect of half-life extension and solubility, PEGylated proteins were oftentimes reported to show an increased stability as observed for IFN-β-1b where 40 kDa PEGylation prevented the protein from precipitation with simultaneous maintenance of the secondary structure and thermal stability [115], PEGylated trypsin was significantly stabilized against thermal denaturation whereas a greater stabilization was obtained by using higher molecular weight PEG [116], PEGylation of α-Chymotrypsin led to a 6°C increase in protein melting temperature which was associated with reduced structural dynamics as determined by H/D-exchange experiments [117], and mono-PEGylation of recombinant human endostatin resulted in better resistance against temperature and pH-induced aggregation. [118]

Recently, non-covalent PEGylation was estimated as promising and novel concept to stabilize proteins via hydrophobic interactions between linker moieties at the PEG-chain and hydrophobic sites on the protein surface. First studies showed that 2 kDa dansyl-PEG was
effective in reducing buffer-mediated aggregation of salmon calcitonin whereas the best stabilization was observed by a 1:1 ratio [119], tryptophan-mPEGs lead to a significant reduction in the aggregation of salmon calcitonin, and the benefit was more pronounced for the 2 kDa compared to the 5 kDa PEG-derivative [120], and 2 and 5 kDa cholesteryl-PEGs showed a comparable, effective protection of hen egg-white lysozyme against aggregation. [121] In a further approach, PEG was modified with a polyanion block polymer which led to noncovalent PEGylation of keratinocyte growth factor-2 (KGF-2) by electrostatical complexation, and which caused significant protein stabilization against thermally induced aggregation. [122]

Besides these promising reports about PEGylation, the use of polyethylene glycol also implicates several disadvantages. Firstly, the chemical synthesis of PEG yields in a relatively broad molecular weight distribution [113], and differences in the PEG size were reported to affect the stability of the PEGylated protein [120], and to cause divergent biological properties. [113] Secondly, water accumulation leads to a 3 to 5-fold increase in the hydrodynamic volume of PEG and a reduced excretion especially at higher molecular weights. [113] Thirdly, PEG is reported to potentially induce immune response by the formation of anti-PEG antibodies [123], and finally, the contamination of PEG with peroxides compromises protein stability due to auto-oxidation processes. [124]

Because of these disadvantages in the use of PEG, hydroxyethyl starch offers a promising novel approach for protein stabilization by covalent or non-covalent HESylation. The polymeric nature of HES might implicate similar stabilizing properties as seen for polyethylene glycol; however, the starch-based structure would overcome the PEG-disadvantages such as accumulation in the human body and peroxide formation. Currently, HESylation is regarded as a promising technique for the half-life extension of proteins [125], and the HESylation of proteins would be an advanced novel concept for protein stabilization.
5. Objectives of the thesis

The present thesis comprises different aspects in the stabilization and characterization of therapeutic proteins, whereas main focus of the work lies on the role of hydroxypropyl-beta-cyclodextrin in liquid antibody formulations. By this, chapter 2 describes the effect of different cyclodextrins on the stability of highly concentrated antibody formulations. Furthermore, the influence of HPβCD on the colloidal as well as conformational protein stability was extensively studied by using two monoclonal antibodies in dilute and concentrated formulations.

Chapter 3 investigates the hypothesis that HPβCD stabilizes a monoclonal antibody against mechanical stress in a comparable mechanism as polysorbate 80. For that reason, surface tension measurements by drop profile analysis, and dilational shear rheology were applied to analyze the composition of the interfacial layer in simultaneous and sequential adsorption experiments.

A further hypothesis suggests that protein stabilization by cyclodextrins is based on a direct interaction between the circular oligosaccharide and hydrophobic parts on the protein surface. In order to examine this theory, chapter 4 describes measurements of antibody-HPβCD interactions by using quartz-crystal microbalance and static / dynamic light scattering.

Since highly concentrated protein formulations are often associated with an increase in solution viscosity, material saving viscosity-measurements are relevant in the development of concentrated protein pharmaceuticals. In chapter 5, two techniques - namely dynamic light scattering and microfluidic rheometry - were investigated regarding their applicability in a highly concentrated IgG solution.

Finally, the evaluation of a new concept for protein stabilization which is based on non-covalent HESylation is presented in chapter 6. For this, a HES-based linker was synthesized, binding to the protein was analyzed, and the effect of non-covalent HESylation on protein stability was investigated under agitation stress.
6. References


50. Serno, T., Inhibition of therapeutic protein aggregation by cyclodextrins, in Pharmaceutical Technology and Biopharmaceutics. 2010, LMU Munich: Munich.
57. Saluja, A., et al., Ultrasonic Storage Modulus as a Novel Parameter for Analyzing Protein-Protein Interactions in High Protein Concentration Solutions:


Chapter II
Stabilization of highly concentrated antibody-formulations by cyclodextrins

1. Introduction

In the recent years, therapeutic antibodies gained more and more importance for treatment of chronic diseases such as cancer or autoimmune disorder. [1-6] Contrary to other therapeutic proteins, antibodies often require high single doses with more than 100 mg per dose. [7, 8] In order to minimize the inconvenience of parenteral application, the injection volume should be kept at a minimum which is obtained by increasing the antibody concentration to values of 100 mg/mL or even higher. [9] However, formulation of highly concentrated protein solutions is reported to exhibit several challenges, most notably a reduced protein stability due to a higher aggregation rate, and a dramatic increase in solution viscosity. [7]

A rise in protein concentration is reported to correlate with an increase in non-covalent intermolecular protein interactions. [10-12] Those protein-protein interactions are held responsible for the concentration-dependent increase in the protein-solution viscosity [12-14], and were ascribed to multiple intermolecular binding between F_ab^*-residues of the antibody molecules. [15] The presence of intermolecular protein-interactions in the highly concentrated state could be furthermore correlated to a rise in protein aggregation upon storage experiments [16], and the formation of protein aggregates is generally supposed to depend on the rate of intermolecular collision which is increased with higher concentrations. [7, 17, 18]

In recent studies, cyclodextrins emerged as promising excipient-candidate to be used in low concentrated liquid protein formulations. [19-21] However, to the best of our knowledge there is no experience about the capability of cyclodextrins to stabilize highly concentrated protein solutions, and in particular highly concentrated antibody formulations. Therefore, aim of the current study was

1. to evaluate the effect of different cyclodextrins on the stability of a 100 mg/mL IgG-formulation,
2. to compare the stabilizing effect of hydroxypropyl-β-cyclodextrin on dilute and concentrated solutions of two monoclonal antibodies, and
to investigate the effect of sulfobuthylether-β-cyclodextrin as viscosity-reducing excipient.

2. Materials and Methods

2.1 Materials

The influence of cyclodextrins on protein stability was investigated by using three different monoclonal antibodies which below are named as IgG A, IgG B, and IgG C. IgG A is a monoclonal antibody of the IgG1 class, which was initially formulated at a concentration of 5 mg/ml in 10 mM phosphate buffered saline pH 6.2. IgG B belongs to the IgG4 class and was a gift from Roche Diagnostics GmbH (Penzberg, Germany). The second antibody was formulated in 20 mM histidine buffer pH 5.8 with an original concentration of 22.9 mg/mL. Bulk material of IgG C had an original concentration of roughly 15 mg/mL, and the formulation buffer was 10.5 mM phosphate pH 6.4.

Before usage, the protein bulk material was filtered through sterile 0.2 µm PES membrane syringe filters (VWR International). To obtain highly concentrated formulations, the antibody solution was concentrated by Vivaspin 20 (30.000 MWCO) centrifugal tubes with PES-membrane (Sartorius Stedim Biotech GmbH, Goettingen, Germany) up to a concentration of roughly 200 mg/mL. Exact protein concentration after the concentration step was measured spectroscopically. Therefore 5 µL of the solution were diluted 1:100 with the respective formulation buffer, and concentration was determined at 280 nm using a Nanodrop 2000 - device (Thermo Scientific) in the setup for IgG measurements. Mass extinction coefficients were 1.370 mL*mg⁻¹*cm⁻¹ for IgG A, 1.40 mL*mg⁻¹*cm⁻¹ for IgG B, and 1.499 mL*mg⁻¹*cm⁻¹ for IgG C.

Hydroxypropyl-β-cyclodextrin (HPβCD) with a molar substitution of 0.59 - 0.73 per anhydroglucose unit, and methyl-β-cyclodextrin (MBβCD) were supplied by Wacker Chemie AG (Burghausen, Germany) in pharmaceutical grade. Sulfobuthylether-β-cyclodextrin (SBEβCD) was obtained from CyDex Inc. (Lenexa, KS). Super refined polysorbate 80 was a gift from Croda GmbH (Nettetal, Germany). Sucrose with purity > 99.5% was purchased from Sigma-Aldrich GmbH. All other reagents were at least of analytical grade.

Phosphate buffered saline at pH 6.2, 20 mM histidine buffer pH 5.8, and 10.5 mM phosphate buffer pH 6.4 were used to prepare the respective excipient stock solutions for the different antibody formulations. Buffer and excipient stock solutions were filtered through sterile 0.2 µm cellulose acetate syringe filters before use. Final protein formulations were obtained by mixing adequate volumes of concentrated protein, excipient stock solution, and pure
buffer. Protein concentration was kept constant at 100 mg/mL for highly concentrated formulations, and at 1.8 mg/mL for low protein concentrations. All stability experiments were performed and analyzed as triplicates.

2.2 Stress-methods

2.2.1 Stirring

Stirring stress was performed at room temperature in 1.5 mL glass vials (VWR International) using a Variomag Telesystem stirrer (Thermo Scientific). A Teflon-coated micro stirring bar of 6 x 3 mm (VWR International) was placed into each vial and the vessel was closed with an 8 mm screw cap (VWR International). Stirring speed was adjusted to 200 rpm for highly concentrated protein solutions, and 400 rpm for low protein concentrations. For SEC measurements, 500 µL of 1.8 mg/mL protein solution were stirred and 100 µL samples were drawn after 0, 3, and 7 hours. For the 100 mg/mL protein formulations, a much lower sample volume was used for material saving. Accordingly, 65 µL were stirred and 5 µL samples were drawn after 0, 24, 48, and 72 hours. Before taking samples, each vial was placed vertically into a 15 mL tube and centrifuged at 3000 g for 2 minutes. This procedure was necessary in order to avoid measurement errors due to evaporated and re-condensed liquid at the vessel top.

Turbidity and particle count of 1.8 mg/mL IgG solutions was performed after stirring 250 µL for one hour. Samples of 100 µL were taken and pipetted into 1900 µL highly purified water for further analysis. For analyzing turbidity and cumulative particles of the 100 mg/mL IgG-formulations, 50 µL were stirred for one hour. After this, samples were gently homogenized by mixing with a pipette, 5 µL were taken and pipetted into 1995 µL highly purified water for further analysis. Additional turbidity and particle count measurements were performed with placebo-formulations containing no protein. Treatment of placebo formulations was identical to the formulations with protein.

For FTIR-analysis of stirred 100 mg/mL IgG A-formulations, 50 µL of the respective sample were stirred for 24 hours at 200 rpm. In order to separate the generated precipitates from supernatant, 100 µL of the respective protein-free excipient-solution were added to the stirred sample, and the mixture was gently homogenized. Afterwards, the precipitated protein was separated from the supernatant by centrifugation for 10 minutes at 15000 g, and diluted supernatant was taken and analyzed by FTIR-spectroscopy. The centrifuged pellet was washed for three times with 300 µL protein-free excipient solution by gently mixing with a pipette. After each washing step, insoluble aggregates were separated from the mixture by
centrifugation for 5 minutes at 15000 g, and the supernatant was removed by a pipette. Finally, the washed pellet was resuspended in 20 µL of the respective excipient-containing buffer for FITR-analysis.

2.2.2 Shaking

For analyzing protein stability during shaking stress, 1 mL of the respective 1.8 mg/mL IgG-formulations were filled into 1.5 mL micro-centrifuge tubes (VWR International), and vertically placed onto an Eppendorf Thermomixer. Throughout the stress time, samples were kept at a temperature of 20°C, and shaking speed was adjusted to 1000 rpm for the IgG A -, and 1200 rpm for the IgG B-formulations. For SEC-analysis, 100 µL samples were drawn at specific time points for further processing. For turbidity and light obscuration measurements, samples were shaken for 48 hours and 100 µL samples were taken and pipetted into 1900 µL of highly purified water.

2.2.3 Storage at elevated temperature

To test the influence of elevated temperature on protein stability, 500 µl of the low concentrated protein formulations were stored in 1.5 mL glass vials (VWR International) at 50°C. At specific time points, samples of 100 µL were taken and analyzed by SEC. For 100 mg/mL samples, 60 µL of each antibody formulation were filled into a 1.5 mL glass vial by using 0.1 mL micro-inserts (VWR-International), and stored at 50°C. For SEC-analysis, samples of 5 µL were taken after specific time intervals.

2.2.4 Light exposure

Light stress experiments for 100 mg/mL IgG-samples were performed in 1.5 mL glass vials (VWR International) by using 0.1 mL micro-inserts (VWR-International). 60 µL of each formulation were filled into the insert, and the vials were closed by 8 mm screw caps (VWR International). The samples were placed under the Suntest CPS (Atlas Material Testing), and light intensity was adjusted to 60 W/m². Measurement temperature was kept at 45°C throughout the sample exposure time. For SEC-analysis, 5 µL samples were taken after 0, 4, 8, and 24 hours of light stress. For analyzing the secondary structure of the light-exposed protein by FTIR-spectroscopy, samples were identically prepared and placed under the Suntest CPS for 24 hours.
2.3 Analytical methods

2.3.1 Size-exclusion chromatography

For SEC analysis, 5 µL of the highly concentrated protein sample were pipetted into 495 µL of SEC mobile phase and gently mixed. Low concentrated protein formulations were treated without further dilution. Samples were centrifuged for 10 minutes at 15000 g to remove insoluble aggregates. Supernatant was analyzed by injection on a Tosoh TSKgel G3000SWXL column using an Ultimate 3000 HPLC system (Dionex Softron GmbH, Germering, Germany). Autosampler temperature was kept at 5°C, and eluting protein was detected by UV-measurement at 280 nm.

For IgG A, injection volume was 50 µL for highly concentrated protein solutions after 1:100 dilution, and 30 µL for undiluted low concentrated formulations. Elution buffer was composed of 50 mM NaH₂PO₄ and 600 mM NaCl, adjusted by sodium hydroxide to pH 7.0. For IgG B, 35 µL of the 100 mg/mL formulations were injected after 1:100 dilutions, and for the 1.8 mg/mL samples, 20 µL were injected without dilution. Elution buffer consisted of 250 mM potassium phosphate and 200 mM potassium chloride which was adjusted to a pH of 7.0 by potassium hydroxide solution. For IgG C, injection volume was 50 µL for the 100 mg/mL samples after 1:100 dilutions. Mobile phase consisted of 50 mM sodium phosphate and 300 mM sodium chloride at a pH of 7.0. Flow rate was kept at 1.0 mL/min for IgG A, and 0.5 mL/min for IgG B and IgG C.

2.3.2 Turbidity

Turbidity measurements were carried out at NEPHLA laboratory turbidimeter (Hach Lange GmbH, Duesseldorf, Germany). The instrument operates in the 90° scattered light method at 860 nm, and results are obtained in formazine nephelometric units (FNU). Sample dilutions were performed immediately before measurement.

2.3.3 Light obscuration

Particle count was determined by light obscuration at a PAMAS SVSS-C instrument (PAMAS GmbH, Rutesheim, Germany). Before each measurement, the device was cleaned with highly purified water until less than 50 particles in the range from 1 – 16 µm, and no particle larger than 16 µm were detected. After that, the device was pre-flushed with 0.5 mL of sample, and subsequently three samples of 0.3 mL are analyzed for particle number. Mean
value of these three measurements is extrapolated to a sample volume of 1 mL. Sample dilutions were performed immediately before measurement.

2.3.4 Differential scanning calorimetry

Protein melting temperature of 1.8 mg/mL protein solutions was determined using VP-DSC (Microcal Inc., MA). For each IgG-formulation, 500 µL were loaded in the respective sample cell and measured against 500 µL protein-free reference \((n = 3)\). Samples were heated from 30°C up to 100°C with a scan rate of 60°C per hour. Before starting the measurement, sample-temperature was equilibrated for 15 minutes. An identically performed baseline run with buffer in the sample as well as reference cell was subtracted from each data set. For obtaining the melting temperature, protein concentration was normalized and data were analyzed using Origin DSC data analysis software.

Melting temperature of highly concentrated antibody formulations was determined using Phoenix DSC 204 (Netzsch GmbH, Selb, Germany). To this end, 60 µL of 100 mg/mL protein formulation were filled into 100 µL aluminum crucibles and cold-sealed. For each sample, a reference of 60 µL protein-free excipient solution was prepared in the same way as the protein samples. At the beginning of each measurement, sample and reference were tempered at 15°C for five minutes, and afterwards heated up to 95°C with a heating rate of 2°C / min. The obtained thermograms were processed using the Netzsch Proteus - thermal analysis software, and apparent melting points \((T_m)\) are reported at the endothermic peak maximum in the obtained thermograms \((n = 3)\).

2.3.5 Fourier-transformed infrared spectroscopy

FTIR-spectroscopy was performed using a Tensor 27 FTIR-spectrometer (Bruker Optics) with Bio-ATR measurement cell. Sample volume was 20 µL, and spectra were recorded by taking the average of 148 scans in the range between 4000 cm\(^{-1}\) and 850 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\). Each protein sample was measured against the respective protein-free buffer or excipient solution as reference. Data were processed by applying the OPUS-software (Bruker Optics). For the resulting absorbance spectra, additional water- and atmospheric-compensation were performed, and after vector-normalization, second derivative spectra were obtained using a 17-point Savitzky-Golay derivative function.

FTIR-measurements were additionally used to directly study the thermodynamic stability of highly concentrated IgG A-formulations. To this end, 20 µL of the 100 mg/mL protein sample were placed into the Bio-ATR II cell and heated from 25°C to 95°C by connection to a
Haake DC 30 thermostat. IR-spectra of reference and sample were recorded at specific temperature points, namely in 10°C steps from 25°C to 55°C, and in 2°C steps from 55°C to 95°C. Equilibration time before starting the measurement were 90 seconds for 10°C steps, and 60 seconds for 2°C steps. To circumvent perturbing water signals, spectra were corrected by atmospheric compensation. Second derivative analysis showed a shift in the IgG A main peak from 1636 cm\(^{-1}\) to 1622 cm\(^{-1}\) when heating the antibody up to 95°C. Therefore absorbance ratios of 1636 cm\(^{-1}\)/1622 cm\(^{-1}\) were plotted against the respective temperature to determine the melting curve. The melting point was obtained by copying the values into Origin8G, and estimating the inflection point of the smoothed melting curve.

2.3.6 Viscosity-measurements

Viscosities of 100 mg/mL IgG A- and IgG C-formulations were measured using an m-VROC viscosimeter (RheoSense Inc., San Ramon, CA) equipped with an A05 chip with 50 µm flow channel. The instrument was connected to a water bath (Lauda RK8KP) and tempered at 25°C. Formulations were filled into a 100 µL Hamilton syringe, and temperature was equilibrated for two minutes before starting each measurement. Solutions exhibited a Newtonian-flow-behavior (data not shown); therefore all measurements were performed as triplicate at one single flow rate which was 100 µL/min for the IgG A-, and 50 µL/min for the IgG C-solutions.

3. Results and Discussion

3.1 Physical stability of a 100 mg/mL IgG-formulation in the presence of different cyclodextrins

For the following experiments, protein stability of 100 mg/mL IgG A-formulations was investigated in the presence of three different cyclodextrins, namely hydroxypropyl-β-cyclodextrin (HPβCD), sulfobuthylether-β-cyclodextrin (SBEβCD), and methyl-β-cyclodextrin (MβCD) in the concentrations 2.5 mM and 25 mM, respectively. By way of comparison, 2.5 mM corresponds to a 0.35% (HPβCD), 0.55% (SBEβCD), and 0.33% (MβCD) solution. The formulations were exposed to different stress conditions and compared to the formulations containing polysorbate 80 (which is known to stabilize proteins by competitive adsorption) and sucrose (which leads to a preferential hydration of the protein). [22]
3.1.1 Stirring stress

Stirring of protein solutions is described as a stress method simulating the mechanical stresses imposed on the protein during processing, potentially leading to significant aggregate-formation in case of susceptible proteins. [23-25] By this, stirring represents a harsh stress method compared to shaking stress [23], especially when using bottom-magnetic-type mixer. [25] Several factors were discussed as causes for protein aggregation during stirring, for instance shearing forces, cavitation, or thermal heating. [23] Most probably, aggregation is caused by forces in the small gap between the stirring bar and the bottom of the vessel, since top-entering-type mixer did not result in any remarkable protein aggregation. [25]

To evaluate the effect of different cyclodextrins on a highly concentrated IgG-solution, formulations were stirred by a magnetic stirring bar for 72 hours, and results of HP-SEC-analysis are illustrated in Figure II.1. Here, the 100 mg/mL IgG-sample “without excipient” (which was formulated in pure buffer) showed a strong reduction in the monomer content with only 20% remaining monomer at the end of the stirring time. Addition of 2.5 mM and 25 mM SBEβCD neither leads to stabilization nor to destabilization of the protein. By contrast, HPβCD and MβCD caused a significant rise in remaining monomer, whereas the 25 mM cyclodextrin-concentration tends to a better stabilization compared to the 2.5 mM. However, the difference between the 2.5 mM and the 25 mM cyclodextrin concentration is not significant due to large standard deviations. The presence of these large error bars can be ascribed to the low sample volume, which was used for material saving as well as the large dilution factor. [24]

Both polysorbate 80 concentrations lead to a stabilizing effect, which is similar to the 25 mM HPβCD and MβCD-samples. However, none of the tested formulations was capable of achieving a complete stabilization. A similar protective effect of polysorbate 20 during stirring stress was reported earlier but also here, even the highest polysorbate concentration tested could not completely prevent the protein from generating some aggregates. [23] In a different study, the presence of polysorbate 20 was shown to be sufficient in reducing antibody-precipitation during shear stress by a cone-and-plate rheometer. [26] Contrary to the polysorbate samples, the stirred 250 mM sucrose formulation resulted in similar values to the formulation without excipient what can be explained by the mechanism of stabilization. Since the presence of sugars leads to preferential hydration of dissolved proteins [22], a good stabilizing effect for sugars was seen during quiescent storage studies [27], but no stabilization was achieved during mechanical stress. [20]
Figure II.1: Remaining monomer [%] after 0, 24, 48, and 72 hours of stirring different 100 mg/mL IgG A-formulations as determined by HP-SEC.

Besides analysis of soluble aggregates by HP-SEC, presence of insoluble aggregates was investigated by turbidity and light obscuration measurements, and the results are illustrated in Figure II.2 and Figure II.3. In contrast to the three-days stirring experiment for HP-SEC analysis, both methods required a much shorter duration of stirring (60 minutes) to cause measurable protein aggregation which is another example for the high sensitivity of particle counting methods in the detection of aggregates species. [28]

After 60 minutes stirring and 400-fold sample dilution with highly purified water, the 100 mg/mL IgG-formulation without excipient resulted in turbidity-values of roughly 2.0 FNU (Figure II.2). Unstirred references, which were diluted in the same rate, resulted in values of roughly 0.5 FNU (data not shown). No significant differences to the formulation without excipient were seen for all stirred cyclodextrin- and the sucrose sample, but a clear reduction was observed for both polysorbate 80 concentrations. In order to exclude that the presence of excipient itself leads to a rise in turbidity during stirring, respective protein-free placebo formulations were stirred and analyzed in identical way (Figure II.2). Here, no increase in turbidity was observed in any formulation which substantiates that the rise in turbidity after stirring the protein-samples was indeed caused by protein aggregation.
Figure II.2: Turbidity of 100 mg/mL IgG A-formulations (bars) and their respective protein-free references (points) after 60 minutes stirring and 400-fold sample dilution prior to the measurement.

After turbidity-measurements, the very same samples were analyzed by light obscuration, and the numbers of cumulative particles > 1 µm are illustrated in Figure II.3. Despite a 400-fold dilution with highly purified water, there were still about 80,000 particles/mL counted in the formulation without excipient which can be explained by the harsh stress condition of the stirring-setup. [23] None of the tested cyclodextrin samples was capable of causing a significant reduction in the measured particle amount, and also the sucrose formulation showed a comparable particle value. By contrast, a drastic reduction in particles was seen for both polysorbate-formulations with less than 5,000 particles in the 0.01%-formulation, and less than 1,000 particles in the 0.1% formulation. Also here, protein-free placebo formulations were identically analyzed after stirring, and each of these formulations resulted in less than 1,000 particles (data not shown). This further confirms the placebo-results of turbidity-measurements (Figure II.2), where the protein-free solutions indicated the absence of a significant amount of particles.

Summing up the stirring-stress results of HP-SEC, turbidity, and light obscuration, polysorbate 80 resulted in the best stabilization whereas both, soluble and insoluble aggregates were effectively reduced. HPβCD and MβCD showed a positive effect on soluble aggregates, but no influence on the formation of protein particles, and addition of SBEβCD and sucrose did not cause any measurable changes in protein stability. The positive effect of
HPβCD and MβCD is in contrast to prior stirring-studies where no significant stabilization was observed after adding those cyclodextrins to a low concentrated IgG-formulation. [19]

![Cumulative particle size distribution](image)

**Figure II.3:** Cumulative particles > 1 µm per milliliter of after 60 minutes stirring of 100 mg/mL IgG A-formulations and 400-fold sample dilution prior to the measurement.

To obtain further information about potential differences between protein aggregates in HPβCD and polysorbate 80 formulations, FTIR spectroscopy was applied to investigate changes in secondary structure after stirring (Figure II.4). By this, sample preparation after stirring was different for precipitates and the supernatant since the centrifuged pellet (which represents the precipitated protein) required several washing steps with protein-free formulation buffer to remove signal contribution of the adhesive protein solution. The spectrum of the native protein exhibits a main band at 1636 cm⁻¹ which represents the predominant intramolecular β-sheet structure of the antibody. [29, 30] In both stirred formulations (HPβCD and polysorbate 80), protein secondary structure in the supernatant was roughly identical to the native form which means that stirring has no effect on the structure of soluble protein. However, washed precipitates exhibit some differences in secondary structure compared to the native state. A slight shift and asymmetry of the main band at 1636 cm⁻¹ indicate heterogeneity of intramolecular β-sheet structures. At the same time, the main peak of the native protein is still present indicating the almost native like structure of precipitates after stirring which was also reported for a monoclonal antibody before. [23] Differences between native protein and precipitates at 1622 cm⁻¹ are typical for the formation of intramolecular hydrogen bonds between β-strands, which often occurs in
When comparing the precipitates of the HPβCD and polysorbate 80 formulations, both exhibit nearly the same spectrum; therefore, no essential differences in the secondary structure of the generated aggregates are visible for both investigated formulations.

**Figure II.4:** Second derivative FTIR-spectra of unstressed 5 mg/mL IgG A-samples (solid line), supernatant (dashed line), and precipitates (dotted line) obtained after 24 hours stirring of 100 mg/mL IgG A-formulations with addition of 25 mM HPβCD (Figure A) or 0.1% polysorbate 80 (Figure B).

### 3.1.2 Light exposure

Light stress is well known to activate various degradation pathways in proteins such as oxidation, deamidation, formation of new disulfide bonds, and aggregation. [31-34] In order to investigate the effect of different cyclodextrins on protein aggregation during light exposure, 100 mg/mL IgG samples were stored up to 24 hours under a Suntest CPS, and soluble aggregates were analyzed at specific time points by HP-SEC. One additional control sample was tested which was formulated as the sample without excipient, but protected from light by coating the sample vessel with aluminum foil. Results in Figure II.5 show a gradual reduction in remaining monomer (A) and a simultaneous increase in dimer content (B) for all formulations other than the control sample. There was no significant difference between each of the light-stressed formulations which indicates, that the presence of all the investigated excipient has no influence on protein stability during light exposure. The light-protected control sample retained the original state; therefore, secondary effects on protein stability within the experimental setup were excluded. Additionally, formulations with very high HPβCD-concentrations (50 mM and 100 mM) were tested but also here, no effect on the formation of soluble aggregates was apparent since the percentage of remaining monomer (A) and fraction of dimer (B) were identical to the excipient-free sample (Figure II.6).
Due to the variable protein degradation routes under light exposure, the development of photo-stable protein formulations represents an almost insurmountable hurdle. Light exposure of the monoclonal antibody HER2 was reported to cause significant protein oxidation, but oxidation could be effectively reduced by the addition of antioxidants; however, the extent of protein oxidation had no effect on the aggregation-level which was determined by HP-SEC. [35] Further light-stress studies with soluble tumor necrosis factor receptor showed that addition of sucrose, L-methionine, L-tryptophan, and L-cystine had no or – in the case of tryptophan – even a negative effect on protein aggregation. [31] These reports and our own observations conform to the statement of Kerwin et al., that only light-protection of proteins is capable of preventing light-induced damage. [32]

Figure II.5: Remaining monomer (A) and fraction of dimer (B) after 0, 4, 8, and 24 hours of light exposure for different 100 mg/mL IgG A-formulations.

Figure II.6: Remaining monomer (A) and fraction of dimer (B) after 0, 4, 8, and 24 hours of light exposure 100 mg/mL IgG A-formulations without excipient and with addition of high HPβCD-concentrations.
Protein secondary structures in light-stressed 100 mg/mL IgG-formulations were measured by FTIR-spectroscopy for the formulations without excipient, and with addition of 100 mM HPβCD (Figure II.7). Both light-stressed formulations showed the same spectra which indicates that the presence of HPβCD has no influence on the protein structure. Compared to the spectrum of native protein, the two light stressed formulations revealed no significant difference which demonstrates the maintenance of the native-like protein structure during light exposure.

Comparable results were reported from Rajsekhar et al., where isolated, light-stress induced antibody dimers exhibited the same FTIR-spectra compared to the unstressed monomer. [33] A similar protein conformation after photodegradation was also seen for lyophilized bovine growth hormone [36], and UV-illumination of α-Lactalbumin resulted in an altered tertiary but retained secondary protein structure. [37]

![Figure II.7: Second derivative FTIR-spectra of different IgG A-samples. The solid line illustrates unstressed 5 mg/mL IgG A, the dashed line 100 mg/mL IgG A without excipient after 24 hours of light exposure, and dotted line 100 mg/mL IgG A with addition of 100 mM HPβCD after 24 hours of light exposure.](image)

### 3.1.3 Storage at elevated temperature

Protein stability in 100 mg/mL IgG-samples was further investigated by storing the different formulations at 50°C (Figure II.8). Elevated temperature is known to trigger the formation of soluble aggregates [38, 39], and protein storage at higher temperature serves as a predictor for protein stability under real storage conditions. [40]
In our case, incubating the antibody at 50°C for 14 days led to roughly 10% decrease in remaining monomer (Figure II.8A) and a significant increase in soluble protein aggregates (Figure II.8B). Furthermore, visible turbid solutions and a lower total protein recovery by HP-SEC analysis were detected in each formulation (data not shown) which demonstrates the additional formation of insoluble aggregates during storage at 50°C. Comparing the effect of different excipients on protein aggregation, the formulations containing no excipient or sucrose showed the best stability, and all other excipients – especially SBEβCD and polysorbate – promote an enhanced degradation. Addition of 2.5 mM and 25 mM of HPβCD showed no effect on protein aggregation which was also reported earlier for storage of a low concentrated IgG-formulation. [19] The 25 mM SBEβCD- and MβCD-formulations resulted in a slight monomer decrease compared to the excipient-free formulation, whereas the 25 mM SBEβCD sample exhibited a strong increase in soluble aggregated species. The sucrose-reference itself had no destabilizing effect at all. Sugars such as sucrose and trehalose are well known to increase the stability of proteins under storage experiments [27, 41, 42], whereas the stabilizing mechanism is ascribed to the phenomenon of preferential exclusion. [42, 43] A slight decrease in protein stability - which was seen for some cyclodextrin-containing samples - indicates that cyclodextrins and sucrose have a different effect on thermodynamic properties of the protein. The negative effect of polysorbate on protein stability during storage (Figure II.8) was also reported in the literature earlier. [19, 41] Presence of surfactants is often held responsible for a reduced thermodynamic stability of proteins [44], and the PEG-residues of the polysorbate-molecule are reported to serve as trigger for destabilization due to protein-oxidation. [35, 45]
3.2 Influence of hydroxypropyl-beta-cyclodextrin on the stability of dilute and highly concentrated IgG-formulations

The effect of cyclodextrins on stability of highly concentrated IgG-solutions was further investigated by focusing on the hydroxypropyl-beta-cyclodextrin (HPβCD) derivative in a broad concentration range from 2.5 mM to 100 mM. HPβCD was chosen since it is already approved as pharmaceutical excipient [46], and a promising protein-stabilizing effect was reported for low concentrated protein formulations. [19, 20, 47, 48] Two different monoclonal antibodies (IgG A and IgG B) were used for the following experiments, and protein stability was investigated in dilute (1.8 mg/mL) as well as highly concentrated (100 mg/mL) formulations in the presence of HPβCD. Polysorbate 80 and sucrose were again utilized as reference stabilizers against mechanical (polysorbate) or thermal (sucrose) stress.

3.2.1 Shaking stress

Agitation is well known to provoke protein aggregation [20, 23, 24], and shaking stress-experiments are often applied to investigate the physical stability of proteins. [49] The experimental shaking-setup strongly influences the magnitude of protein aggregation and has to be chosen carefully to enable visible differences between the tested formulations [49]. Different shaking conditions with varied filling volume, vessel adjustment (horizontal and vertical), shaking speed, and shaking device have been tested for IgG A formulations in dilute and concentrated solutions, but the antibody remained stable under all tested conditions (data not shown). Exemplarily results for shaking-stress of 1.8 mg/mL IgG A-solutions (which was performed according to the stress-parameters indicated in the “Materials and Methods”-section) are shown in Figure II.9 where all formulations remained stable within a shaking period of 14 days.

Contrary to IgG A, seven days shaking of low concentrated IgG B-formulations without any additional excipient resulted in a decrease of remaining monomer to roughly 70% (Figure II.10). All of the tested HPβCD-concentrations as well as 0.1% polysorbate lead to a good aggregation-protection, since no loss in monomer could be detected. Protein-stabilization against shaking stress was previously reported for low HPβCD-concentrations [19, 20, 47], and the present data show no difference in the stabilizing potential for all tested cyclodextrin formulations. Addition of 250 mM sucrose is equivalent to the formulation without excipient which was already demonstrated in earlier agitation studies. [20, 50]

For the very same antibody (IgG B), shaking stress experiments with highly concentrated formulations (50 mg/mL) showed that addition of 2.5 mM HPβCD was effective in stabilizing
the protein against agitation induced aggregation. [19] Furthermore, it was demonstrated that the effect of HPβCD on protein stabilization during shaking was rather dependent on the absolute concentration of the cyclodextrin than on the protein-cyclodextrin ratio since an increase in the HPβCD-concentration did not result in further increase of the stabilizing effect. [19]

Figure II.9: Remaining monomer [%] after 0, 1, 3, 7, and 14 days of shaking 1.8 mg/mL IgG A-formulations with addition of different excipients as determined by HP-SEC.

Figure II.10: Remaining monomer [%] after 0 and 7 days of shaking 1.8 mg/mL IgG B-formulations with addition of different excipients as determined by HP-SEC.

Protein aggregation after shaking was additionally analyzed by light obscuration and turbidity-measurements after 20-fold sample dilution with highly purified water (Figure II.11).
Here, the formulation without excipient revealed the highest level of insoluble aggregates with roughly 135,000 particles > 1 μm, and a turbidity value close to 3.0 FNU. Addition of HPβCD leads to a great reduction in cumulative particles as well as solution-turbidity. Increasing the HPβCD-concentration from 2.5 mM to 100 mM leads to a stepwise particle-decrease from 4,000 particles (2.5 mM HPβCD) to 400 particles (100 mM HPβCD). By this, particle amount in the 100 mM HPβCD-formulation equals the result of the polysorbate-reference which had only 300 particles. Congruent to light obscuration, turbidity-values of the 2.5 mM HPβCD-formulation showed a clear reduction compared to the sample without excipient, and increasing HPβCD-concentrations lead to a further reduction in turbidity from about 0.70 FNU (2.5 mM HPβCD) to 0.53 FNU (100 mM HPβCD). Interestingly, also the sucrose formulation leads to a slight reduction in turbidity and a significant decrease in cumulative particles which is in contrast to the result of HP-SEC, where no stabilizing effect of sucrose was visible (Figure II.10). Therefore, sucrose was effective in reducing the amount of insoluble particles > 1 μm while the remaining monomer was unaffected compared to the excipient free formulation. The relatively high turbidity value in the sucrose formulation compared to the HPβCD and polysorbate formulations (Figure II.11) indicates the presence of larger amounts of insoluble aggregates < 1 μm which could not be detected by light obscuration but which are presumably co-responsible for the monomer loss (Figure II.10).

Figure II.11: Cumulative particles > 1 μm per milliliter (bars) and turbidity (points) after 48 hours shaking of different 1.8 mg/mL IgG B-formulations. Samples were diluted 20-fold directly before the respective measurement.
Comparing the shaking-results for low concentrated IgG A and IgG B, a different aggregation-sensitivity was observed for both proteins since only IgG A showed a great resistance against agitation-stress. The mechanism of protein aggregation during shaking is ascribed to (1) the higher collision-rates of the protein to the air-liquid interface, and (2) the surface activity of proteins which leads to partial protein unfolding at the interface. [51] By this, susceptibility of proteins to form aggregates during shaking was already reported earlier as highly protein- and formulation-specific. [51]

3.2.2 Stirring stress

Dilute and highly concentrated IgG solutions containing increasing concentrations of HPβCD were stirred for different periods to evaluate HPβCD’s stabilizing effect against mechanical stress. The 100 mg/mL protein formulations were stirred for a longer time compared to the dilute ones to allow better discrimination between the different formulations, since the concentrated protein formulations are known to be more stable against mechanically-induced aggregation. [17] Results of SEC measurements for monomer recovery are shown in Figure II.12. In both protein concentrations, IgG A was more susceptible for protein aggregation, since the monomer recovery is always below the recovery of IgG B. This behavior is in contrast to the shaking stress experiments where IgG A showed a high resistance against agitation induced aggregation. One possible explanation would be a different surface activity of both antibodies since aggregation processes during shaking are mainly ascribed to processes at the air-water interface. [52] Furthermore, stirring causes additional stress such as shear or cavitation and represents a harsher stress method compared to shaking. [23] Results for dilute and concentrated IgG solutions of both MAbs show that addition of HPβCD leads to protein stabilization. Increasing the concentration of HPβCD from 2.5 mM to 100 mM increased the monomer recovery progressively from approximately 40 % to 80 % for IgG A, and from 65 % to 85 % for IgG B after 7 h of stirring the 1.8 mg/ml protein solutions. For 100 mg/ml protein, remaining monomer increased from 30 % to 70 % for IgG A, and from 70 % to 90 % for IgG B after 72 h of stirring. In all tested formulations, the control experiments showed that 0.1 % of polysorbate 80 was sufficient to significantly enhance the monomer recovery, at least as good as HPβCD, in many cases even better, while sucrose was not effective in protecting IgG from mechanical stress.
Figure II.12: (Left) Remaining monomer [%] after 0, 3, and 7 hours of stirring 1.8 mg/mL IgG A (Figure A) and IgG B (Figure C) formulated with different excipients as determined by HP-SEC. (Right) Remaining monomer [%] after 0, 24, 48, and 72 hours of stirring 100 mg/mL IgG A (Figure B) and IgG B (Figure D) formulated with different excipients as determined by HP-SEC.
Figure II.13 demonstrates the turbidity values for dilute and concentrated protein formulations after stirring for 1 hour. Stirring of the formulations without excipient leads to visibly turbid samples, and significant increase in turbidity was measured after 20-fold (for 1.8 mg/mL IgG) or 400-fold (for 100 mg/mL IgG) dilution of the stirred sample. For instance, the turbidity of unstressed IgG A without excipient resulted in roughly 0.4 FNU for the 1.8 mg/mL, and 0.7 FNU for the 100 mg/mL samples, whereas one hour of stirring resulted in 6.5 FNU for the 1.8 mg/mL, and 2.5 FNU for the 100 mg/mL IgG A samples after dilution, respectively (Figure II.13A and B). In agreement with the results from SEC (Figure II.12), sucrose did not reduce the protein aggregation level and polysorbate 80 led to a significantly lower turbidity. In dilute protein solutions, all the applied HPβCD concentrations show a significantly lower turbidity as compared to the control protein sample, though there is no significant between the investigated HPβCD-concentrations. The relatively high error bars might be ascribed to process-related impreciseness due to the low sample volume and the high dilution factor. Meanwhile, increasing the concentration of HPβCD in the 100 mg/mL protein samples results in a stepwise lower turbidity. Here, highly concentrated IgG A (Figure II.13B) was more susceptible for aggregation than IgG B (Figure II.13D) and therefore, the protective effect of HPβCD is more pronounced for the IgG A samples. However, also concentrated IgG B was stabilized by high cyclodextrin-concentrations, and the 100 mM HPβCD sample resulted in even lower turbidity-values compared to the standard excipient polysorbate (Figure II.13D). To exclude possible particle-generating effects of the pure excipient solutions, placebo formulations without protein were identically stressed and measured. Independent of the excipient class or concentration, each placebo formulation resulted in low turbidity values, close to the turbidity of highly purified water (Figure II.13B). Therefore, higher turbidities in protein-containing formulations are caused by aggregated protein particles and not by the excipient itself.
Figure II.13: (Left) Turbidity of 1.8 mg/mL IgG A (Figure A) and IgG B (Figure C) after 60 minutes of stirring and afterwards **1:20 dilutions** with highly purified water. (Right) Turbidity of 100 mg/mL IgG A (Figure B) and IgG B (Figure D) after 60 minutes of stirring and afterwards **1:400 dilutions** with highly purified water. Stirred placebo formulations with the same excipient concentration but without protein are shown as points in Figure B. Reference shows turbidity of unstressed IgG without excipient (dilution-factor was the same as in the stirred samples).
In addition to turbidity measurements, light obscuration was applied (Figure II.14) which provides more information regarding particle numbers. [24] Here, stirring generated a remarkable increase in particle count where the 1.8 mg/mL IgG samples (Figure II.14A and C) resulted in roughly 200 000/mL cumulative particles after 20-fold dilution. Due to the low sample volume of the 100 mg/mL IgG solutions, a 400-fold dilution step was applied before each measurement. Despite that high dilution factor, there were still 60 000/mL to 80 000/mL cumulative particles detected in the 100 mg/mL IgG samples (Figure II.14B and D). For comparison, unstressed IgG A (which was analogously diluted and measured) showed less than 250/mL particles for the low, and less than 2000/mL particles for the concentrated sample. Similar as for the results of SEC and turbidity measurements (Figure II.12 and Figure II.13, respectively), the sucrose-containing formulation was comparable to the formulation without excipient (no statistically significant difference) indicating neither a stabilizing nor a destabilizing effect, while a significant stabilizing effect is shown when increasing the cyclodextrin concentration up to 100 mM for both the low- and high-protein-concentration formulations. It is worth noting that even the lowest tested HPβCD-concentration (2.5 mM) led to a significant reduction in cumulative particles for the 1.8 mg/mL IgG formulations (Figure II.14A and C). By contrast, a higher HPβCD-amount is required to significantly reduce the particle content in the 100 mg/mL IgG samples (Figure II.14B and D). Meanwhile, the polysorbate 80 sample resulted in a very low particle count (less than 1000/mL particles for IgG A, and less than 5000/mL particles for IgG B after dilution), far below the level that could achieved by the addition of CD and similar to the particle content of the starting material. The possibility of particle formation due to self-assembly of HPβCD, as reported earlier [53, 54], was excluded by control measurements for placebo-formulations (with the same excipient concentrations but without protein), which were treated in the same way as the protein-samples. All the tested controls had less than 1000 particles/mL, which was similar to the pure buffer sample (data not shown).
Summing up the results of the stirring experiments, stirring was demonstrated to be more aggressive compared to shaking [23] since a huge amount of particles was generated after a short stirring time of only 60 minutes. None of the tested formulations was capable to offer complete protein stabilization for both, soluble and insoluble aggregated protein species. The greatest difference between the respective formulations was evident by using light obscuration, and the presence of protein particles was already reported earlier as a very sensitive predictor of further aggregation. [28]

Both antibodies showed a high sensitivity to stirring stress which was seen in a sharp decrease in remaining monomer and a massive increase in particle numbers. By this, the higher concentrated formulations were more resistant against stirring stress since a much longer stirring time was required to induce a distinct monomer loss (Figure II.12). This observation is accordance to Treuheit et al. who demonstrated an inverse relationship...
between protein concentration and aggregation during mechanical stress. [17] It was concluded that the ratio between air/water interface and protein plays a more crucial role than the concentration of the protein itself. [17] Comparing both antibodies, IgG A showed a higher susceptibility for stirring-induced aggregation than IgG B – independent on the respective protein concentration. Regarding the effect of increasing concentrations of HPβCD on stabilization against mechanical stress, it is interesting to notice the same trend for the stirred dilute and concentrated IgG solutions, namely that increasing the concentration of HPβCD up to 100 mM (which is close to the solubility limit of HPβCD) leads to a progressive increase in monomer recovery and reduction of turbidity and particle count. This observation is in accordance to the shaking-stress experiment with low concentrated IgG B where a stepwise reduction of particles was seen with increasing HPβCD-concentrations (Figure II.11). In 1.8 mg/mL protein solutions, even the lowest HPβCD-concentration (2.5 mM) leads to significant protein stabilization which was, however, less distinct as observed during shaking stress (see Figure II.10 and Figure II.11). Increasing the cyclodextrin-concentration from 2.5 mM to 100 mM leads to a stepwise better stabilization against stirring stress. Similar to the 1.8 mg/mL samples, also highly concentrated IgG-formulations showed a slight stabilization after addition of only 2.5 mM HPβCD and a stepwise reduction in aggregation with increasing the cyclodextrin amount. Therefore, the stabilization effect seems to be independent of the protein:HPβCD ratio but more on the concentration of HPβCD itself. The good protein stabilization by HPβCD during shaking, and the lower protein stabilization during stirring indicates that stirring provokes additional effects such as shear or cavitation [23] which have a significant contribution to the mechanism of protein aggregation, and which are less affected by the presence of HPβCD. In each sample set, the polysorbate 80 formulation achieved the lowest amount of particles > 1 µm and the best monomer recovery by HP-SEC, but compared to that, relatively high turbidity-values. Differences between turbidity and light obscuration were already reported earlier [24] and are ascribed to the presence of larger amounts of small aggregates which was also previously seen for polysorbate containing protein formulations after mechanical stress. [23]

3.2.3 Viscosity measurements

Solutions of high polysaccharide concentrations are known to increase solution viscosity, which can reduce protein diffusion, and thus reduce aggregation. Additionally, high viscosity can pose a syringeability problem and reduce patient compliance, particularly for highly concentrated antibody solutions.[7] Accordingly, the viscosity of the concentrated IgG A solutions stabilized with different stabilizers was measured using the microchip technology of
the mVROC system. Results in Figure II.15 show that viscosities were generally rather low, where the 100 mg/mL protein formulation without excipient had a viscosity of only 2.6 mPas. Addition of HPβCD leads only to a moderate increase of solution viscosity up to 4.3 mPas for the highest tested HPβCD-concentration (100 mM). 250 mM sucrose in 100 mg/mL antibody solution leads to a viscosity level of roughly 4.0 mPas. The slightly higher viscosity of the 100 mM HPβCD ($M_W$ = 1400 g/mol) formulation compared to the 250 mM sucrose ($M_W$ = 342 g/mol) formulation is attributed to the higher molar mass of the former, since higher molar mass sugars increase the viscosity of MAb more than the smaller ones. [55]

In general, the increase in viscosity after addition of HPβCD is rather low and cannot be responsible for the observed reduction in aggregation. The sucrose formulation had a similar viscosity compared to the highest HPβCD concentration tested and did not show any stabilizing effect. Accordingly, one can exclude increased viscosity as an underlying mechanism for the reduction of aggregation that is observed with HPβCD.

![Figure II.15: Viscosity of 100 mg/mL IgG A-solutions in the formulation without excipient, with increasing HPβCD-concentrations, or with sucrose, as determined by an m-VROC viscometer at 25°C.](image)

### 3.2.4 Storage at elevated temperature

The influence of elevated temperature (50°C) on the aggregation of 1.8 mg/mL and 100 mg/mL antibody formulations was evaluated by storage for 7 or 14 days, respectively (Figure II.16). Results show that in low and high concentrations, IgG A was more susceptible for aggregation than IgG B which is consistent to the aggregation behavior in stirring stress experiments (Figure II.12). Furthermore, both mAbs exhibit a higher tendency for
aggregation in the highly concentrated formulations which can be seen in a monomer loss of approximately 20% after 7 days of storage, vs. 5% or even no monomer loss in case of diluted IgG A- and IgG B samples.

This effect is in contrast to the protein stability during stirring stress, where the low protein concentrations were more susceptible to the formation of aggregates (see above). Treuheit et al. reported a concentration-dependent difference between dilute and highly concentrated protein formulations during mechanical stress (agitation) and storage experiments, where low concentrated formulations were more prone to aggregation under mechanical stress, and highly concentrated formulations more during storage. [17] It was concluded, that for interfacially-driven aggregation such as agitation-stress, the protein-interface ratio plays a crucial role, whereas under quiescent storage, the frequency of protein collision (which is increased in higher concentrated protein-formulations) determines the rate of aggregation. [17] A further study by Saluja et al. demonstrated, that a 50 mg/mL protein solution generates significant higher amounts of soluble aggregates compared to a 5 mg/mL formulation under quiescent storage conditions, which was attributed to an increase in intermolecular protein interactions with higher protein concentrations. [16]

Figure II.16: Remaining monomer [%] of dilute and highly concentrated formulations of IgG A and IgG B without excipient after 0, 7, and (in case of 100 mg/mL IgG formulations) 14 days of storage at 50°C as determined by HP-SEC.

The effect of HPβCD, polysorbate, and sucrose on the stability of 1.8 mg/mL and 100 mg/mL IgG A- and IgG B-formulations during storage at 50°C is illustrated in Figure II.17. Here, both protein concentrations show the same trend, namely that 1) polysorbate does not influence protein aggregation over the studied time intervals, 2) 250 mM of sucrose lead to a
significant stabilization of the highly concentrated protein but not the dilute protein solutions, and 3) HPβCD concentrations ≥ 50 mM resulted in a progressive reduction of the monomer recovery with increasing cyclodextrin concentration both in case of 1.8 and 100 mg/mL IgG solutions. The decreased protein stability for the 50 mM and 100 mM HPβCD-formulations can be seen as hint for a direct interaction between the cyclodextrin and partially unfolded protein where a more denatured protein conformation is favored in the presence of HPβCD. The phenomenon of decreased thermal IgG-stability in the presence of HPβCD will be discussed in detail in the next section about thermal analysis. Anyhow, the results of the storage studies clearly demonstrate a difference between the disaccharide sucrose and the cyclodextrin-derivative HPβCD. A protein stabilizing effect similar to the preferential exclusion phenomenon of sugars such as sucrose or trehalose [27, 41-43] is therefore unlikely in the presence of HPβCD.
Chapter II

3.2.5 Thermal analysis

The conformational stability of both IgGs in the presence of different excipients was analyzed by determining the respective protein melting behavior. Firstly, melting points of 100 mg/mL IgG A-formulations were measured by FTIR-analysis, which is known as material saving, non-invasive method, and applicable for direct measurement at high protein concentration. [56, 57] Melting point analysis by FTIR was considered most suitable for proteins with initially high intramolecular β-sheet structures. [56] Second derivative spectra of 100 mg/mL IgG A in the native (25°C) as well as thermally denatured (95°C) conformation are illustrated in Figure II.18. The main band at 1636 cm$^{-1}$ in the native form indicates the predominant presence of intramolecular β-sheets. With increasing temperature, the main band at 1636 cm$^{-1}$
disappears, and a new band at 1622 cm\(^{-1}\) is formed which refers to the origin of intermolecular β-sheet structures. [56] For determination of the melting curves, respective adsorption ratios between 1636 cm\(^{-1}\) and 1622 cm\(^{-1}\) are calculated for each measured temperature point, and were plotted against the corresponding temperature (Figure II.19).

![Figure II.18: Changes in second derivative FTIR-spectra of 100 mg/mL IgG A-solution without excipient before (solid line) and after (dotted line) heating from 25°C to 95°C.](image)

The melting curves for each of the tested 100 mg/mL IgG A-formulations (without excipient, with addition of 25 mM HPβCD, and with addition of 0.1% polysorbate 80) show a perfect overlay (Figure II.19) which is also mirrored in the corresponding melting temperatures in Table II-1. Therefore, neither the addition of 25 mM HPβCD, nor the presence of 0.1% polysorbate affects the conformational stability of IgG A in the highly concentrated solution.
Figure II.19: Melting curves of 100 mg/mL IgG A-formulations containing no excipient, HPβCD 25 mM, and polysorbate 0.1% as determined by FTIR-measurements.

<table>
<thead>
<tr>
<th>sample</th>
<th>melting temperature $T_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>without excipient</td>
<td>74.45°C ± 0.20</td>
</tr>
<tr>
<td>HPβCD 25 mM</td>
<td>74.32°C ± 0.08</td>
</tr>
<tr>
<td>polysorbate 0.1%</td>
<td>74.44°C ± 0.56</td>
</tr>
</tbody>
</table>

Table II-1: Melting points of 100 mg/mL IgG A-formulations as determined from the inflection point of the melting curves in Figure II.19.

In general, measuring the protein melting behavior by FTIR-temperature ramps provides the great benefit of low sample volumes, and the capability to measure in a broad concentration range. [56] However, the applicability is limited by a comparably long duration of analysis and therefore, further measurements with highly concentrated IgG-formulations were performed by using a common DSC-device. Additionally, low concentrated protein samples were investigated by Microcal VP-DSC.

The thermograms of 1.8 mg/mL and 100 mg/mL samples (Figure II.20) show two melting peaks, which are related to the $F_{ab}$ and $F_c$ parts of the antibody. [58] Interestingly, IgG A exhibited a predominant first ($T_{m1}$) and a much smaller second ($T_{m2}$) melting point whereas IgG B shows a smaller $T_{m1}$ and a dominating $T_{m2}$. Thermograms of the respective samples containing 100 mM HPβCD are integrated into the graphs to illustrate the influence of the cyclodextrin on the melting behavior (Figure II.20). In all tested formulations, the cyclodextrin-formulation resulted in a shift of the melting-temperature to lower values.
The main melting-temperatures (that of the predominant peak) of all tested formulations are illustrated in Figure II.21. The results show that sucrose increased the $T_m$ - except in the 100 mg/mL IgG A formulation (Figure II.21B) - which is in accordance with the preferential exclusion phenomenon. [57] By contrast, polysorbate had no or only a minor influence on $T_m$. Meanwhile, the low concentrations of HPβCD did not or only marginally affect the melting temperature, but a continuous decrease in $T_m$ was observed with increasing cyclodextrin concentrations. Here, the decrease in melting temperature was more obvious for IgG A than for IgG B with 1.5°C - (Figure II.21A) and 3°C-decrease (Figure II.21B) in the 100 mM formulation for IgG A, and roughly 0.4°C-decrease in the 100 mM formulations for IgG B (Figure II.21C and D). Since the decrease in melting temperature by addition of HPβCD might be ascribed to an interaction between the cyclodextrin and the IgG, different melting
behavior of IgG A and IgG B after addition of cyclodextrin indicates a possible difference in the interaction between the two IgGs and HPβCD. Comparing the T_m of dilute solutions without excipients vs. concentrated protein solution, no significant difference was seen for IgG B whereas for IgG A, T_m is lower for the dilute sample compared to the concentrated one. A similar observation was reported for thermal analysis of two monoclonal antibodies, which showed a 2-3°C higher melting point when increasing the protein concentration from 1 to 100 mg/mL. [18] Stabilization of proteins due to higher concentrations was ascribed to the excluded volume theory in crowded environments which hinders the protein from unfolding.

The results of the melting temperature (Figure II.21) are reflected in the outcome of quiescent storage at 50°C (Figure II.17), where the presence of increasing HPβCD-concentrations lead to a gradually destabilization of the protein. Predictability of protein stability during long time storage based on Tm-measurements was already shown before for a monoclonal antibody. [60] Interestingly, despite the gradual decrease in conformational protein stability in the presence of increasing HPβCD-concentrations, the resistance against mechanically induced aggregation (see previous shaking and stirring experiments) was increased.

The mechanism of protein stabilization against mechanical agitation observed with cyclodextrins is highly debated in the literature. Many investigators report the binding of cyclodextrins to hydrophobic residues as the underlying cause of stabilization. [61, 62] For instance, cyclodextrins can form host-guest complexes with the hydrophobic amino acids (mainly Phe, Tyr and Trp) of some proteins, such as hGH and LHRH [21, 63-66], leading to inhibition of aggregation. Others hold the surface activity of some cyclodextrins (e.g. HPβCD) responsible for their stabilizing activity. For instance, the effectiveness of HPβCD in reducing interfacially-induced precipitation of porcine growth hormone was ascribed to the surface activity of HPβCD. [67] In another study, the proposed relationship between the interfacial stabilization of rh-GH by HPβCD and surface activity of HPβCD was substantiated by correlating increasing degrees of substitution of HPβCD (that translate into increasing surface activity) to reduced amounts of aggregates in vortexed rh-GH formulations. [47] In the current study, the progressive increase in stability with increasing concentrations of HPβCD concentrations point out to the possibility of a direct interaction between IgG and the cyclodextrin rather than a surface effect.

A cyclodextrin-induced decrease in melting temperature was reported for globular proteins in the presence of α-cyclodextrin, whereas the shift in melting temperature was ascribed to a weak non-covalent interaction between the cyclodextrin and hydrophobic side chains of the unfolded protein. [68] In a further study with lysozyme, presence of hydrophilic cyclodextrins in a concentration of 100 mM lead to a significant reduction of the protein melting
temperature via stabilizing an unfolded protein conformation, but chemically induced protein-aggregation was significantly reduced. [69] The hypothesis of a binding between HPβCD and native/unfolded IgG A and IgG B was studied in detail by quartz crystal microbalance and light-scattering experiments, and will be extensively discussed in chapter IV.

Figure II.21: Protein melting temperature of (Left) 1.8 mg/mL IgG A (Figure A) and IgG B (Figure C) and (Right) 100 mg/mL IgG A (Figure B) and IgG B (Figure D).
Highly concentrated protein solutions often implicate an increased solution viscosity which limits their syringeability and usability for parenteral application. [7, 70] The formation of an intermolecular protein network by protein-protein interactions is held responsible for the increase in viscosity and was studied in detail by rheological measurements. [10, 11, 71] A further study with concentrated and higher viscous antibody-solutions showed that the intermolecular protein-association is mainly driven by interactions between the Fab-parts of the antibodies. [15] The extent of intermolecular protein interactions is strongly dependent on the ionic strength of the solution; therefore, addition of salts is commonly applied to reduce the protein solution viscosity. Besides the salt concentration, also the nature of the ion represents a crucial factor for reducing the viscosity whereas chaotropic anions showed the most pronounced effect. [14, 15] In the following experimental series, the sodium salt of sulfobutylether-beta-cyclodextrin (SBEβCD) was investigated by measuring the viscosity of a 100 mg/mL IgG formulation to get information about the potential chaotropic nature of the SBEβCD-anion and its effect on the solution viscosity.

**3.3.1 Viscosity-measurements**

A third monoclonal antibody (IgG C) was chosen for the present study since IgG C exhibits high viscosity-values with increasing protein concentration which needs to be improved. Figure II.22 shows an initial viscosity-value of roughly 12.5 mPa*s for 100 mg/mL IgG C when formulated in pure buffer (10 mM phosphate) without further excipient. Addition of 25, 50, and 75 mM sodium chloride causes a stepwise decrease in solution viscosity to about 6.5 mPa*s for the highest salt concentration tested. By contrast, addition of HPβCD showed a moderate viscosity increase, which was also apparent in 100 mg/mL IgG A-solutions (Figure II.15). Interestingly, despite the similar molecular weight of HPβCD and SBEβCD, addition of SBEβCD leads to an opposite effect, and causes a clear reduction in solution viscosity (Figure II.22). Comparing equimolar concentrations of SBEβCD ($M_w = 2200$ g/mol) and sodium chloride ($M_w = 58$ g/mol), the cyclodextrin was more effective in reducing the viscosity of the highly concentrated protein formulation.

The reduction of protein solution viscosity by addition of sodium chloride was frequently reported elsewhere [7, 14, 15, 55, 72], and can be explained by the Hofmeister series of ions. Hofmeister’s theory categorizes ions into large, less hydrated (chaotropic) ions and small, tightly hydrated (kosmotropic) ions. [73] The lower hydration of chaotropic ions facilitates an interaction between the ion and apolar sites on the protein surface. [73, 74] By
contrast, kosmotropic ions (which are also declared as “salting-out agents”) are strongly hydrated which hinders them from interaction with the protein surface. [75] The preferential ion-interaction with water is also held responsible for the protein stabilizing effect of kosmotropes. [75] Due to the interaction between chaotropic ions and the protein surface, the intermolecular protein-network is disrupted and the protein-viscosity in high protein concentrations is reduced. [10, 15] At this, protein-interaction with ions was mainly seen for anions, whereas cations showed a much lower effect. [15, 74] Therefore, reduced solution viscosity in the presence of SBEβCD is a hint for the chaotropic nature of the SBEβCD-anion, and an interaction between SBEβCD and the protein surface seems presumable. By contrast, addition of 25 mM SBEβCD resulted in a slight viscosity-increase for a 1.8 mg/mL IgG-formulation [19] which shows the dominating contribution of sugar concentration on the solution viscosity in low protein concentrations. [76]

Figure II.22: Sample viscosity of 100 mg/mL IgG C in the presence of increasing concentrations of SBEβCD, HPβCD, and NaCl, as determined by mVROC-measurements.

3.3.2 Influence of SBEβCD on protein stability

Applicability of SBEβCD as viscosity-reducing excipient was further investigated in stress-experiments to evaluate the protein stability in presence of the cyclodextrin. For this, mechanical and thermal stress was applied for the 100 mg/mL IgG C-formulations with addition of 10 and 25 mM SBEβCD (which was the minimum concentration to achieve the greatest reduction in viscosity). As a control, the same SBEβCD-concentrations were tested in combination with 0.1% polysorbate 80 (mechanical stress) and 250 mM sucrose (thermal
stress) to see, if SBEβCD has an influence on the protein-stabilizing properties of both excipients.

3.3.2.1 Stability against mechanical stress

Protein stability under mechanical stress was analyzed by stirring the highly concentrated IgG C-solutions for 72 hours (Figure II.23). Comparable to IgG A and IgG B, stirring the formulation without excipient leads to significant protein aggregation, and roughly 40% monomer-loss was detected after three days (Figure A). At the same time, dimer-content increases from 2% to 4.5% over the duration of the experiment (Figure B). Addition of both SBEβCD-concentrations showed no effect on monomer- and dimer-amount compared to the formulation without excipient. This is in accordance to the stirring-results for IgG A where no effect on protein stability was seen in the 25 mM SBEβCD-formulation (Figure II.1). Furthermore, also stirring of low concentrated IgG with 25 mM SBEβCD was reported to be without impact on protein stability. [19] By contrast, polysorbate 80 was effective in protecting the protein from aggregation which is seen in a higher monomer recovery and a lower dimer-formation compared to the formulation without excipient (Figure II.23). The protein-stabilizing effect of polysorbates during mechanical stress was extensively discussed above, and was not affected by the presence of SBEβCD since the formulations containing SBEβCD and polysorbate resulted in the same monomer- and dimer-amounts as the formulations with polysorbate only.

![A) Monomer](image1)

![B) Dimer](image2)

Figure II.23: (A) Remaining monomer [%] and (B) fraction of dimer [%] after stirring 100 mg/mL IgG C-formulations for 0, 24, 48, and 72 hours, as determined by HP-SEC.
3.3.2.2 Stability against thermal stress

For evaluating protein stability in different formulations during thermal stress, 100 mg/mL IgG C-formulations were analyzed in the formulation without excipient, and in presence of 10 and 25 mM SBEβCD, 250 mM sucrose, and respective excipient-combinations. Samples were stored for 10 days at 50°C, and remaining monomer and soluble aggregates were analyzed after 0, 3 and 10 days by HP-SEC (Figure II.24). Comparing the monomer-recoveries of the different formulations (Figure A), a slight loss in monomer was determined for the 2.5 mM SBEβCD-formulation, but all the other formulations showed no significant trend indicating protein stabilization or destabilization. However, a stepwise rise was seen in the percentage of dimer when increasing the SBEβCD-concentration to 25 mM (Figure B). The negative effect of SBEβCD on proteins’ thermal stability was already reported before [19], and can also be seen in the storage-experiments of IgG A (Figure II.8). Even the presence of sucrose was not effective to counterbalance the negative effect of the cyclodextrin which indicates that the chaotropic nature of the SBEβCD-anion leads to a destabilization of IgG C, and the preferential exclusion effect of sucrose was not sufficient for preventing the protein from an interaction with the cyclodextrin.

![Figure II.24](image_url): (A) Remaining monomer [%] and (B) fraction of dimer [%] after storage 100 mg/mL IgG C-formulations at 50°C for 0, 3, and 10 days, as determined by HP-SEC.

The fundamental effect of SBEβCD on thermal stability of IgG C was further evaluated by microcalorimetry. Since the occurrence of an interaction between cyclodextrin and protein is expected to be independent on the protein concentration (which was also shown for HPβCD (see Figure II.20)), low concentrated IgG-formulations were investigated for material saving reasons. Thermograms of 1.8 mg/mL IgG C without excipient and with addition of 25 mM
SBEβCD are illustrated in Figure II.25 and show a slight reduction of the melting temperature from 76.6°C (formulation without excipient) to 76.3°C (with addition of 25 mM SBEβCD). Despite similar protein melting points in both formulations, the graphs show that the onset of protein unfolding is shifted to lower temperatures in the presence of SBEβCD. Therefore, the interaction between SBEβCD and the antibody leads not only to a disruption of the intermolecular protein network and a reduction in solution viscosity, but also to a reduction in the resistance of the protein against thermal stress.

**Figure II.25:** Melting curve of 1.8 mg/mL IgG C-solution with / without addition of 25 mM SBEβCD as determined by Microcal measurements. The shown curves are obtained as average of three measurements.
4. Conclusions

In the present chapter, the influence of cyclodextrins on different aspects of protein stability was discussed. In the beginning, protein aggregation of a 100 mg/mL IgG-formulation was investigated in the presence of three different cyclodextrins (HPβCD, SBEβCD, MβCD) in low and medium concentrations (2.5 and 25 mM). Here, HPβCD and MβCD were effective in stabilizing the protein against mechanical stress. However, the polysorbate 80 reference was significantly superior in preventing the protein from the formation of insoluble particles. No influence of any tested excipient was observed for the light-induced aggregation rate, but a slight protein-destabilization was observed for the higher concentrated cyclodextrin formulations during thermal stress.

Afterwards, the stabilizing properties of HPβCD were extensively studied by using two different monoclonal antibodies. Here, the effect of a broad concentration range of HPβCD (2.5 mM to 100 mM) on antibody stability was investigated in low (1.8 mg/mL) as well as high (100 mg/mL) protein concentrations. All different stress-studies resulted in similar observations for HPβCD regarding protein stability, independent on protein concentration. During mechanical stress, the cyclodextrin showed a stabilizing effect on protein aggregation whereas the stabilizing potential gradually increased with increasing cyclodextrin concentrations. However, for both proteins and for high and low protein concentration, the control-formulation containing polysorbate 80 was more effective in protein stabilization, as clearly seen in the number of insoluble aggregates. The marginal increase in solution viscosity upon addition of HPβCD refutes the assumption that the protein-stabilizing effect of HPβCD against mechanical stress is solely caused by the rheological properties of the formulation. Contrary to the stabilizing effect of HPβCD during mechanical stress, increasing HPβCD-concentrations lead to a stepwise reduction in the resistance against thermal stress which was mirrored in a decrease in protein melting temperature. In general, the effect of increasing concentrations of HPβCD on the mechanical and thermal stability of both IgGs was the same for dilute and concentrated IgG solutions and seems independent of the protein : HPβCD ratio but more on the concentration of HPβCD itself. The decrease in respective protein melting temperature indicates an interaction between HPβCD and both antibodies. Whether this is a direct interaction through complexation, or an effect of cyclodextrin on the water structure is not clear at the moment.

Finally, the sodium salt of SBEβCD was demonstrated to be effective in reducing the viscosity of a 100 mg/mL IgG-solution, and the viscosity-reduction in presence of SBEβCD was more pronounced compared to an equimolar concentration of sodium chloride. Stability tests with highly concentrated IgG showed that SBEβCD had no effect on the protein stability during mechanical stress, but a slight protein-destabilization was seen under thermal stress.
conditions. It is speculated, that there is an interaction between the surface of the antibody and SBEβCD which leads on the one hand to a reduction of intermolecular protein interactions, and on the other hand to a reduced conformational protein stability.

Based on the results of this study and on previous studies performed in our lab regarding the stabilization of proteins using HPβCD [19, 20, 77, 78], one can conclude that high concentration of cyclodextrins (such as HPβCD) is probably not a viable option to stabilize high concentration MAb formulations, and that polysorbate 80 represents a better option, so long its degradation issue is controlled. Additionally, the observed differences regarding the effect of HPβCD on the stability of IgG A and IgG B point to the different cyclodextrin-interaction with the proteins, probably due to differences in structure, surface distribution of hydrophobic amino acids, and/or charge pattern. This highlights the need to investigate the effect of CD on the protein on a case by case basis. Finally, the observation made by Serno et al., that low concentration HPβCD can stabilize dilute protein solutions against mechanical (shaking) stress without negatively affecting the thermal stability [20] remains a promising application for the use of cyclodextrins and the circumvention of polysorbate-associated protein-instability issues. [79]
5. References


Chapter III

The role of polysorbate 80 and HPβCD at the air-water interface of IgG solutions

Tim Serno, Elisabeth Härtl, Ahmed Besheer, Reinhard Miller, Gerhard Winter

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Experimental work was performed by Elisabeth Härtl and Tim Serno, writing by Elisabeth Härtl, Tim Serno, and Ahmed Besheer, and corrections by Reinhard Miller and Gerhard Winter.

Declaration:

The present work was performed in cooperation with Dr. Tim Serno. Following data of the present chapter were not generated by my own experimental work but extracted from his dissertation:

- Figure 3
- Figure 4A
  data points of polysorbate $5 \times 10^{-7}$M, $1 \times 10^{-5}$M, $3 \times 10^{-5}$M
- Figure 4B
  data points of HPβCD $1 \times 10^{-4}$M, $2.5 \times 10^{-4}$M, $1 \times 10^{-3}$M
- Figure 4C
- Figure 5A
  data point of IgG $1 \times 10^{-6}$M
  data points of polysorbate $1 \times 10^{-7}$M, $5 \times 10^{-7}$M, $1 \times 10^{-5}$M, $3 \times 10^{-5}$M
  data points of IgG $1 \times 10^{-6}$M + polysorbate $1 \times 10^{-7}$M, $5 \times 10^{-7}$M, $1 \times 10^{-6}$M, $1 \times 10^{-5}$M
- Figure 5B
  data point of IgG $1 \times 10^{-6}$M
  data points of HPβCD $1 \times 10^{-4}$M, $2.5 \times 10^{-4}$M, $1 \times 10^{-3}$M
  data points of IgG $1 \times 10^{-6}$M + HPβCD $1 \times 10^{-4}$M, $2.5 \times 10^{-4}$M, $1 \times 10^{-3}$M
**Abstract**

**Purpose** To test the hypothesis of surface displacement as the underlying mechanism for IgG stabilization by polysorbates and HPβCD against surface-induced aggregation.

**Methods** Adsorption/desorption kinetics of IgG-polysorbate 80-/HPβCD were monitored. Maximum bubble pressure method was used for processes within seconds from surface formation. Profile analysis tensiometry was applied over long periods and to assess surface rheologic properties. Additionally, the kinetics of adsorption, desorption and surface displacement was followed by a double-capillary setup of the profile analysis tensiometer, allowing drop bulk exchange.

**Results** Weak surface activity for HPβCD vs. much higher surface activity for polysorbate 80 was shown. Protein displacement when exceeding a polysorbate 80 concentration close to the CMC and a lack of protein displacement for HPβCD was observed. The drop bulk exchange experiments show IgG displacement by polysorbate 80 independent of the adsorption order. In contrast, HPβCD coexists with IgG at the air-water interface when the surface layer is built from a mixed IgG-HPβCD-solution. Incorporation of HPβCD in a pre-formed IgG-surface-layer does not occur.

**Conclusions** The results confirm surface displacement as the stabilization mechanism of polysorbate 80, but refute the frequently held opinion, that HPβCD stabilizes proteins against aggregation at the air-water interface in a manner comparable to non-ionic surfactants.

**KEY WORDS**

antibody, drop profile analysis, hydroxypropyl-beta-cyclodextrin (HPβCD), polysorbate, surface dilational rheology
1. Introduction

Polysorbates as well as cyclodextrins (CDs) are valuable excipients for the prevention of surface-induced protein aggregation, as encountered for example during agitation of liquid protein formulations associated with exposure of the protein to the air-water interface [1-7]. While polysorbates are well established stabilizers already present in many marketed formulations, they suffer from a number of shortcomings, such as in some cases an increased tendency of protein oxidation and aggregation upon quiescent storage at elevated temperature [2, 3, 8]. In contrast, the stabilizing potential of the CD-derivative hydroxypropyl-β-cyclodextrin (HPβCD) against surface-induced aggregation is not accompanied by protein oxidation or compromised by aggregation during storage [6]. Furthermore, HPβCD possesses a favorable toxicological profile as excipient for parenteral administration, considering that doses as high as 8-16 g/day are administered to patients in approved parenteral products [9]. Therefore HPβCD can be considered a valuable alternative to non-ionic surfactants.

From a mechanistic point of view, the stabilizing effect of polysorbates has been extensively studied, and most studies link protein stabilization against surface-induced aggregation to the competition between protein and surfactant at the air-water interface [2, 3, 8, 10-13]. For example, the adsorption of polysorbate 80 in the presence of recombinant Factor VIII (280 kDa) was studied using a Wilhelmy Plate tensiometer, where the steady state interfacial behavior was shown to be entirely governed by surfactant adsorption [13]. Another study investigated the rheological, structural and mechanical properties of mixed adsorption layers of bovine serum albumin (BSA) and polysorbate 80 [14]. The study confirmed competitive adsorption between BSA and polysorbate 80, with almost complete displacement of the protein at high polysorbate 80 concentrations. However, to the best of our knowledge no detailed studies are available for monoclonal antibodies (mAbs) in the presence of polysorbates, with mAbs currently being by far the most widespread class of therapeutic proteins [15]. Polysorbate 80 was frequently reported to stabilize monoclonal antibodies [16, 17] and stabilization by competition at the air-water interface was implicitly assumed, however never directly demonstrated.

In comparison to polysorbates, very little is known so far about the stabilizing mechanism of HPβCD against surface-induced aggregation. Two possible mechanisms are discussed in the literature. In the first, the ability of CDs to bind to proteins and incorporate hydrophobic protein residues in their interior cavity is held accountable for their stabilizing effect [9, 18]. However, for the model proteins IgG and rh-GCSF, direct binding to CD-derivatives in bulk solution as a reason for aggregation inhibition was rendered unlikely by previous studies [6, 19].
Chapter III

The second possible stabilization mechanism points to the surface activity of some CD-derivatives [20], thereby potentially being able to compete with proteins at the air-water interface similar to non-ionic surfactants [1, 6, 7, 20]. For example, the surface activity of HPβCD was reported to strongly depend on the degree of substitution [7, 21, 22], with surface tension values between 69 mN/m and 52 mN/m reported for degrees of substitution ranging from 2.5 to 11.3. This surface activity was held by Charman et al. as the reason for the effectiveness of HPβCD in reducing interfacially-induced precipitation of porcine growth hormone with a mechanism analogous to that of polysorbate 20 [1]. In another study, the proposed relationship between the interfacial stabilization of rh-GH by HPβCD and surface activity of HPβCD was substantiated by correlating the increasing degrees of substitution of HPβCD (that translate into increasing surface activity) to reduced amounts of aggregates in vortexed rh-GH formulations [7]. Finally, a study measuring the surface-tensions of pure HPβCD and IgG-solutions as well as mixed IgG-HPβCD solutions using a Wilhelmy-plate tensiometer confirmed that both IgG and HPβCD are surface-active [6], and thus there is a high likelihood for competition at the air-water interface, though the ability of HPβCD to displace IgG at the interface was not shown.

It can be concluded that it is of high interest to study in detail the surface characteristics of IgG-polysorbate and of IgG-HPβCD solutions in order to get more insight into the stabilizing mechanisms of both excipients. In this study, the hypothesis of a competitive-displacement as the most likely mechanism of aggregation inhibition at the air-water interface is tested. To this end, surface tension measurements by maximum bubble pressure method for solutions of polysorbate 80, HPβCD, IgG and mixtures of IgG-stabilizer were performed to investigate the surface adsorption at short time scales. Moreover, surface tension measurements using drop profile analysis were performed to investigate surface adsorption at equilibrium. Additional drop profile analysis studies were performed using a special double-capillary-setup, which allows exchange of the bulk sample solution, thus shedding more light on the surface-displacement mechanisms and adsorption/desorption-kinetics at the air-water-interface. Concurrently, surface rheological properties were determined by surface dilational rheology in order to verify actual surface layer composition. In all the experiments, the adsorption behavior of polysorbate 80 with/without IgG was compared to that of HPβCD with/without IgG, and mechanistic conclusions on the stabilization principles of both excipients were drawn.
2. Materials and Methods

2.1 Materials

A monoclonal antibody (mAb) of the IgG class, that was also used in a previous stability study [6], was kindly donated by Roche Diagnostics GmbH, Penzberg, Germany. The IgG bulk material provided for this work was formulated in a 20 mM histidine buffer at pH 5.8. Bulk concentration was 2.4 mg/ml. Protein solutions were filtered through Acrodisc® 0.2 μm PVDF syringe filter units (Pall GmbH, Dreieich, Germany) prior to usage in all solutions. The total molecular weight of this particular antibody is 146.3 kDa as determined by MALDI mass spectrometry. HPβCD (pharmaceutical grade, average molecular weight 1400 g·mol⁻¹) was kindly donated from Wacker Chemie AG, Burghausen, Germany. Polysorbate 80 (average molecular weight 1312 g·mol⁻¹) was kindly donated from Croda Inc. (Edison, NJ, USA) in super-refined quality and used as received. Histidine was from Merck KGaA, Darmstadt, Germany.

2.2 Methods

2.2.1 Preparation of dilutions

All dilutions of the IgG were carried out into histidine buffer at a concentration of 20 mM and a pH of 5.8. Mixed IgG-HPβCD and mixed IgG-polysorbate 80 solutions were prepared from stock solutions of the respective excipients in 20 mM histidine buffer. Solutions were prepared with Milli-Q deionised water and the glassware used for preparation of the solutions was cleaned with concentrated sulphuric acid.

2.2.2 Maximum bubble pressure measurements

The dynamic surface tension of solutions of polysorbate 80, HPβCD or mAb alone as well as of mixed solutions of the mAb with either polysorbate 80 or HPβCD at short adsorption times was measured using the maximum bubble pressure technique. The basic principle of this analytical technique is the determination of the maximum pressure of a bubble that is growing at the end of a thin steel capillary (inner diameter 0.25 mm) which is immersed into the solution under investigation. The calculation of the surface tension using the maximum bubble pressure method is based on the Laplace equation:
\[ \gamma = \frac{(P - P_h) \cdot r}{2} \]

Here \( P \) is the maximum bubble pressure, \( P_h \) the hydrostatic pressure of the liquid and \( r \) the capillary radius. By determining the surface tension at different life times of the bubble, the dynamic surface tension is obtained. The advantage of the method over other methods for the determination of the dynamic surface tension is the possibility to measure already after a few milliseconds of surface age. The instrument used for these studies was the BPA-1P (Sinterface Technologies, Berlin, Germany).

2.2.3 Drop profile analysis and dilational shear rheology

Drop profile analysis was employed for detailed characterization of the dynamic surface tension of surface layers of pure excipients or IgG and also of mixed IgG-HPβCD as well as of IgG-polysorbate 80 solutions. The instrument used for these investigations was a Profile Analysis Tensiometer (PAT 1, Sinterface Technologies, Berlin, Germany). Some single capillary-measurements with polysorbate and IgG/polysorbate-samples were performed using PAT 2P (Sinterface Technologies, Berlin, Germany) which operates in analogous mode as PAT 1.

As indicated in Figure II.1, the basic principle of drop profile analysis is that the coordinates of the shape of a pendant drop of the studied solutions are recorded by a video camera and compared to its theoretical profile which can be calculated from the Gauss-Laplace equation. Thereby the dynamic surface tension, as the only free variable in the theory, can be obtained [14]. There is a balance of capillary and gravitational forces; whereas the surface tension acts to form a spherical drop, gravity acts oppositely giving the drop a prolonged shape.
2.2.4 Double-capillary experiments

A special setup of the drop profile analysis instrument (PAT I), using 2 concentric capillaries (Figure III.2), allows the exchange of the droplet bulk without changing its volume. While measuring the surface tension using the CCD camera, the internal capillary can (slowly) pump fresh liquid into the drop while the outer capillary drains an equivalent amount of the fluid, thus maintaining a constant drop volume [23-25].

Figure III.2: Double capillary setup for drop bulk exchange at PAT-I instrument.

For drop bulk exchange experiments, a droplet with a 13 mm$^3$ volume was first formed by the outer capillary. At specific time points, new solution was pumped into the drop via the inner capillary. Parameters for bulk exchange were $\Delta V=0.067$ mm$^3$ and $\Delta t=0.1$ s in which $\Delta V$ describes the exchanged volume per pulse and $\Delta t$ the time between two pulses. For all
exchange experiments, the drop was flushed in total with 2000 mm$^3$ new solution, which represents more than 150 fold of the actual drop volume. Duration of the exchange process was volume controlled and varied between 6500 and 7500 s. Different parameters may have an influence on exchange effectiveness [26], therefore the setup of the aforementioned parameters for exchange-procedure was initially adjusted by flushing a 13 mm$^3$ droplet of C$_{12}$DMPO (10$^{-4}$ M) with pure water.

The same setup was used to determine dilational rheological properties of the surface layers. For this purpose harmonic area oscillations of the drop at low frequency (0.01, 0.02, 0.04, 0.1, and 0.2 Hz) were performed by the dosing system, with droplet size oscillation from 12 mm$^3$ to 14 mm$^3$. The corresponding response of the surface-tension is measured and the elastic as well as the viscous contributions can be determined separately. Low frequencies of the oscillations are important in order to maintain the Laplacian shape of the drop [27].

3. Results

3.1 Maximum bubble pressure experiments at short adsorption time scales

Measurements using the maximum bubble pressure method (MBPM) were performed to evaluate the surfactant and protein adsorption directly after the formation of the air-water interface. Results in Figure III.3 show the dynamic surface tension of HPβCD, polysorbate 80 and the IgG solutions in histidine buffer at concentrations that were previously used in an earlier study demonstrating the effectiveness of the excipients against surface-induced aggregation [6]. It can be seen that polysorbate 80 alone lowers the surface tension much faster and to a higher extent as compared to HPβCD. Even at the first value that was recorded (33 ms) the surface tension of the polysorbate 80 solution (65.04 mN/m) is already substantially lower than the surface tension of the pure histidine buffer (72.6 – 73.4 mN/m). This is an indication that the de novo surface is very rapidly occupied by polysorbate 80 when employed at this concentration (3 x 10$^{-5}$ M = 0.004%).

By contrast, HPβCD only leads to a very slight decrease of surface tension during the experiment, which is probably due to its lower surface activity. Interestingly, in the mixed IgG-stabilizer solutions, the adsorption of polysorbate 80 exhibits a lag phase of about 1 s before a measurable decay of the surface tension is observed. In contrast, the IgG-HPβCD solution does not show a significant reduction in the surface tension directly after the formation of a new air-water interface.
Chapter III

3.2 Surface tensiometry by drop profile analysis

In order to gain a better understanding of the adsorption behavior of IgG-polysorbate 80 and IgG-HPβCD drop profile analysis was chosen as a different experimental approach. The basic idea was to investigate more diluted solutions as compared to the actual formulations’ concentrations used earlier [6], in order to create conditions under which the adsorption processes and possible competition mechanisms occur at a slower time scale, which can then actually be followed in detail. By making the adsorption behavior “visible” at lower concentrations, it was expected to obtain conclusions which also apply on the actual formulations by extrapolating to higher concentrations and hence faster adsorption rates.

Figure III.3: Dynamic surface tension of solutions of polysorbate 80, HPβCD and the IgG as well as their respective mixtures as determined by the maximum bubble pressure technique. Note that x-axis is in logarithmic time scale.
Before studying the mixtures of HPβCD or polysorbate 80 with IgG, every component was investigated separately, as seen in Figure III.4A–C. Equilibration at the air-water interface is rather slow, however all equilibrium surface tensions that were observed in this experiment lie in the same range as the values that were determined earlier by different experimental methods [7, 21, 22, 28]. Figure III.4A shows the adsorption profile of different concentrations of polysorbate 80 in histidine buffered solution. Polysorbate 80 shows a strong surface activity, with a significant decrease in surface tension with increasing concentrations. At concentrations above $1 \times 10^{-5}$ M the surface tension slightly increases again, which refers to
the critical micellar concentration (CMC) value of the system. The CMC-values for polysorbate 80 that are reported in literature vary significantly due to the chemically heterogeneous nature of polysorbate 80. The concentration of $1 \times 10^{-5}$ M determined for the present system is well in the (lower) range of reported values [12, 14, 29, 30]. Meanwhile, the adsorption kinetics of increasing concentrations of HPβCD are shown in Figure III.4B. The data confirm that HPβCD possesses (comparably weak) surface activity as evidenced by the drop in surface tension with increasing concentrations. Contrary to polysorbate 80, HPβCD does not show a CMC in the studied range (up to $7.5 \text{ M} \approx 1\% \text{ w/v}$), in accordance with previous studies, which reported that HPβCD does not show a CMC at concentrations up to 7% [31].

In Figure III.4C, the dynamic surface tension of the pure IgG at different concentrations is shown. It can be seen that at the lowest investigated concentration ($1 \times 10^{-8}$ M) a long induction period (approximately 80,000 s, which corresponds to 22 h) precedes measurable adsorption to the air-water interface. In contrast, lysozyme in comparable concentration showed a relatively short induction period of about 10,000 s as determined by the same method at comparable concentrations [32]. One possible reason for the difference in induction period could be size of both proteins (146 kDa for IgG vs. 14.3 kDa for Lysozyme). Because of its large molecular weight, the diffusion of IgG to the subsurface from which adsorption to the air-water-interface takes place might occur rather slowly [33]. It can be speculated that also the different degree of charge repulsions between the protein molecules could influence the diffusion time to the subsurface. In addition, the induction period also depends on the structural stability of the investigated molecule, because unfolding of the adsorbed proteins at the interface contributes to the surface pressure. More flexible, non-globular proteins such as β-casein partially unfold faster and therefore show shorter induction periods [32, 34]. It is also worth noting, that the observed adsorption profile shows differences to the published adsorption profile of another IgG [33]. Whereas for the IgG investigated in the current study the equilibrium surface tension reaches a steady value of about 53 mN/m beginning at concentrations of $1 \times 10^{-7}$ M, the published results reveal a saturation of the interface at concentrations as high as $2 \times 10^{-5}$ M also at a surface tension of about 53 mN/m. However, it has to be taken into account that IgG adsorption was followed for different time periods in the two studies which renders comparison of the surface tension values difficult.
For the analysis of the mixed solutions of the IgG with polysorbate 80 or HPβCD, a constant IgG-concentration of $1 \times 10^{-6}$ M was chosen. This concentration was considered as a compromise between a reasonable time to achieve equilibrium conditions (80,000 s) and an initial adsorption that is slow enough to allow mechanistic observations without interference from multilayer protein adsorption. The equilibrium surface tension of the IgG in the absence of any excipients is indicated by a straight line in Figure III.5, and the equilibrium surface tension of the pure polysorbate 80-solution and the pure HPβCD-solution at different concentrations are also included into Figure III.5 for comparison. As observable from Figure III.5A, at low surfactant concentrations, the surface tension of the polysorbate 80-IgG mixture is lower than that of the pure surfactant solution. However, the values of the mixture more or less match the value of the pure IgG solution (about 53 mN/m). Increasing polysorbate 80 concentrations from $1 \times 10^{-7}$ M to $1 \times 10^{-6}$ M does not lower the surface tension of the mixture. These findings indicate the dominating contribution of the IgG to the composition of the adsorption layer of the mixture in this concentration range. When the polysorbate 80 concentration is further increased, the surface tension of the mixed solution drops to a value that is very close to that of the pure polysorbate 80 solution and significantly below that of the pure IgG solution, which strongly suggests that beginning from a concentration of $1 \times 10^{-5}$ M, polysorbate 80 dominates the surface layer of the IgG-polysorbate 80 mixture, and this concentration is close to the CMC of the pure polysorbate 80 solution as discussed above.
For the mixed IgG-HPβCD solution, a very different surface-tension isotherm than for the IgG-polysorbate 80 system is obtained, as shown Figure III.5B. No matter how high the concentration of HPβCD, the surface tension of the mixture changes only slightly. Moreover, the surface tension of the mixed IgG-HPβCD solutions is higher than the surface-tension of the pure IgG, even at the lowest HPβCD-concentrations.

3.3 Double capillary experiments

The behavior of polysorbate 80, HPβCD, and IgG was further analyzed by PAT-measurements using the double-capillary-setup, with drop bulk-exchange during surface tension measurement. PAT experiments with the double-capillary-setup were performed as single- and double-exchange studies. In the single-exchange studies, the droplet bulk was exchanged with new solution once, while in the double-exchange studies, two different solutions are consecutively exchanged with the droplet bulk. The IgG concentration was kept constant at $1 \times 10^{-6}$ M. Polysorbate 80 was measured at $1 \times 10^{-5}$ M and $2.5 \times 10^{-5}$ M; HPβCD at $2 \times 10^{-4}$ M and $1 \times 10^{-3}$ M. Results for both excipient concentrations provided the same conclusions, therefore only the results of one dataset are shown.

3.3.1 Single exchange studies

Single exchange experiments allow drawing conclusions about the reversibility of adsorption for each single component as well as the excipient-IgG-mixtures. The timeline for the measurements is shown in Figure III.6.
Figure III.6: Timeline for single-exchange experiments. Sample solution is illustrated by black color and pure histidine buffer by light grey color. Surface tension of the sample solution is measured for 5000 s. From 5000 s to 6550 s, the first oscillation was performed to measure the rheological properties of the surface. Afterwards, the droplet bulk was replaced by pure histidine buffer between 6600 and 13000 s, and finally rheological properties of the surface were measured again between 14550-16100 s.

As seen in Figure III.7A and B, the surface tension of pure IgG solution showed a rapid initial reduction followed by a slower reduction till 5000 s, where the bulk was exchanged against histidine buffer. This bi-phasic reduction in surface tension probably reflects the diffusion of the protein to the surface in the first phase followed by a slower diffusion of the protein to the already occupied surface and/or possible protein unfolding or interfacial rearrangements at the interface. The bulk exchange with pure histidine buffer did not affect the IgG adsorption process, which is demonstrated by the unmodified monotonous decrease of the surface tension. This continuous reduction of the surface tension despite the depletion of the protein from the bulk might be due to slow conformational changes and unfolding of the already adsorbed protein at the interface, leading to exposure of its hydrophobic residues.
The surface tension of pure polysorbate 80 solutions is shown in Figure III.7A. Polysorbate 80 turns out to be a relatively “sticky” surfactant, so that even after flushing the drop with 2000 mm$^3$ histidine buffer (>150 fold droplet volume), the surface tension reaches a plateau value which is lower than that of the pure buffer, indicating the presence of traces of polysorbate at the surface. Interestingly, the mixture of IgG and polysorbate 80 exhibits a very similar behavior as compared to the pure polysorbate solution. For the mixture the drop in dynamic surface tension is much steeper than in the case of pure IgG and identical to that of the pure polysorbate. After exchanging the droplet bulk with buffer, the surface tension increases similar to the pure polysorbate and does not maintain the monotonous reduction as in the case of pure IgG.

Contrary to polysorbate 80, adsorption of the pure HPβCD solution was completely reversible (Figure III.7B). After bulk exchange, surface tension of the pure buffer is recovered rather rapidly (within approximately 1000 s). However, the IgG-HPβCD-mixture behaves differently from either the pure IgG or HPβCD solutions. Before bulk exchange, the reduction in the dynamic surface tension is relatively steeper at the beginning compared to pure IgG or HPβCD. After buffer exchange, an increase of surface tension similar to pure HPβCD solution is observed and lasts approximately 1000 s, nearly the same period required to re-establish the surface tension of the histidine buffer in case of the pure HPβCD solution. After that, a strong kink occurs and surface tension starts to decrease analogous to the pure IgG.
solution. Hence it can be speculated that HPβCD was washed out by buffer exchange while IgG remains on the surface. This points out to the concomitant presence of both components in the surface layer before starting the exchange process.

The drop oscillations used to measure surface rheological properties are visible in the surface tension curves as large fluctuations. Large amplitudes are characteristic of less flexible molecules, such as protein [23], which form a very thin “membrane” on the surface that is compressed and expanded during the oscillation process. Components with lower surface adsorption energy compensate changes in surface area by fast adsorption/desorption processes. Therefore a surface layer covered by surfactant results in low amplitudes during oscillation. Samples containing HPβCD, polysorbate 80, and IgG-polysorbate 80-mixture show low magnitude of surface tension changes during oscillation. This further confirms the absence of protein in the surface layer for mixed IgG/polysorbate 80 solutions. In contrast, the amplitude of IgG-HPβCD-mixtures at the first oscillation before buffer exchange shows a medium amplitude between pure IgG and HPβCD, and increases significantly in the second oscillation after buffer exchange. These observations additionally illustrate that the 2 components coexist at the interface before washing, and that HPβCD but not the protein is washed out during buffer exchange.

The above qualitative assessment was quantified by evaluating the surface rheology at five different oscillation frequencies: 0.01, 0.02, 0.04, 0.1, and 0.2 Hz. Results for surface elasticity and viscosity are illustrated in Figs. 8 and 9. The protein exhibits the highest elasticity values, while HPβCD has the lowest value and polysorbate 80 is somewhere in between. Comparable to the results of surface tension measurements, the mixture of IgG/polysorbate 80 exhibits the same rheological properties as the pure polysorbate 80 solution before and after buffer exchange. In contrast, the IgG-HPβCD-mixture exhibits values between the pure IgG-and HPβCD-solutions during the first oscillation (Figure III.8A and Figure III.9A) whereas after buffer exchange, the rheological properties are quite similar to those of the pure IgG.
Figure III.8: Surface elasticity of polysorbate 80 2.5x10^{-5} M, HPβCD 1x10^{-3} M, IgG 1x10^{-6} M, and respective mixtures before (A) and after (B) buffer exchange.

Figure III.9: Surface viscosity of polysorbate 80 2.5x10^{-5} M, HPβCD 1x10^{-3} M, IgG 1x10^{-6} M, and respective mixtures before (A) and after (B) buffer exchange.
3.3.2 Double exchange studies

To draw further conclusions about surface-displacement of IgG by excipients, sequential adsorption experiments were performed in double-exchange studies, with the timeline shown in Figure III.10.

![Timeline for double exchange experiments](image)

**Figure III.10:** Timeline for double exchange experiments. IgG solution is illustrated by black color, excipient solution by light grey, and pure histidine buffer by dark grey color. Pure IgG solution was used to build the surface layer at the beginning. After the first oscillation (5000-6550 s), polysorbate 80- or HPβCD-solution was pumped into the drop. Oscillations were performed again (14550-16100 s) and droplet bulk was exchanged afterwards with histidine-buffer. Measurement was finished at 25650 s after the third oscillation.
Figure III.11: Dynamic surface tension curves for double exchange studies using polysorbate 80 2.5x10\(^{-5}\) M (A) or HP\(\beta\)CD 1.0x10\(^{-3}\) M (B).

Figure III.11A shows that upon exchange of the bulk IgG with polysorbate 80, the latter rapidly displaces the IgG from surface and reduces the surface tension dramatically reaching values similar to pure polysorbate 80 (c.f. Figure III.7A). The second exchange against buffer resembles the results of the single exchange experiments for IgG/polysorbate mixture (Figure III.7A), which indicates a rather complete protein replacement from the surface layer. The low oscillation amplitudes during the second and third oscillation further confirm the absence of protein on the surface.

On the other hand, HP\(\beta\)CD seems not to affect the adsorption process of IgG (Figure III.11B), where the drop bulk exchange of IgG against HP\(\beta\)CD results in similar surface tension values as seen for exchange of IgG against pure histidine buffer (Figure III.7). The second washing with pure buffer did not result in a rapid and short increase in surface tension as seen for the pre-mixed IgG/HP\(\beta\)CD-solutions (Figure III.7B). Furthermore the amplitudes of the second and third oscillations (Figure III.11B) did not decrease in intensity, indicating that an inclusion of HP\(\beta\)CD into an already-adsorbed protein layer on the surface probably did not take place.

Information about surface rheology from the drop oscillations, before and after drop-bulk-exchange, corroborate the above results, where flushing the IgG droplet with polysorbate 80 leads to a clear reduction of surface elasticity and viscosity (Figure III.12A and Figure III.13A - second oscillation). Hence a displacement of IgG from the air-water interface by polysorbate 80 could be proven also after sequential adsorption. In contrast, pumping HP\(\beta\)CD into the IgG droplet does not reduce elasticity, or change viscosity (Figure III.12B and Figure III.13B, respectively). These results confirm that cyclodextrin did not displace the
protein from surface, and that surface elasticity and viscosity are always determined by IgG once the IgG has adsorbed to the interface.

Figure III.12: Surface elasticity of first, second, and third oscillation for double exchange experiments. First oscillation was performed with pure IgG 1x10^{-6} M solution, second oscillation after drop bulk exchange against polysorbate 80 2.5x10^{-5} M (A) or HPβCD 1x10^{-3} M (B), and third oscillation after subsequent drop bulk exchange against pure buffer.

Figure III.13: Surface viscosity of first, second, and third oscillation for double exchange experiments. First oscillation was performed with pure IgG 1x10^{-6} M solution, second oscillation after drop bulk exchange against polysorbate 80 2.5x10^{-5} M (A) or HPβCD 1x10^{-3} M (B), and third oscillation after subsequent drop bulk exchange against pure buffer.
4. Discussion

This work tests the validity of the theory of surface displacement as the underlying mechanism for the observed stabilization effect of polysorbate 80 and HPβCD against surface-induced aggregation of mAbs. To this end, several methods were used to monitor the surface tension at very short time periods directly after the formation of a new interface (the maximum bubble pressure method, MBPM), as well as for long time periods until reaching equilibrium (drop profile analysis tensiometry). Additionally, a special setup of the drop profile tensiometry applying a concentric capillary system (double-capillary setup) was used to follow dynamics of adsorption/desorption and displacement of the different components upon exchange of the droplet bulk. Surface rheological measurements using the double-capillary setup provided additional information about the elastic and viscous behavior of the surface layer.

In the literature discussing aggregation at the air-water interface, it is sometimes assumed that during agitation processes a constant “renewal” of the air-water interface takes place [4, 16, 35, 36]. In this context, renewal refers to mechanical destruction of the surface and subsequent formation of a fresh surface. Accordingly, MBPM was chosen to monitor the surface tension directly after surface formation. MBPM can measure surface tension over a surface lifetime ranging from few milliseconds to several seconds [37-42]. It is thus a valuable tool to monitor the adsorption of polysorbate 80 and HPβCD to newly formed interfaces in the presence and absence of IgG. Results in Figure III.3 show that polysorbate 80 adsorbs within a few milliseconds to a newly formed interface, much faster than IgG and HPβCD. Interestingly, this rapid adsorption is slightly delayed (on the order of 1 s) in the presence of IgG in the solution, while the mixture of IgG and HPβCD does not show any significant changes. Hence rapid adsorption of HPβCD does not explain stabilization of the IgG by HPβCD.

Contrary to the MBPM, the drop profile analysis tensiometry was used to monitor dynamic changes in the surface tension over long time periods (sometimes up to 80,000 s) until reaching equilibrium. This technique has several advantages over ring tensiometry, including the fact that it is a contactless method, i.e. no further interface (e.g. the platinum-water interface in the Wilhelmy-plate instruments) is introduced into the investigated system, leading to more accurate results [43]. As described earlier, the experiments using drop profile analysis tensiometry were carried at lower protein concentrations than the MBPM concentrations. Therefore, care has to be taken when relating these results to processes in more highly concentrated protein formulations.

Measurements with the drop profile analysis tensiometry confirm the surface activity of all three components, where the surface tension decreases with increasing concentration, with
the equilibrium surface activity of polysorbate 80>IgG>HPβCD. Mixtures of IgG with increasing concentrations of polysorbate 80 show a constant surface tension similar to that of pure IgG below a concentration of $1 \times 10^{-5}$ M of polysorbate, but this surface tension decreases dramatically above this concentration approaching the surface tension value of pure polysorbate 80. This is an indication that above this concentration, the surfactant displaces IgG from the interface. Such a behavior is however not seen for HPβCD, despite the relatively high concentrations used (2 orders of magnitude higher than polysorbate).

Single-exchange experiments using the double-capillary-setup of the profile analysis tensiometry and the associated surface rheological measurements show that exchanging the bulk of the droplet containing single components with buffer leads to rapid desorption of HPβCD and polysorbate 80 (though it is not complete in case of the later), while IgG remains bound to the surface. This observed irreversibility of adsorption of IgG is probably due to large adsorption energy as already shown for several proteins [23, 24, 44]. In the meantime, IgG-polysorbate behaves nearly the same as pure polysorbate before and after buffer exchange, indicating that at this concentration ($2.5 \times 10^{-5}$ M) polysorbate did replace IgG at the surface as already seen in the aforementioned equilibrium measurements. In contrast, the mixture of IgG and HPβCD showed a rapid washing out of the latter, while IgG remained at the surface, indicating that both components coexisted at the interface.

These results were corroborated by the double exchange experiments, which showed that addition of polysorbate 80 to a solution of IgG leads to rapid displacement of the latter from the surface (Figure III.11A). On the other hand, addition of HPβCD to a solution of IgG showed that the former was not only unable of displacing IgG from the surface, but also probably excluded from the surface.

The above results provide direct evidence that polysorbate 80 displaces IgG from surface in both simultaneous and sequential adsorption. This characteristic behavior of non-ionic surfactants is well known and was already shown elsewhere [14, 23, 24, 45]. The mechanism of protein replacement by ionic and non-ionic surfactants after sequential adsorption was explained by the orogenic displacement model [46]. Another explanation for protein displacement after subsequent buffer exchange describes adsorption of surfactant molecules onto the protein via hydrophobic interaction [24], which leads to a hydrophilisation of the protein. Despite high protein surface adsorption energy, hydrophilic protein/surfactant complexes possess lower surface activity and lead to a protein displacement from the air-water interface.

However, HPβCD exhibits a rather different behavior. The single exchange experiments could show that, for a pre-mixed IgG-HPβCD-solution, the protein and the cyclodextrin coexist in the surface layer. Meanwhile, the double exchange experiments showed that an integration of HPβCD in an already adsorbed IgG-layer did not occur. In contrast, formation
of a protein/polysaccharide layer after sequential adsorption was shown for a β-
lactoglobulin/pectin-system [47]. However, HPβCD did not show similar behavior.
Results of the current study provide supporting evidence for the surface displacement theory
as a mechanism for the observed stabilizing effect of polysorbate 80 against the surface
induced aggregation of IgG. In concentrations > 1×10⁻⁵ M, which are in accordance with the
concentrations used previously [6], polysorbate 80 displaces IgG from the interface.
Meanwhile, despite the fact that we used the same HPβCD concentrations as those which
elicited a protein stabilizing effect in a previous study [6], our observations refute the surface
displacement theory as the underlying mechanism for the protein stabilization observed for
HPβCD. Probably a different mechanism takes place at the interface, namely an interaction
between HPβCD and the partially unfolded protein, preventing further protein unfolding or
aggregation. The investigation of this hypothesis is currently underway.

5. Conclusions

In this study, the mechanism of stabilization of polysorbate 80 and HPβCD against surface-
induced IgG-aggregation was investigated. Accordingly, the surface tension of IgG in the
absence and presence of polysorbate 80 or HPβCD was monitored using the maximum
bubble pressure method, drop profile analysis tensiometry with different concentrations,
double-capillary drop profile analysis tensiometry with single and double bulk exchange, as
well as surface dilation rheometry. Results show that polysorbate 80 displaces IgG from the
surface, with this effect starting very rapidly after the formation of new surface (after approxi-
mately 1 s) as shown by the maximum bubble pressure method. Additionally, using different
concentration of polysorbate 80 showed that this replacement takes place when the
concentration of the later exceeds 1×10⁻⁵ M. Drop bulk exchange experiments showed that
this replacement takes place from a comixture of polysorbate and IgG, or even upon addition
of polysorbate to a preformed surface film of IgG. Meanwhile, HPβCD could not displace IgG
despite its surface activity. Mixtures of IgG and HPβCD coexisted at the interface. Single
exchange experiments showed that HPβCD was rapidly washed from the interface leaving
the protein film at the surface, while the double exchange experiments showed that HPβCD
was excluded from a preformed IgG film. These results support the theory of surface
displacement as the underlying mechanism for the stabilization effect of polysorbate 80, but
refute the frequently held opinion that HPβCD stabilizes proteins against aggregation at the
air-water interface in a manner comparable to non-ionic surfactants.
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7. References


Chapter IV

Weak antibody-cyclodextrin interactions determined by quartz crystal microbalance and dynamic/static light scattering

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Abstract

In a quest to elucidate the mechanism by which hydroxypropyl β-cyclodextrin (HPβCD) stabilizes antibodies against shaking stress, two heavily debated hypotheses exist, namely that stabilization is due to HPβCD’s surface activity, or due to specific interactions with proteins. In a previous study by Serno et al. (Pharm. Res. 30 (2013) 117), we could refute the first theory by proving that, although HPβCD is slightly surface active, it does not displace the antibody at the air-water interface, and accordingly, its surface activity is not the underlying stabilizing mechanism. In the present study, we investigated the possibility of interactions between HPβCD and monoclonal antibodies as the potential stabilization mechanism using quartz crystal microbalance (QCM) and static as well as dynamic light scattering. In the presence of HPβCD, the adsorption of IgG antibodies in the native state (IgG A) and the unfolded state (IgG A and IgG B) on gold-coated quartz crystals was studied by QCM. Results show that HPβCD causes a reduction in protein adsorption in both the folded and the unfolded states, probably due to an interaction between the protein and the cyclodextrin, leading to a reduced hydrophobicity of the protein and consequently a lower extent of adsorption. These results were supported by investigation of the interaction between the native protein and HPβCD using static and dynamic light scattering experiments, which provide the protein-protein interaction parameters, $B_{22}$ and $k_D$, respectively. Both $B_{22}$ and $k_D$ showed an increase in magnitude with increasing HPβCD-concentrations, indicating a rise in net repulsive forces between the protein molecules. This is further evidence for the presence of interactions between HPβCD and the studied antibodies, since an association of HPβCD on the protein surface leads to a change in the intermolecular forces between the protein molecules. In conclusion, this study provides evidence that the previously observed stabilizing effect of HPβCD on IgG antibodies is probably due to direct interaction between the cyclodextrin and the protein.

KEY WORDS
Quartz crystal microbalance (QCM), Dynamic light scattering (DLS), Static light scattering (SLS), Second virial coefficient ($B_{22}$-value), IgG antibody, Unfolded protein, Hydroxypropyl-beta-cyclodextrin (HPβCD)
1. Introduction

Formulation of liquid protein pharmaceuticals usually requires addition of suitable stabilizers to overcome instability problems – most notably protein aggregation [1]. For this purpose, surfactants and sugars are the most commonly used excipients, where the former stabilize mainly against mechanical stress, and the latter against thermal stress [2]. Cyclodextrins represent another class of excipients that can inhibit protein aggregation, with a recent review article summarizing findings in this respect [3]. For instance, several cyclodextrins were able to prevent the aggregation of human growth hormone [4]. Similarly, a protein stabilizing effect of hydroxypropyl-beta-cyclodextrin (HPβCD) comparable to polysorbate 80 was shown during the shaking stress of monoclonal antibodies [5].

The protein stabilizing mechanism of surfactants and sugars has been widely investigated and is currently attributed to competitive displacement at the air-water interface in the case of surfactants [6-9], or preferential exclusion in the case of sugars [10, 11]. Meanwhile, cyclodextrin’s mechanism of protein stabilization is still controversial. For instance, Samra et al. suggest a combined stabilizing mechanism for different cyclodextrins based on binding to the protein and preferential exclusion of the cyclodextrins from the protein surface [12]. Similarly, melittin - a 26 amino acid polypeptide - was shown to interact with HPβCD via its water-exposed hydrophobic amino acids, probably through formation of inclusion complexes [13]. On the other hand, Tavornvipas et al. propose the surface activity of cyclodextrin as the driving force for protein stabilization [14].

After the observation made by Serno et al. that HPβCD reduces antibody aggregation upon shaking [5], we set out to investigate the underlying mechanisms of this stabilizing effect. Accordingly, the interactions between an IgG antibody and HPβCD at the air-water interface was recently examined by drop profile analysis [15], where the results rule out competitive inhibition as the mechanism of antibody stabilization. Accordingly, the present study was undertaken to investigate whether an interaction between HPβCD and IgG is responsible for the observed antibody stabilization. For this purpose, the interaction between two monoclonal antibodies (IgGA and B) and different concentrations of HPβCD was investigated by quartz crystal microbalance (QCM) and both static and dynamic light scattering methods.

QCM is a sensitive technique to measure the adsorption of molecules on solid surfaces with nanogram sensitivity [16, 17] and was used previously for the investigation of antibody and Fc-fusion protein adsorption [18-20]. For instance, measurable adsorption of a monoclonal antibody onto gold surface was reported for a protein concentration lower than 1 µg/mL [21]. The method was also used to determine adsorption of an Fc-fusion protein to silicone-oil coated crystals [18]. Furthermore, two monoclonal antibodies were examined by QCM
regarding their affinity to four different surfaces as well as the (ir)reversibility of the bound protein layer [19]. Additionally, many studies describe the use of QCM to measure interactions between two different molecular species [20, 22-24]. However, in the last-mentioned references, one of the molecular species was immobilized onto the quartz chip before measurement, and afterward, the binding partner was introduced. A similar experimental setup with immobilization of one binding partner is described for Surface Plasmon Resonance (SPR) [25], and outcomes of QCM- and SPR-experiments are reported to be comparable [24, 26].

In our case, no interaction between protein and HPβCD could be determined by SPR when immobilizing IgG B onto the chip surface [27]. This might be due to inaccessibility of the protein binding site after immobilization for SPR measurements or due to very low affinity interaction. Therefore, the present study describes the use of QCM for qualitative evaluation of possible binding between IgG and cyclodextrins without immobilization. Since it is assumed that cyclodextrins are able to bind to exposed hydrophobic amino acids and therefore lead to a reduction in the surface hydrophobicity of the protein [4], a lower protein adsorption to the hydrophobic gold surface of the QCM-chip is expected for protein-cyclodextrin mixtures in the case of a binding interaction between them.

In addition, static and dynamic light scattering was used in this study for investigating the IgG-HPβCD interaction. Both are useful techniques to assess protein-protein interactions by determination of the second virial coefficient $B_{22}$ (by static light scattering, SLS) and interaction parameter $k_D$ (by dynamic light scattering, DLS) [28-30]. Different factors can influence protein-protein interactions such as solution pH [28] or alteration in the solution ionic strength [29, 30]. Amongst other factors, hydrophobicity of the protein’s surface is frequently held responsible for the attractive protein-protein interactions. In this context, it was shown that hydrophobic amino acids are preferentially present at the interface between protein-homodimers [31]. For the present study, SLS and DLS were chosen as the methods to assess interactions between the above mentioned IgGs and HPβCD, since a possible binding of HPβCD to hydrophobic amino acids on the protein’s surface would reduce the surface hydrophobicity of the interacting proteins and therefore can lead to an increase in $B_{22}$ and $k_D$-values.
2. Materials and Methods

2.1 Materials

Two monoclonal antibodies of the IgG class were used. IgG A was formulated in 10 mM PBS-buffer with pH adjusted to 6.2 by phosphoric acid. IgG B (which was also used for the shaking stability study [5] and the surface activity study [15]) was kindly donated by Roche Diagnostics GmbH (Penzberg, Germany) and formulated in 20 mM histidine buffer pH 5.8. Protein and buffer solutions were filtered through sterile 0.2 µm PES membrane syringe filters before usage. Hydroxypropyl-beta-cyclodextrin was obtained from Wacker Chemie AG (Burghausen, Germany), maltoheptaose from CycloLab (Budapest, Hungary), L-histidine from Sigma-Aldrich (Steinheim, Germany), guanidinium chloride from Merck KGaA (Darmstadt, Germany), while toluene, ammonium hydroxide 25% solution, and hydrogen peroxide 30% were from Fisher Scientific (Fairlawn, NJ). All reagents were at least of analytical grade.

2.2 QCM-measurements

QCM-measurements were performed using QCM200 Quartz Crystal Microbalance Digital Controller and QCM25 5 MHz Crystal Oscillator (Stanford Research Systems, Sunnyvale, CA, USA). Protein adsorption on gold surface was studied using gold-plated quartz crystals with a fundamental resonant frequency of 5 MHz. The QCM-assembly (illustrated in Figure IV.1) included a syringe pump (NE-1010X, New Era Pump Systems Inc., Farmingdale, NY), enabling constant flushing of the device with buffer. Sample was injected via a 6 port injection valve and pumped onto the crystal, which was connected to a QCM25 Crystal Oscillator and tempered by dipping into a 25.0°C water bath. Data were recorded by QCM200 Digital Controller.

![Figure IV.1: Experimental setup of the QCM device.](image-url)
A constant flow rate of 100 µL/min was applied for approximately 15 min to flush the crystal with the buffer to remove air bubbles. Afterward, the flow rate was reduced to 50 µL/min and the crystal holder was dipped into a 25°C water bath with the buffer flow inside the crystal holder directed against gravity to minimize any further air bubble entrapment. Frequency and resistance shifts were recorded until a stable baseline was reached (approximately 45 min), after which 250 µL sample solution was injected with a flow rate of 50 µL/min. Once the entire injected sample reached the crystal holder (~400 s), flow was stopped so that protein adsorption reaches equilibrium. In some cases, (ir)reversibility of adsorbed protein amount was tested by flushing the crystal after equilibration with 50 µL/min buffer. The running buffer composition was identical to the sample formulation buffer. This was necessary to exclude undesired effects due to different viscosities of sample and running buffer. After each measurement, the gold crystal was cleaned with a solution composed of H₂O₂ 30% (1 part), NH₄OH 25% solution (1 part), and distilled water (5 parts). The solution was heated to 70°C, and the crystal was dipped into it for 5 min. Afterward, the crystal was flushed with water and ethanol and finally blown dry by nitrogen gas.

Calculation of the adsorbed mass using frequency shifts followed the Sauerbrey equation

$$\frac{\Delta F}{F_0} = -\frac{\Delta m}{\rho_0 \cdot A \cdot d}$$

where \(\Delta F\) is the measured change in frequency, \(F_0\) the resonant frequency of the crystal, \(\Delta m\) the mass of the adsorbed layer, \(\rho_0\) the density of quartz, \(A\) the area of the electrodes, and \(d\) the crystal thickness [32]. The Sauerbrey equation is only valid for rigid layers bound onto the crystal surface; non-rigid layers require simultaneous analysis of resistance shifts to calculate the adsorbed mass. Furthermore, changes in resistance enable conclusions about the nature of adsorbed layer, where an increase in resistance indicates a soft, viscoelastic layer, whereas no change in resistance is observed for rigid layers [18]. Since a change in resistance was observed after injection of IgG A or B, the adsorbed mass \(\Delta m\) was calculated using the following equations:

$$\Delta m = \Delta F_{\text{measured}} \cdot (1 - \frac{R}{F_{\text{elastic}}}) \cdot C$$

with

$$\frac{R}{F_{\text{elastic}}} = \frac{\Delta R_{\text{measured}} / \Delta F_{\text{measured}}}{\Delta R_{\text{dry/wet}} / \Delta F_{\text{dry/wet}}}$$

Here, \(\Delta F_{\text{measured}}\) and \(\Delta R_{\text{measured}}\) are the measured frequency and resistance differences before and after protein adsorption. \(\Delta F_{\text{dry/wet}}\) and \(\Delta R_{\text{dry/wet}}\) are frequency and resistance differences of the crystal when measured against air (dry) and measured in equilibrated wet condition immediately before sample injection (wet). The crystal specific constant \(C\) related to 1 cm² electrode area is calculated by
\[ C = \frac{A \cdot \sqrt{\rho_Q \cdot \mu}}{2 \cdot F_0^2} \]  

(4)

where \( A \) is the active electrode area (0.40 cm\(^2\)), \( \rho_Q \) the density of quartz (2.648 g/cm\(^3\)), \( \mu \) the shear modulus of quartz (2.947*10\(^{11}\) g/(cm\(^2\)*s\(^2\))), and \( F_0 \) the crystal resonant frequency (Hz).

### 2.3 Light scattering

For light scattering experiments, protein bulk solutions were concentrated by Amicon Ultra Centrifugal Filters with regenerated cellulose membrane and 10.000 MWCO (Millipore, Billerica, MA, USA) up to a concentration of 25 mg/mL. Each protein was measured in its respective formulation buffer with addition of 0, 2.5, 25, 50, and 100 mM HPβCD. Sample solutions were filtered through Millex-GV 0.22 µm hydrophilic Durapore PVDF membrane (Millipore, Billerica, MA, USA). For every HPβCD-concentration, IgG dilution series were measured as duplicate with protein concentration of 10, 8, 6, 4, and 2 mg/mL. SLS and DLS measurements were performed with a Zetasizer Nano-ZS (Malvern Instruments) using a 75 µL glass cuvette. Prior to measurement, samples were centrifuged at 10.000 rpm for 5 min to eliminate dust. Temperature was set to 25.0 ± 0.1°C with 2 min temperature equilibration before analysis.

Light scattering measurements require knowledge of refractive index and viscosity of the respective sample. Refractive index of the different concentrated HPβCD-buffer solutions was measured using an Abbe-refractometer (Carl Zeiss) at 25°C. Viscosity was determined by mVROC-viscosimeter (RheoSense Inc.) using an A05 chip with 50 µm flow channel connected to a 250 µl Hamilton syringe. Temperature was set at 25°C with 2 min equilibration time. Viscosity was determined at a constant flow rate of 150 µL/min for 18 s.

#### 2.3.1 Static light scattering (SLS)

For SLS measurements, toluene was used as a standard with well-known Rayleigh ratio. Sample Rayleigh ratios \( (R_\theta) \) were calculated using the following equation:

\[ R_\theta = \frac{I_A \cdot n_0^2}{I_T \cdot n_T^2} \cdot R_T \]  

(5)

Here, \( I_A \) is the difference between sample scattering intensity and respective protein-free buffer scattering intensity, \( n_0 \) is the sample refractive index, \( I_T \) the scattering intensity of toluene, \( n_T \) the refractive index of toluene, and \( R_T \) the Rayleigh ratio of toluene.
Rayleigh equation (Eq. (6)) allows calculation of the second virial coefficient ($B_{22}$) and sample molecular weight ($M_w$) by using the Debye Plot.

$$\frac{K \cdot c}{R_\theta} = \left( \frac{1}{M_w} + 2 \cdot B_{22} \cdot c \right)$$  \hspace{1cm} (6)

For the Debye Plot, $Kc/R_\theta$ is plotted against sample concentration ($c$). $B_{22}$ values are obtained from the slope of the straight line and $M_w$ from the reciprocal intercept. In this context, the optical constant $K$ is defined by

$$K = \frac{4 \cdot \pi^2}{\lambda_0^4 \cdot N_A} \cdot \left( n_0 \cdot \frac{dn}{dc} \right)^2$$  \hspace{1cm} (7)

where $\lambda_0$ is the wavelength of the laser, $N_A$ the Avogadro’s number, $n_0$ the solvent refractive index, and $dn/dc$ the differential refractive index increment.

### 2.3.2 Dynamic light scattering (DLS)

Dependent on HPβCD- and IgG-concentration, light scattering measurements showed one or two peaks. One peak was obtained for high protein concentrations, but an additional HPβCD-peak was visible in samples with lower IgG and high HPβCD-concentration. In order to determine the diffusion coefficient of the protein peak ($D_m$) (and not the smaller-sized HPβCD), it was calculated from the measured protein size ($d_H$) using the Stokes-Einstein equation:

$$D_m = \frac{k \cdot T}{3 \cdot \pi \cdot \eta \cdot d_H}$$  \hspace{1cm} (8)

Here, $k$ is the Boltzmann constant, $T$ the absolute temperature, $\eta$ the sample viscosity, and $d_H$ the solute hydrodynamic diameter.

Correlation between $D_m$ and sample concentration ($c$) is given by

$$D_m = D_s \cdot (1 + k_D \cdot c)$$  \hspace{1cm} (9)

where $D_s$ represents the self-diffusion coefficient of an infinitely diluted sample and $k_D$ as interaction parameter is defined by

$$k_D = 2 \cdot B_{22} \cdot M_w - \xi_1 - 2 \cdot \nu_{sp}$$  \hspace{1cm} (10)

with $\xi_1$ as coefficient of the linear term in the virial expansion of the frictional coefficient, and $\nu_{sp}$ as partial specific volume of the solute [28, 33]. $B_{22}$ is the second virial coefficient and $M_w$ the molecular weight of the solute.

For the determination of $k_D$, $D_m$-values were plotted against IgG-concentration. Dividing the slope by the intercept (= $D_s$) results in $k_D$. 

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3. Results

3.1 QCM-measurements

For IgG A, protein-HPβCD-interactions were examined for the native and the unfolded protein. Complete protein unfolding was achieved by the addition of 6 M guanidine-HCl. The adsorption profile from a 100 µg/mL IgG A solution in the native and the unfolded state is illustrated in Figure IV.2. Results show different adsorption behaviours depending on the protein’s folding state. Native protein causes a frequency decrease in roughly 37 Hz and an increase in resistance of 1.1 Ω, which starts immediately after protein injection. On the contrary, unfolded protein in the same concentration leads to a significantly smaller frequency shift of 22 Hz and a significantly larger increase in resistance (approx. 3 Ω), which starts with a delay of roughly 400 s after injection. The lower frequency shift for unfolded IgG A demonstrates a lower protein adsorption compared to the native IgG A (600 ng/cm² for the native, and 260 ng/cm² for the unfolded protein). At the same time, the higher resistance increase represents a less rigid and a rather loosely bound viscoelastic layer. In both cases, protein adsorption was irreversible since flushing the crystal with buffer did not remove any adsorbed protein (as evidenced by the lack of changes on the measured frequency, Figure IV.2A). Such irreversibility of protein adsorption to solid surfaces is commonly observed and reported in the literature [34, 35]. Meanwhile, flushing the crystal with the running buffer caused a slight increase in resistance values for both the native and unfolded proteins (Figure IV.2B), indicating a reduction in the rigidity of the adsorbed layers. This might be due to the effect of the fluid flow, leading to loosening of the adsorbed protein layer.
Figure IV.2: Adsorption of 100 µg/mL native and unfolded IgG A on gold-coated quartz crystal. Diagrams show results of frequency (A) and resistance (B) shifts as well as the corresponding adsorbed mass (C). Arrows mark the time point when flushing the crystal with 50 µl/min running buffer started.

To determine the bulk protein concentration achieving saturation of the gold surface, protein adsorption was studied as a function of increasing protein concentration. Figure IV.3 shows that a surface saturation is reached at a concentration of approx. 500 µg/mL for the native IgG A solution, which results in a maximum adsorbed mass of roughly 850 ng/cm². Increasing the injected protein concentration to 1000 µg/mL did not cause an increase in the amount of adsorbed protein. For the following adsorption experiments with HPβCD, and in order to show the (positive or negative) effect of HPβCD on IgG-adsorption, we had to choose a solution concentration well below that leading to gold surface saturation. Accordingly, the HPβCD-protein adsorption experiments were conducted at a concentration of 100 µg/mL.
The effect of different concentrations of HPβCD on IgG A adsorption can be seen in Figure IV.4. It is worth noting that the injection of pure HPβCD leads to a negligible adsorption onto the crystal (data not shown); therefore, adsorbed mass is only related to the protein. Results show a gradual reduction of the adsorbed mass when increasing the HPβCD-concentration from 25 mM to 100 mM. The decrease in adsorbed mass after addition of HPβCD indicates a reduction in protein hydrophobicity, which gives a hint to an interaction between HPβCD and the protein.

For the unfolded IgG A, addition of HPβCD to the formulation and running buffer leads to significant decrease in the amount of the adsorbed protein (Figure IV.5). Even the lowest
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HPβCD-concentration tested (25 mM) caused a notable reduction of >40% in adsorbed mass, with roughly 100 ng/cm² decrease compared to the HPβCD-free sample. This indicates an interaction between the unfolded antibody and HPβCD, which reduces the protein adsorption to the hydrophobic gold surface.

Figure IV.5: Frequency- (A) and resistance- (B) shifts of 100 µg/mL solutions of unfolded IgG A containing different HPβCD-concentrations. Unfolding was performed by the addition of 6 M guanidine-HCl to the protein formulation as well as the QCM-running buffer. The resulting adsorbed mass is shown in (C).

To evaluate if the interactions between unfolded IgG A and HPβCD are caused by specific binding between HPβCD and the protein, maltoheptaose was used as a negative control. Maltoheptaose is a linear oligosaccharide composed of seven glucose subunits without the characteristic ring structure of cyclodextrin. Results in Figure IV.6 show that addition of 25 and 100 mM maltoheptaose to the unfolded IgG A did not affect the adsorbed protein amount compared to the sugar-free reference. This implies a lack of interaction between maltoheptaose and the protein. Based on the above results, the protein-cyclodextrin interaction is assumed to be due to a specific interaction between HPβCD and the protein and not merely due to its polyol nature.
Figure IV.6: Adsorbed mass of 100 µg/mL unfolded IgG A with addition of different Maltoheptaose-concentrations. Unfolding was performed by addition of 6 M guanidine-HCl to sample formulation as well as QCM-running buffer.

Protein-HPβCD interactions were also studied for IgG B. The protein adsorption in native and unfolded conformation (Figure IV.7) without HPβCD shows a similar profile as seen for IgG A (Figure IV.2). Also here, the unfolded protein leads to a smaller frequency decrease compared to the native antibody but to a larger resistance increase. Therefore, the native IgG B forms a comparatively rigid protein layer, whereas a more viscous (less viscoelastic) layer is observed for the unfolded protein (see Ref. [36]). The resulting adsorbed mass for the native and unfolded IgG B (Figure IV.7C) is slightly lower compared to IgG A (Figure IV.2C), and the gold surface is saturated at a lower bulk IgG-concentration (Figure IV.8, c.f. Figure IV.3). These discrepancies could be attributed to the differences in the surface hydrophobicity of the two IgGs; however, the differences in the molecular surface charge or different buffers used in the studies can also be the contributing factors [37, 38].
Figure IV.7: Adsorption from 100 µg/mL native and unfolded IgG B solutions on gold-coated quartz crystal. Diagrams show results of frequency (A) and resistance (B) shifts as well as the corresponding adsorbed mass (C).

Figure IV.8: Adsorption-isotherm of different concentrations of native IgG B in 20 mM Histidine buffer pH 5.8 on gold-coated quartz crystal.

The interaction between HPβCD and IgG B (which was used for the shaking stability [5] and the surface activity studies [15]) was examined for 100 µg/mL unfolded protein. Results show a reduction in frequency similar to IgG A, indicating a reduction in the adsorbed mass and a
possible protein-HPβCD interaction (Figure IV.9). Contrary to IgG A, addition of 25 mM HPβCD did not show any reduction in frequency, while higher concentrations led to concentration-dependent reduction in frequency (and accordingly the adsorbed mass). These results can be explained by a possibly lower affinity of HPβCD for the unfolded IgG B compared to the unfolded IgG A. Meanwhile, the resistance curve for the unfolded IgG B, with different HPβCD-concentrations, shows a similar pattern irrespective of the HPβCD-concentration; namely a steep increase followed by a slow drop down. This indicates that the initially elastic protein layer gradually collapses to form a rigid layer independent of the presence of HPβCD. During that process, the overall adsorbed protein mass does not change, since the frequency values remain constant.

![Figure IV.9](image)

**Figure IV.9:** Frequency (A) and resistance (B) shifts during adsorption of 100 µg/mL unfolded IgG B with addition of different HPβCD-concentrations. Unfolding was performed by addition of 6 M guanidine-HCl to sample formulation as well as QCM-running buffer.
3.2 Light scattering measurements

Interactions between native antibody and HPβCD were further analyzed by SLS and DLS, which are widely used to determine protein-protein interactions in solution [28-30, 39]. Both antibodies were investigated in the presence of different HPβCD-concentrations (from 0 to 100 mM), and changes in intermolecular protein interactions were determined by calculating the interaction parameters, $B_{22}$ and $k_D$.

3.2.1 SLS

The slopes of the Debye Plots of IgG A and B (Figure IV.12 in Supporting information) provide the corresponding $B_{22}$-values (Figure IV.10). Negative values indicate attractive protein-protein interactions, whereas positive values are obtained for repulsive interactions [40]. The Debye Plot of IgG A (Figure IV.12A) shows a slightly negative slope in the absence of cyclodextrin. Increase in the HPβCD-concentration leads to a gradual increase in the slope which even turns positive starting from 50 mM HPβCD (Figure IV.10A). Therefore, addition of HPβCD up to a concentration of 100 mM leads to a change in intermolecular protein interactions from attractive to repulsive forces. For IgG B, weak repulsive interactions are obtained in pure histidine buffer (Figure IV.12B and Figure IV.10B). Similar to IgG A, addition of HPβCD leads to an increase in $B_{22}$-values for IgG B, indicating an increase in repulsive intermolecular protein interactions. For both native antibodies, the presence of HPβCD changes intermolecular protein interactions leading to an increase in the net repulsive interactions. This can be seen as further conformation for a HPβCD-IgG interaction.

Figure IV.10: $B_{22}$-values of IgG A (A) and IgG B (B), measured in their respective formulation buffer with addition of different HPβCD-concentrations.
3.2.2 DLS

The protein interaction parameter, $k_D$, is obtained from the DLS studies. Negative $k_D$-values indicate attractive protein-protein interactions, and positive $k_D$-values indicate repulsive interactions [39]. Plots of the protein mutual diffusion coefficient, $D_m$, measured in different HPβCD-concentrations are shown in Figure IV.13A and B. Corresponding $k_D$-values which are obtained from the quotient between the slope and the intercept of the $D_m$-plots are illustrated in Figure IV.11. Similar to the SLS results, addition of HPβCD leads to an increase in $k_D$-values for both antibodies, indicating an increase in protein-protein repulsive interactions. For IgG A, $k_D$-values are negative up to 50 mM HPβCD and become positive for the highest HPβCD-concentration tested (100 mM). In contrast, $B_{22}$-values of IgG A turn positive starting from 25 mM HPβCD (Figure IV.10A). A similar apparent discrepancy of negative $k_D$ and positive $B_{22}$-values was already reported for several antibodies [40] and can be explained by the fact that $k_D$-values are affected by thermodynamic as well as hydrodynamic contribution and can thus be different from the $B_{22}$-results [41]. IgG B formulated in pure buffer without cyclodextrin shows a slightly positive $k_D$, which correlates with the positive $B_{22}$-value (Figure IV.10B). Increasing the HPβCD-concentration up to 100 mM leads to an increase in $k_D$, also indicating an increase in protein repulsion.

Figure IV.11: $k_D$-values of IgG A (A) and IgG B (B), measured in their respective formulation buffer with addition of different HPβCD-concentrations.
4. Discussion

In the present study, antibody adsorption on hydrophobic gold surfaces was measured in the presence and absence of HPβCD. Generally, proteins are amphiphilic in nature, and hence, surface active and tend to adsorb onto hydrophobic interfaces [34, 35]. During this process, proteins undergo structural reorganization with a loss in their ordered super-structure and exposure of hydrophobic residues [42]. This could result in their irreversible adsorption to interfaces. In this work, the irreversibility of protein adsorption was observed for IgG A from the native as well as the unfolded state. In both cases, no protein desorption could be detected (Figure IV.2). The reason of irreversibility is explained by structural changes in protein conformation, leading to a multipoint attachment between protein and the hydrophobic surface after exposing buried hydrophobic protein moieties [43, 44]. Oom et al. reported that reversibility of IgG-adsorption to different surfaces depends on the respective protein concentration, with a high amount of reversibly bound protein observed for 50 mg/mL protein concentration, and low reversibility for 1 mg/mL protein solutions [19]. Since the present experiments were performed with a very low protein concentration (100 µg/mL), the observed irreversibility of protein adsorption is consistent with the literature reports.

It is worth noting that the amount of adsorbed native protein is lower than that of the guanidine-HCl-unfolded one. A similar behavior was reported for different proteins when adsorbing to a hydrophilic TiO₂-surface [36]. Here, it was shown that the adsorption of unfolded protein (achieved by addition of 6 M urea) leads to a reduced amount of adsorption and a lower density of the resulting protein layer. This phenomenon was explained by the random coil structure of the unfolded protein which occupies a larger space above the surface and consequently leads to a lower amount of adsorbed protein with lower density of the formed layer [36]. In addition, guanidine-HCl is known to disrupt protein networks [45], and is able to enhance protein solubility as salting-in salt [46] which might be further reasons for the lower amount of adsorbed protein and the looser protein network on the gold surface (Figure IV.2).

In order to evaluate a possible binding between HPβCD and IgG, an experimental approach was chosen in which protein adsorption in the presence/absence of cyclodextrin was investigated. Since it is assumed that cyclodextrins interact with the protein, probably through the latter’s exposed hydrophobic amino acids [4, 47, 48], reduced protein hydrophobicity, and consequently, a lower adsorption is expected in the presence of HPβCD. Compared to classical SPR- or QCM-binding experiments, which require immobilization of one binding partner [20, 22-26], the present setup allows direct analysis of the interaction occurring in the bulk solution. For native IgG A, a concentration-dependant reduction in protein adsorption was observed after addition of HPβCD in concentrations from 25 mM up to 100 mM (Figure
Since HPβCD itself did not result in any measurable adsorption, a binding between native IgG A and HPβCD in the bulk seems to be the underlying cause. The binding to the protein’s hydrophobic sites was further confirmed by studying the adsorption of the unfolded proteins in the presence of increasing concentrations of HPβCD. Protein unfolding was achieved by the addition of 6 M guanidine-HCl, which is sufficient for complete protein unfolding [49]. The results show a decrease in adsorbed protein amount after addition of HPβCD. Comparing the results from the native and unfolded IgG A (Figure IV.4 and Figure IV.5), one notices that 1) the percentage reduction in adsorption caused by HPβCD is higher for the unfolded protein compared to the folded one (approx. 40% vs. 15%, respectively, at HPβCD-concentration of 25 mM), and 2) addition of increasing concentration of HPβCD to unfolded IgG A did not cause a concentration-dependent reduction in protein adsorption, as it is the case with native IgG A, but rather a constant decrease in adsorbed mass of roughly 100 ng/cm² was observed (Figure IV.5C). The first phenomenon points to a higher affinity of HPβCD to the unfolded protein compared to the folded one, so that even at the same concentration, the effect of CD is more prominent with the unfolded protein. This is probably due to the exposure of the hydrophobic groups in the unfolded state, to which CD readily binds [50, 51]. The lack of effect of higher CD-concentrations on protein adsorption in the case of unfolded protein also points to the high affinity, where lower concentrations are sufficient to completely occupy the hydrophobic binding site on the protein. Another possible influencing factor is the presence of guanidine-HCl with the unfolded protein, which interacts with hydrophobic sites on the protein, leading to a lesser amount of binding sites. Meanwhile, there was no effect of increasing concentrations of maltoheptaose on protein adsorption (Figure IV.6), showing that indeed, the observed effects on IgG-adsorption are due to specific interactions between HPβCD and the antibodies. Therefore, QCM-measurements were effective in showing the presence of an interaction between HPβCD and IgG even in the absence of prior immobilization of one binding partner to the chip surface.

To further prove the interaction between HPβCD and native IgG, SLS and DLS measurements were performed. SLS provides the second virial coefficient, $B_{22}$, which provides information about the nature of protein-protein interaction (repulsive or attractive) [29, 33, 39]. DLS determines the concentration-dependent diffusion coefficient of the protein molecules, which depends on the occupied volume fraction as well as repulsive or attractive intermolecular forces [30]. Measuring the mutual diffusion coefficient by DLS provides the interaction parameter, $k_D$. In the present study, both antibodies were investigated in formulations with different HPβCD-concentrations to evaluate whether the presence of cyclodextrins causes changes in protein-protein interactions. For both antibodies, the addition of increasing amounts of HPβCD (from 2.5 mM to 100 mM) leads to an increase in $B_{22}$ and $k_D$-values, which implies an increase in net repulsive protein-
protein interactions. Since hydrophobicity of proteins represents one main factor in intermolecular protein interactions [31], the presence of HPβCD probably alters the protein surface, leading to a more hydrophilic protein. This seems to be due to a HPβCD-induced shielding/complexation of the hydrophobic amino acids on the protein surface.

5. Conclusions

The present study investigated the interaction between HPβCD and two monoclonal antibodies using QCM as well as SLS and DLS. QCM was performed with the native form (IgG A) and the unfolded form (IgG A and IgG B) of IgG antibodies to study their adsorption on gold-coated quartz crystals in the presence of increasing amounts of HPβCD. Results show that HPβCD leads to a reduction in protein adsorption, probably due to an interaction between the (un)folded protein and cyclodextrin, leading to a reduced hydrophobicity of the protein and consequently a lower adsorption. The interaction was further evaluated by SLS and DLS, which provide information about the protein-protein interaction parameters, $B_{22}$ and $k_D$, respectively. $B_{22}$- and $k_D$-values increased with increasing HPβCD-concentrations, indicating a rise in net repulsive forces between the protein molecules. This can be seen as a further evidence of an interaction between HPβCD and both antibodies since an association of HPβCD on the protein surface leads to a change in the protein intermolecular forces. Finally, the used methods provided useful information about the interplay between HPβCD and the tested IgGs and can be valuable tools in studying protein-excipient interactions.
6. Appendix A. Supplementary material

Figure IV.12: Debye Plot of IgG A (A) and IgG B (B), measured in their respective formulation buffer with addition of different HPβCD-concentrations.

Figure IV.13: $D_m$ Plots of IgG A (A) and IgG B (B), measured in their respective formulation buffer with addition of different HPβCD-concentrations.
7. References

18. Dixit, N., K.M. Maloney, and D.S. Kalonia, *Application of quartz crystal microbalance to study the impact of pH and ionic strength on protein–silicone


27. Serno, T., Inhibition of therapeutic protein aggregation by cyclodextrins, in Pharmaceutical Technology and Biopharmaceutics. 2010, LMU Munich: Munich.


Chapter V

A critical eye on methods for the determination of the viscosity of highly concentrated antibody solutions

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Pharmaceutical Development and Technology, 2013 (article in rebuttal)

Experimental work and writing were performed by Elisabeth Härtl, corrections were from Ahmed Besheer and Gerhard Winter.
Abstract

Highly concentrated antibody solutions are often associated with an increase in viscosity and require time-saving and low volume methods for viscosity measurements. In this study, the applicability of dynamic light scattering (after addition of standard-sized beads) and microfluidic viscosimetry was investigated and compared by measuring the viscosity of a highly concentrated antibody solution. The results show that in our case, dynamic light scattering was not applicable due to the occurrence of interactions between standard-sized beads and the protein whereas microfluidic viscosimetry worked well. Finally, the combination of both techniques is suggested as a promising approach for a time-saving viscosity-screening.

1. Introduction

The characterization of highly concentrated antibody solutions presents a number of challenges to the formulation scientist, including the need to measure the protein solution viscosity by using sample-saving analytical methods during early development. In this study, we compared two methods for the determination of the sample viscosity of highly concentrated antibody solutions, namely dynamic light scattering (DLS) and microfluidic viscosity measurements.

The former has been discussed lately as a high-throughput screening method for the determination of viscosity and particle aggregation. It operates with addition of standard particles (usually polystyrene latex particles), and exhibits several benefits such as the small sample volume of less than 100 μL, the determination of zero-shear viscosity, and the capability to be applied as high-throughput technique. However, there are limitations which restrict the applicability of dynamic light scattering for viscosity measurements. For instance, the maximum measurable viscosity is limited at a value of roughly 30-50 mPa·s and – what is even more important – buffer-latex and protein-latex interactions can occur which lead to particle aggregation, inhomogeneous particle distribution, and irrelevant data.

The mVROC is an instrument that determines viscosity by pumping the sample solution through a miniaturized rectangular slit and measuring the pressure at three different sensor positions. Viscosity is simultaneously calculated by plotting the measured pressure against the sensor position (see also ). The method also offers a number of benefits, including the small required sample volume of about 100 μL and a design which reduces the effect of interfacial tension frequently observed with cone and plate rheometers. Limitations are the
higher sample requirements if one measures different shear rates, and its more medium than high throughput.

In the present study, the applicability and limitations of both low volume methods were investigated and compared by measuring the solution viscosity of a monoclonal antibody in different concentrations.

2. Materials and methods

2.1 Materials

The protein for the following study was an antibody of the IgG1 class which was initially formulated in a concentration of 5 mg/mL in PBS-buffer pH 6.2, and filtered through 0.2 µm PES membrane syringe filters (VWR-International). Vivaspin 20 with 30,000 MWCO PES membrane (Sartorius Stedim Biotech) was used to obtain highly concentrated protein stock solutions with roughly 150 mg/mL. The exact protein content after the concentration step was determined spectroscopically using a Nanodrop 2000 (Thermo scientific) device. Final protein concentration was obtained by diluting the highly concentrated stock solution with PBS-buffer pH 6.2.

2.2 Dynamic light scattering

Measuring the viscosity by DLS is based on spiking the sample solutions with a bead-suspension of well-known and monodisperse bead-size. Suchlike standard particles with narrow size distribution in the nanometer-range are currently available on polystyrene- or silica-basis. For the following study, we used 50 nm and 200 nm polystyrene latex particle-suspensions (Nanosphere Size Standards, Duke Scientific Corporation), and a 50 nm silica microsphere-suspension (Polysciences, Inc.). Each particle-suspension was used in three different concentrations, namely without dilution, after 1:10, and 1:100 dilution with highly purified water. For viscosity-measurements, 50 µL IgG-samples were prepared and spiked with 0.5 µL of the respective particle suspension. Afterwards, the spiked samples were filled into a 45 µL low volume glass cuvette (Hellma Analytics, Müllheim, Germany), and measured by using a Zetasizer Nano-ZS (Malvern Instruments, Herrenberg, Germany) in the 173° backscatter mode with multiple narrow analysis. Before the respective measurement, samples were tempered at 25.0°C for 120 seconds, and each sample was measured in three consecutive runs.
The Zetasizer software calculates the particle hydrodynamic diameter \( D_H \) by using the Stokes-Einstein equation with \( D_H = kT/3\pi\eta_D \) whereby \( k \) is the Boltzmann constant, \( T \) the absolute temperature, \( \eta \) the solution viscosity, and \( D \) the Diffusion coefficient. By measuring the diffusion coefficient of the particles in a dispersant of well-known viscosity, particle sizes are calculated.

For determining solution viscosities by dynamic light scattering, measurements were performed assuming a dispersant viscosity of pure water. Therefore an increase in spiked bead size is detected for actual higher viscosities due to lower Brownian motion of the particles. Viscosities of highly concentrated formulations can be calculated from respective measured bead size using the following correlation \( D_{\text{water}}/\eta_{\text{water}} = D_{\text{measured}}/\eta_x \) with \( D_{\text{water}} \) as particle size measured in pure water, \( \eta_{\text{water}} \) as viscosity of water, \( D_{\text{measured}} \) as size of bead particles as measured in the sample solutions, and \( \eta_x \) as unknown viscosity of the sample solution.

### 2.3 mVROC measurements

Sample viscosity was additionally determined using the mVROC device (RheoSense Inc.) equipped with an A05-chip. Measurement temperature was set at 25.0°C and controlled by an external water bath (Lauda RK8KP). The sample solutions were filled into a 100 µl Hamilton syringe, connected to the measurement chip, and tempered for two minutes. Measurements were performed with 150 µL/min flow rate for 10 seconds.

### 3. Results

#### 3.1 Dynamic light scattering

Initially, compatibility between the three different particle species and IgG was tested in low concentrated 5 mg/mL IgG-solutions, and exemplarily results for the 50 nm polystyrene beads are illustrated in Figure V.1. The pure 5 mg/mL IgG-solution (Figure A) showed a diameter for the IgG of roughly 11 nm which is characteristic for monoclonal antibodies. [7] Homogeneity and narrow size distribution of the standard particles in PBS-buffer pH 6.2 is demonstrated in Figure B. However, addition of undiluted as well as diluted 50 nm polystyrene standard particles to the 5 mg/mL IgG-solution resulted in a significant protein-particle interaction since the original particle size of 50 nm was no longer present (Figures C and D). Viscosity-contribution to the incorrect, measured particle sizes was excluded by
measuring the actual sample viscosity by a conventional cone-and-plate rheometer (data not shown).

Comparable aggregation behavior was observed after addition of 200 nm polystyrene beads and 50 nm silica-beads for each tested particle concentration (data not shown). Moreover, also test-measurements with 100 mg/mL IgG resulted in protein-particle interactions which demonstrated the inapplicability of the DLS-method for the viscosity-determination in our case. Similar observations with polystyrene-beads were reported recently and ascribed to the formation of protein-latex particles. [5]

Figure V.1: Intensity-results of dynamic light scattering for (A) 5 mg/mL IgG-solution, (B) 50 nm polystyrene particles in PBS-buffer pH 6.2, (C) 5 mg/mL IgG-solution after addition of undiluted 50 nm polystyrene particles, and (D) 5 mg/mL IgG-solution after addition of 100-fold diluted 50 nm polystyrene particles.

3.2 mVROC measurements

In contrast to dynamic light scattering, determination of the protein-sample viscosities worked well with the mVROC device (Figure V.2). Here, sample volumes of roughly 100 µL were required which is only slightly higher compared to the DLS-method. The result in Figure V.2 shows the typical viscosity trend which is generally observed for increasing protein
concentrations. The viscosity increase follows a linear slope at lower protein concentrations (up to 40 mg/mL) and switches to an exponential progression with higher protein concentrations indicating a rise in intermolecular protein interactions. [8] Accuracy of the mVROC results was validated by comparison to a classical cone and plate rheometer (Physica MCR100, Anton Paar). Here, different concentrated PEG-solutions with viscosities ranging from 0 – 200 mPa*s gave equivalent results for both methods (data not shown).

![Figure V.2: Viscosity of IgG-solutions (0 – 100 mg/mL) as determined by mVROC measurements. Each concentration was measured as triplicate and respective error bars are hidden behind the symbols.](image)

### 4. Discussion

Aim of the present work was to compare the applicability of two new material saving methods for the viscosity-measurement of highly concentrated antibody solutions. For that purpose, dynamic light scattering after addition of polystyrene and silica standard beads, and the mVROC device were used. In DLS-measurements, the presence of protein-bead interactions and the formation of aggregates represented a challenging limitation as no distinct size for the spiked standard beads could be detected in any of those samples. Here, formation of aggregation after spiking the samples with beads was independent on bead concentration, bead size, and on the tested bead material (polystyrene and silica) which renders the DLS-method as not applicable for determining the solution viscosity of our antibody formulations. By contrast, the mVROC device was a suitable method to overcome the limitations from DLS-measurements. Its low required sample volume of roughly 100 µl is a big advantage compared to a classical cone-and-plate rheometer which necessitates
several hundred µl for one single data point. A very good reproducibility was given for each measured protein concentration.

Since the development of highly concentrated antibody formulations usually involves the screening of solution viscosity, a timesaving procedure for viscosity measurements is necessary to keep the processing costs at a maintainable range. By this, the dynamic light scattering method offers several benefits [3, 4], and using DLS plate reader devices is invincibly fast since a number of data points are provided within a few minutes. However, protein-bead interactions might occur and were already reported for another antibody. [5] This interaction leads to a significant measurement-slowdown due to the need for verifying and clarifying the observed interaction, searching for alternative standard particles and particle concentrations, and – if other standard particles were not successful – switching to a conventional method for viscosity-measurements such as a cone and plate rheometer. Contrary to that, there are no relevant limitations which hamper the applicability of the mVROC-device for highly concentrated IgG-formulations. The mVROC allows measurements at a broad viscosity range and is not limited as the DLS-method where a solution viscosity of 30 mPa*s was reported as maximum when using 100 nm standard particles. [5] Nevertheless, measurements by the mVROC are more time-consuming and not suitable to be used as high-throughput method.

Based on the pros and cons of both methods, a combination of these two techniques would be very promising as a general approach for viscosity-screening. In a first step, viscosity of the formulations is quickly measured by using the standard setups of the DLS-method which will provide successful results in many cases. [3-5] If obstacles (like aggregation) show up during the first DLS-measurements, switching to the mVROC-method would be the alternative since it avoids laborious search for suitable standard beads and novel setting-adjustment.
5. References


Chapter VI
Estimating the metal coordination between hydroxyethyl starch and proteins as novel protein-stabilizing concept

1. Introduction

PEGylation of proteins by covalent attachment of polyethylene glycol (PEG) to specific amino acid residues represents a capable technique to positively affect the properties of therapeutic proteins - most notably the extension of in-vivo half-life. [1-3] But also protein stability and aggregation-tendency was reported to be positively affected by PEGylation [4, 5], whereas the underlying stabilization-mechanism is ascribed to the excluded volume theory. [5, 6] Besides the covalent binding between PEG and proteins, a significant impact on protein stability was also shown for non-covalent PEGylation, where the attachment between PEG and protein was based on polyanion complexation [7], or hydrophobic interactions via lipophilic head groups attached to the PEG-polymer. [8, 9] Recently, a novel concept of non-covalent PEGylation was reported which is based on metal coordination between modified PEG and the protein. [10] In general, PEGylation by metal coordination requires both, a protein with fairly good affinity for metal complexation, and the chemical modification of PEG by attachment of a metal chelating group. The presence of multivalent cations is facilitating the non-covalent linkage between protein and PEG by formation of a chelate complex with protein and PEG as ligands.

Protein-metal complexes without the presence of polymer have already been estimated before as capable tool for protein stabilization, and the effect was ascribed to a preferential binding of the metal-ion to the protein in its native conformation. [11] Attachment of a large polymer via the concept of metal coordination represents a new approach of protein stabilization, which might additionally improve the protein stability by the theory of preferential exclusion. [12] Big advantages of the method compared to covalent polymer-linkage would be (1) maintenance of the original protein structure, and (2) circumventing the synthesis which might negatively affect the protein stability. To the best of our knowledge, there are no data available about the capability of that concept so far.

Polyethylene glycol is an established polymer in protein formulations, being used also in stabilization of protein lyophilisates, or as a part of many widely used surfactants (such as
tweens and poloxamers). However, PEG is held responsible for the formation of peroxides which lead to protein destabilization by oxidative processes. [13, 14] Furthermore, polyethylene glycol was reported to trigger the formation of anti-PEG antibodies [15], and with increasing molecular weight, PEG tends to accumulate in the liver due to its poor renal clearance. [16] Therefore, we decided to investigate the metal coordination between protein and polymer by using hydroxyethyl starch (HES), since it offers desirable properties such as a good safety profile and enzymatic biodegradability which are controllable by the substitution pattern. [17-19]

To sum up, aim of the present chapter is the investigation of metal coordination between hydroxyethyl starch and proteins regarding its ability for protein stabilization. In a first step, HES-based linker molecules were synthesized as a metal-chelating group was attached to the polymer. Afterwards, metal-based binding between modified HES and protein was investigated to evaluate the binding affinity between both macromolecule species. Finally, the complex consisting of protein, HES, and metal ion was analyzed regarding its potential to stabilize the protein against mechanical stress.

2. Materials and Methods

2.1 Materials

2.1.1 Proteins

The concept of metal coordination was investigated by using two proteins, namely granulocyte colony-stimulating factor (GCSF) and human growth hormone (hGH). GCSF was initially formulated in 10 mM sodium acetate buffer pH 4 containing 0.004% polysorbate 20. Protein bulk concentration was 4.04 mg/mL. Prior to the experiments, protein bulk solution was dialyzed against the buffer for metal coordination, which was composed of 10 mM Tris and 50 mM sodium chloride at a pH of 7.4. Dialysis was performed at 4 - 8°C for 40 hours with a CelluSep® T1 dialysis tube (scienova GmbH, Jena, Germany), and buffer was changed three times within that period. Re-buffered protein solution was filtered through 0.2 µm PES sterile syringe filters (VWR International), and exact protein concentration was determined by UV-spectroscopy using the Nanodrop 2000 - system (Thermo scientific) with a GSCF-extinction coefficient of 0.815 mL·mg⁻¹·cm⁻¹ at 280 nm. For all experiments with GCSF, protein-concentration was set constant at 1.0 mg/mL.

Human growth hormone was a gift from Sandoz GmbH (Kundl, Austria), and protein bulk concentration was 8.1 mg/mL hGH in 10 mM phosphate buffer pH 7.0. Protein concentration
was verified by UV-measurements at a Nanodrop 2000 using an UV-extinction coefficient of 0.859 m^2*mg^{-1}*cm^{-1}. Prior to experiments, the bulk solution was filtered through 0.2 µm PES sterile syringe filters (VWR International). The formulation buffer for analyzing the metal coordination was 5 mM Tris and 150 mM sodium chloride, adjusted to a pH of 7.0; therefore, hGH bulk-solution was diluted by the final formulation buffer to a protein-concentration of 5 µmol/L (which corresponds to approximately 0.11 mg/mL).

Protein formulations with different additives were prepared from respective protein- and excipient-stock solutions by using the corresponding buffer. HES-containing stock solutions were filtered through sterile 0.2 µm PVDF syringe filters (VWR International), and all other stock solutions and buffers were filtered through sterile 0.2 µm cellulose acetate syringe filters (VWR International). Mixing of adequate volumes protein-, excipient-, and pure buffer solution resulted in the final-concentrations.

2.1.2 Chemicals

Hydroxyethyl starch (HES) with a molar substitution of 0.5 and an initial molecular weight of 70 kDa was kindly donated from Serumwerke Bernburg AG (Bernburg, Germany). Hexamethylenediamine (HMDA) with 98% purity, Diethylenetriaminepentaaetic acid (DTPA) > 99%, N_{6}N_{6}-Bis(carboxymethyl)-L-lysine hydrate (NTA) > 97%, and N-(3-Dimethylamino-propyl)-N'-ethylcarbodiimide hydrochloride (EDC) > 98% were purchased from Sigma Aldrich (Taufkirchen, Germany). Sodium cyanoborohydride (NaBH_{3}CN) was from Merck Schuchardt OHG (Hohenbrunn, Germany), and Tris(hydroxymethyl)aminomethane in Ph.Eur.-quality from Merck KGaA (Darmstadt, Germany). All other reagents were at least of analytical grade.

2.2 Methods

2.2.1 Synthesis of HES-based linker molecules

In order to obtain HES in lower molecular weights, 70 kDa HES was subjected to acid hydrolysis, and subsequent dialysis against highly purified water according to Noga et al. [20] After dialysis, the HES-solution was lyophilized for further processing. This procedure resulted in batch-dependent molecular weights between 23 and 35 kDa as determined by multi-angle light scattering after asymmetric flow field flow fractionation. [20] On the basis of hydrolyzed HES, two different HES-linker molecules for metal coordination were synthesized, which are HES-HMDA-DTPA and HES-NTA.
HES-HMDA-DTA was produced in a two-step synthesis. At first, 500 mg HES and 50-fold molar excess of HMDA were dissolved in phosphate buffer pH 9.0 and shaken for two hours, which leads to a coupling between both molecules via Schiff’s base formation. For reductive amination, NaBH₃CN in 5-fold molar excess (compared to HES) was added to the reaction mixture which was then shaken for further 20 hours. After that time, the reaction solution was dialyzed for 24 hours against highly purified water by using CelluSep® T1 dialysis tubes, and the HES-HMDA product was lyophilized. Success of coupling between HMDA and HES was proven by NMR-spectroscopy. In the second step, DTPA was attached to HES via the free primary amino group of the HMDA-linker. For this, 200 mg of the HES-HMDA conjugate, 10-fold molar excess of DTPA, and 5-fold molar excess of EDC (as activating agent) were dissolved in highly purified water, and the mixture was stirred in a 20R vial for 20 hours. Again, the reaction product was dialyzed against highly purified water, and afterwards lyophilized. Coupling between HES-HMDA and DTPA was verified by NMR-spectroscopy and conductometric titration.

For synthesis of HES-NTA, 1000 mg HES and a 50-fold molar excess of NTA were dissolved in phosphate buffer pH 9.0 and shaken for two hours, which leads to the formation of a Schiff’s base between HES and NTA analog to the synthesis of HES-HMDA (see above). Afterwards, a 5-fold molar excess (compared to the HES concentration) of NaBH₃CN was added to trigger the reductive amination of the Schiff’s base, and this reaction solution was shaken for further 20 hours. The solution was then dialyzed for 24 hours against highly purified water with CelluSep® T1 dialysis tubes, and lyophilized. Coupling efficiency of the HES-NTA product was determined by NMR-spectroscopy.

2.2.2 Conductometric titration

Coupling efficiency of HES-HMDA-DTPA-synthesis was analyzed by conductometric titration using a WTW LF 318 Conductivity meter equipped with a TetraCon® 325 Standard-conductivity cell (Weilheim, Germany). A standard solution was prepared by dissolving 100 mg ZnCl₂ in 1 liter of highly purified water. Titration was performed as triplicate, and for each measurement, 10 mg HES-HMDA-DTPA were dissolved in 10 mL highly purified water, and ZnCl₂ standard solution was added in 40 µL steps. After each step, the conductivity was measured and plotted against the concentration of ZnCl₂ in solution.
2.2.3 NMR-spectroscopy

For obtaining the $^1$H-NMR-spectrum of the lyophilized synthesis products, approximately 15 mg substance were dissolved in 1 mL D$_2$O, and spectra were recorded by using a Jeol JNMR-GX500 (500 MHz) spectrometer.

2.2.4 Size-exclusion chromatography for estimating metal coordination

Metal coordination between GCSF and HES-NTA was investigated by size-exclusion chromatography at an Ultimate 3000 HPLC-system (Dionex Softron GmbH) equipped with a TSKgel G3000SW$_{XL}$ column (Tosoh Bioscience). Mobile phase was identical to the formulation buffer, and composed of 10 mM Tris and 50 mM sodium chloride at a pH of 7.4. After preparation of the samples by mixing the respective stock solutions, the formulations were incubated for 60 minutes at room temperature to enable the formation of the metal coordination, and afterwards 60 µL of each sample were injected. Flow rate was set at 0.5 mL/min, and eluting species were UV-detected at 215 and 280 nm.

2.2.5 Microscale thermophoresis

Metal coordination between hGH and HES-NTA was analyzed by microscale thermophoresis using a prototype of the Monolith NT.LabelFree Instrument (NanoTemper Technologies GmbH, Munich, Germany). At first, hGH bulk solution was centrifuged for 10 minutes at 14,000 g to remove any insoluble aggregates. Since the thermophoresis results are incorrect in the presence of protein aggregates, 0.05% of polysorbate 20 was added to the formulation buffer to prevent the protein from aggregation (which was especially needed in the presence of high copper-concentrations). All experiments were performed with hGH at a concentration of 5 µmol/L which leads to an appropriate fluorescence signal. For each titration curve, a concentration series up to 16 samples was prepared with a constant concentration of one (or two), and varied concentration of the second (or third) binding partner. Respective sample volume was 20 µL, and approximately 4 µL of each formulation were sucked into NanoTemper glass capillaries and placed onto the sample tray. Measurements were performed in the UV mode with an excitation wavelength of 280 nm and emission wavelength around 360 nm. LED-power was set at 60%, and temperature within the measurement chamber was 25°C. Thermophoresis was measured by using a laser power of 25% within a laser-on period of 30 seconds and a subsequent laser-off time of 5 seconds. Data processing was performed by using the NanoTemper Data Analysis Software.
2.2.6 Shaking stress

For agitation studies, formulations were prepared by pre-mixing all components except the protein, and finally, the respective protein bulk solution was added in the desired concentration. Sample volumes of 500 µL were filled into 650 µL micro-centrifuge tubes (VWR International), and horizontally placed onto an Eppendorf mixer 5432 (Eppendorf AG, Hamburg, Germany). Each formulation was prepared and analyzed in triplicate. Formulations were shaken at room temperature, and 100 µL samples were drawn in the case of GCSF after 0, 5, 10, and 15 minutes of shaking, and in the case of hGH after 0, 1, 2, and 5 minutes of shaking. The 100 µL samples were centrifuged for 10 minutes at 15,000 g to remove insoluble aggregates, and the supernatant was analyzed for remaining monomer by HP-SEC on an Ultimate 3000 HPLC-system (Dionex Softron GmbH) by using a TSKgel G3000SWxl column (Tosoh Bioscience). For the GCSF-samples, the running buffer was composed of 10 mM Tris and 50 mM sodium chloride at a pH of 7.4, and the flow rate was adjusted to 0.5 mL/min. Injection volume was 60 µL, and UV-detection was carried out at 280 nm. Mobile phase for the hGH-samples was composed of 5 mM Tris and 150 mM sodium chloride at a pH of 7.0, flow rate was set at 0.6 mL/min, and UV-detection was performed at a wavelength of 214 nm. Respective injection volume was 50 µL.

3. Results and Discussion

3.1 Synthesis and characterization of HES-based linker molecules

For enabling metal coordination with hydroxyethyl starch, two different chelating molecules were covalently attached to HES, namely DTPA (Diethylenetriaminepentaacetic acid) and NTA (Nα,Nα-Bis(carboxymethyl)-L-lysine hydrate).

3.1.1 HES-HMDA-DTPA

DTPA (structure illustrated in Scheme VI-2) represents a five-armed chelator with marked capability to form chelate complexes with metal ions. The molecule was attached to HES in a two-step synthesis by utilizing HMDA as a linker. In the first step, HMDA was coupled to the terminal aldehyde function of HES via formation of a Schiff’s base and subsequent reduction to secondary amine (Scheme VI-1). A 50-fold molar excess of HMDA was used in order to facilitate a preferably quantitative reaction. Addition of NaBH₃CN led to reductive amination, and the obtained product was intensively dialysed against highly purified water for
purification. After lyophilization of the HES-HMDA-solution, $^1$H-NMR-spectroscopy was applied to prove the effective binding between HMDA and HES (data not shown). By this, a distinct HMDA-signal was seen in the $^1$H-NMR-spectrum but due to the low molecular weight of HMDA compared to HES, the relatively high signal to noise ratio hampered an exact quantitative interpretation. In the second step, coupling between HES-HMDA and DTPA was achieved by the presence of EDC (Scheme VI-2). The carbodiimide-structure of EDC is facilitating a binding between free primary amine of HES-HMDA and the carboxylic function of DTPA by formation of an acylisourea intermediate. [21]

Scheme VI-1:  Synthesis of HES-HMDA by Schiff’s base formation and subsequent reductive amination.

Scheme VI-2:  EDC-mediated synthesis of HES-HMDA-DTPA.
Coupling efficiency between DTPA and HES was demonstrated by conductometric titration (see also [22]), where the resulting titration curve (Figure VI.1) shows a biphasic increase in conductivity with increasing amount of ZnCl₂. At the beginning of the titration, addition of ZnCl₂ leads to a relatively strong increase in conductivity which is ascribed to the release of H⁺-ions due to complex formation between DTPA and Zn²⁺. After saturation of DTPA with Zn²⁺, only slight conductivity-increase was observed which is merely caused by the increasing ZnCl₂-concentration.

**Figure VI.1:** Conductometric titration of 9.8 mg HES-HMDA-DTPA - dissolved in water - with ZnCl₂.

The amount of successfully modified HES-HMDA-DTPA was calculated via the inflection point in the titration curve, and the results in Table VI-1 demonstrate a 72.4 % modification of HES with DTPA.

<table>
<thead>
<tr>
<th>molecular weight of HES-HMDA-DTPA</th>
<th>amount of Zn²⁺ complexed per 1 mol HES-HMDA-DTPA</th>
<th>percentage modification of HES with DTPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>24,000 g/mol</td>
<td>0.724 ± 0.011 mol</td>
<td>72.4 ± 1.1 %</td>
</tr>
</tbody>
</table>

**Table VI-1:** Results for conductometric titration of HES-HMDA-DTPA with ZnCl₂.

After effective attachment of DTPA to HES, metal coordination between HES and protein was estimated by using human growth hormone as model protein since hGH is known for its affinity to metal ions. [23-25] For this, different concentrations (with up to 50-fold molar excess) of HES-HMDA-DTPA and ZnCl₂ were added to a 0.5 mg/mL hGH-formulation, and
the mixtures were analyzed by using HP-SEC for the presence of various-sized molecule species. Size-exclusion chromatography was chosen based on literature reports which showed successful characterization of covalent and non-covalent PEGylation of proteins by a peak-shift in HP-SEC chromatograms. [10, 26, 27] Formation of a stable complex between hGH and modified HES was expected to reduce the retention time of the protein-peak. However, none of the tested formulations resulted in any shift of the protein signal which indicates the absence of the metal-induced hGH-HES-complex (data not shown).

The failure of complexation could be ascribed to the multiple denticity and high cation-affinity of the DTPA-structure. This might cause a strong complexation and a full embedding of the metal-ion into the chelator which leads to a sterical hindrance for the interaction between the metal-ion and protein. As potential alternative to DTPA, a second approach was performed by using NTA as chelating structure since it possesses only three complexing sites and thus a lower affinity to multivalent cations.

3.1.2 HES-NTA

NTA was coupled to HES according to the reaction in Scheme VI-3, where the primary amine group of NTA and the terminal carbonyl-function of HES formed a Schiff’s base structure which was afterwards reduced by addition of NaBH₃CN. The NTA-residue possesses only three carboxylic functions for the chelate-complex formation and is therefore expected to be less metal-affine compared to DTPA. Due to the lower cation-affinity of HES-NTA, and a minor release of H⁺-ions after complexation with zinc, conductometric titration was not applicable to determine the coupling efficiency for the HES-NTA-synthesis since the inflection-point in the titration curve was too weak for correct analysis.

\[
\begin{align*}
\text{HES} & \quad \text{NTA} \quad \text{NaBH}_3\text{CN} \\
\text{HES-NTA} & \quad \text{Reactive site}
\end{align*}
\]

Scheme VI-3: Synthesis of HES-NTA by Schiff’s base formation and subsequent reductive amination.
As an alternative to conductometric titration, \(^1\text{H}\)-NMR-spectroscopy was applied to verify the success of synthesis. The spectrum in Figure VI.2 exhibits a large signal between 5.3 and 5.8 ppm which is characteristic for the proton at position C\(^1\) of the anhydroglucose units of the HES-polymer. [20, 22] A comparable small signal between 1.5 and 2.0 ppm indicates the presence of NTA and is ascribed to the protons of the CH\(_2\)-chain. Therefore, a binding between HES and NTA actually occurred, but due to the large molecular weight of HES (M\(_w\) around 35 kDa), the signal contribution of NTA is comparatively low which hampers quantitative interpretation of the spectrum.

![NMR-spectrum of HES-NTA](image)

Figure VI.2: NMR-spectrum of HES-NTA

### 3.2 Metal coordination between HES-NTA and GCSF

Granulocyte-colony stimulating factor (GCSF) is an 18.8 kDa sized protein which stimulates the proliferation and activity of granulocytic colonies. [28, 29] Stability of GCSF exhibits a great dependence on the solution pH, and pH-values below 4.5 resulted in the maximum maintenance of secondary structure and lowest propensity for protein aggregation. [28, 30] Interestingly, covalent PEGylation resulted in a good stabilization of GCSF at physiological pH-values since precipitation and aggregation was significantly reduced compared to the non-PEGylated formulations. [27] The stabilizing effect was slightly higher for 20 kDa PEG compared to 5 kDa PEG, and ascribed to sterical-hindrance for the formation of aggregates.
GCSF was furthermore reported as feasible protein for the formation of copper-mediated metal coordination since it was already effectively attached to a flexible PEG-NTA derivative. Due to the positive literature-reports for GCSF regarding covalent and non-covalent PEGylation, GCSF was used as first model protein to study the hypothesis of protein stabilization by metal coordination to hydroxyethyl starch.

3.2.1 Binding analysis by size-exclusion chromatography

Metal-coordination between GSCF and HES was analyzed by HP-SEC since effectiveness of covalent and non-covalent PEGylation was often successfully determined by size-exclusion chromatography where high-molecular-weight PEGylated species are detectable by eluting at shorter retention times. In order to induce non-covalent HESylation of GCSF, copper was chosen as suitable linker metal since GCSF has already been effectively attached to NTA-modified PEG by Cu²⁺-ions. Binding between GCSF and HES-NTA in the presence of Cu²⁺-ions was investigated for 10- and 20-fold molar excess of HES-NTA, and eluting profiles of the tested formulations are illustrated in Figure VI.3. Here, the pure GCSF-formulation shows a sharp monomer peak between 20 and 21 minutes of retention time. Peak-broadening and the appearance of a shoulder were observed in the presence of HES-NTA in 10-fold, and more pronounced in 20-fold molar excess. This broadening indicates the formation of higher-molecular weight species, and might be ascribed to the copper-mediated complexation between HES and GCSF. The broadening of the protein-signal and lack of a distinct second peak for the protein-HES-conjugate can be ascribed to the naturally wide size distribution of the HES-molecules. Protein-free controls with mixtures of copper and HES-NTA showed a comparable small UV-signal at later retention times which was also seen in the respective GCSF-containing samples. Therefore, despite the presence of Cu²⁺-ions in the GCSF-formulations, a distinct amount of HES-NTA remains unbound, or is subjected to dissolution-processes when analyzed by HP-SEC. No UV-signal was observed for the pure Cu²⁺-control.

In general, the results of HP-SEC indicate a low affinity of HES-NTA to GCSF since the eluting profile of the main protein-peak was only marginally affected. The low affinity could be explained by the mono-derivatisation of HES with NTA, and was already observed for Cu²⁺-mediated protein complexation for PEG with only one single NTA-residue. By contrast, significant protein-PEG interactions were seen for flexible PEG-molecules with multiple NTA-moieties. Therefore, one binding site in the HES-molecule seems not sufficient to enable a satisfying interaction with GCSF.
3.2.2 Stability against shaking stress

In order to basically test the hypothesis of protein stabilization by metal coordination, the formulation with 20-fold excess of HES-NTA (which exhibits the best interaction to GCSF - as shown in Figure VI.3) was exposed to mechanical stress by shaking. GCSF is known to be susceptible to agitation stress by the formation of high molecular weight aggregates. [31, 32] Results in Figure VI.4 show that shaking of the pure GCSF-formulation leads to a drastic loss in native protein with only 30% monomer left after 15 minutes. All other formulations resulted in additional protein destabilization which is demonstrated by deterioration of the monomer recovery. The lowest amount of remaining monomer was observed after addition of Cu$^{2+}$ and NTA (without HES). Replacement of NTA by an equimolar amount of HES-NTA leads to a slight protein stabilization which indicates the protective effect of the polymer on the aggregation process. It was already discussed earlier that addition of macromolecules might stabilize proteins against aggregation due to the effect of excluded volume which reduces rapid intermolecular associations. [12, 33] However, presence of 20-fold molar excess of HES was not sufficient to leastwise compensate the negative effect of Cu$^{2+}$ and NTA. Interestingly, also the presence of pure HES without Cu$^{2+}$ showed no stabilizing effect on the
This indicates that HES is not able to stabilize the protein by sterical effects against mechanical stress, but rather mitigates the negative effect of Cu\(^{2+}\) and NTA.

![Graph showing remaining monomer after 0, 5, 10, and 20 minutes of shaking 1 mg/mL GCSF-solutions with no additives (black), addition of CuCl\(_2\) in 10-fold molar excess and HES-NTA in 20-fold molar excess (dark grey), HES in 20-fold molar excess (grey), CuCl\(_2\) in 10-fold molar excess and NTA in 20-fold molar excess (light grey).]

**Figure VI.4:** Remaining monomer after 0, 5, 10, and 20 minutes of shaking 1 mg/mL GCSF-solutions with no additives (black), addition of CuCl\(_2\) in 10-fold molar excess and HES-NTA in 20-fold molar excess (dark grey), HES in 20-fold molar excess (grey), CuCl\(_2\) in 10-fold molar excess and NTA in 20-fold molar excess (light grey).

### 3.3 Metal coordination between HES-NTA and hGH

The concept of metal coordination was further investigated by using human growth hormone (hGH) as second model protein. Affinity of hGH to multivalent metal-ions was frequently reported in literature, where presence of Zn\(^{2+}\)-ions induced dimeric hGH-association [34], and metal-ion affinity chromatography was successfully applied for hGH-purification. [23] Formation of Zn-hGH-complexes was demonstrated as fully reversible after addition of EDTA by maintaining the native protein structure [25], and association between hGH and Fe\(^{3+}\)-ions was considered as suitable for the preservation of hGH-stability. [24] In the present study, metal-mediated coordination between hGH and HES-NTA was analyzed by using the novel technique of microscale thermophoresis, and protein stabilization was afterwards assessed by agitation stress.
3.3.1 Binding analysis by microscale thermophoresis

Currently, various methods are utilized for the investigation of biochemical interactions in macromolecular complexes. [35, 36] By this, microscale thermophoresis offers several advantages such as the low sample consumption with only a few microliters, measurements within the natural environment of the molecules, no need for fluorescence labeling or immobilization of one binding partner, easy sample preparation, and short measurement times. [36] The method is based on the fact that molecules are known to move along a temperature gradient [37, 38], and their motility is highly dependent on any alteration in the solvation shell, molecule size, and surface charge. [36] The principles of microscale thermophoresis measurements are explained in great detail by Jerabek-Willemsen et al. [36] and an illustration is given in Figure VI.5.

![Operating principle of microscale thermophoresis as taken from [36]. A) Schematic illustration of the Monolith instrument consisting of an IR-laser (for generating a local temperature increase within the sample solution) and an optical element (which measures the corresponding fluorescence of the sample molecules). Sample solutions are placed in 4 μL capillaries. B) Measurement signal for one capillary during a single measurement. At the beginning, particles are constantly distributed and show an initial fluorescence value. A locally induced temperature increase leads to movement of the particles until the steady state is reached after approximately 30 s. Turning off the IR-laser provokes back-diffusion of the molecules.](image)

In brief, thermophoretic measurements require a highly precise temperature gradient which is generated by an IR-laser, and which leads to a local temperature increase of 1-10 Kelvin within a radius of 200 μm. Temperature is equilibrated in less than one second, and the
thermophoretic mobility of the sample molecules is monitored by their intrinsic fluorescence intensity over a time period of roughly 30 seconds. Any binding between two molecules (independent on the respective molecule size) leads to alterations in their charge or solvation shell which directly affects the movement along the temperature gradient, and which is measured by time-dependent changes in the local fluorescence intensity. Titration-curves by microscale thermophoresis are obtained by measuring a sample series with up to 16 concentrations, where one binding partner remains a constant value, and the second partner is present in increasing concentrations.

Metal-based interaction between HES-NTA and hGH requires a multivalent cation with sufficient affinity to the protein. Therefore, hGH-metal interactions were investigated in a first step where affinity of hGH to three different cations was estimated. Titration curves of 5 µmol/L hGH with a wide concentration range of Ni$^{2+}$ and Zn$^{2+}$ ions (30.5 nM to 1.0 mM) showed no change in the thermophoresis-signal which indicates the absence of any significant protein-metal interactions (Figure VI.6A and B). By contrast, measuring the same concentration-range with Cu$^{2+}$-ions resulted in a thermophoresis-increase from about 670 to 740 (Figure VI.6C) which is strongly suggestive for a hGH-Cu-association. The preferential interaction of hGH to Cu$^{2+}$-ions in comparison to Ni$^{2+}$- and Zn$^{2+}$-ions was already reported earlier and is ascribed to the conformational status of the binding-relevant histidine residues. [23] This observation additionally explains the failure of Zn-mediated linkage between hGH and HES-HMDA-DTPA (see above). The lack of data points in the Cu-titration curve (Figure VI.6C) is caused by the occurrence of protein aggregation in formulations containing higher concentrations of Cu$^{2+}$ as aggregated species lead to perturbation and miss-interpretation of the thermophoresis signal. However, presence of aggregation is a further hint for the effective metal-complexation since protein-aggregation and metal-induced protein-precipitation were often attributed to the affinity between protein and metal-ion. [25, 34, 39, 40]
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Figure VI.6: Thermophoresis signal for titration series of 5 µmol/L hGH with increasing concentrations of (A) NiCl₂, (B) ZnSO₄, and (C) CuCl₂.

For further interaction-experiments with HES-NTA, a concentration of 50 µmol/L CuCl₂ was chosen which was sufficient to result in a significant interaction with hGH, but was still below the threshold of protein aggregation (see Figure VI.6C). HES-NTA was added to the preformed hGH-Cu-mixture to investigate if the polymer can be attached to the metal-protein complex by that way. The experimental setup solely records the thermophoretic signal of the protein, since there is no fluorescence contribution of HES-NTA. The titration curve in Figure VI.7A shows a thermophoresis-signal of 760 (which means the thermophoresis-induced relative fluorescence change multiplied by a factor of 1,000 [36]) in the low concentrated HES-NTA-formulations which is ascribed to the pure Cu-hGH complex. By increasing the HES-NTA-concentration, an abrupt decrease in thermophoresis occurs at roughly 150 mM HES-NTA, and the signal resembles again the values of the pure hGH-formulation. The result is therefore a hint that indeed no three-component-binding occurred, but rather Cu²⁺ was snatched by the NTA-residue of the HES-molecule. In order to exclude viscosity-mediated effects of HES-NTA on the thermophoresis of hGH, a control experiment without addition of CuCl₂ was performed (Figure VI.7B). Here, thermophoresis of hGH remained
constant at roughly 680 which demonstrates that the presence of HES has no contribution on the thermophoresis-value. Therefore, forming the three-component complex by adding HES-NTA to a preformed hGH-Cu-mixture was not successful.

Figure VI.7: Thermophoresis signal for titration series of (A) a mixture of 5 µmol/L hGH and 50 µmol/L CuCl₂ with increasing concentrations of HES-NTA, and (B) 5 µmol/L hGH without CuCl₂ with increasing concentrations of HES-NTA.

In a second approach to potentially facilitate the Cu²⁺-based coordination between hGH and HES-NTA, increasing Cu²⁺-concentrations were added to a constant mixture of hGH and HES-NTA to circumvent the competition about the metal-ion (Figure VI.8). By this, 250 µM and 2.5 mM HES-NTA were investigated with a CuCl₂-concentration series ranging from 300 nM to 10 mM. For the 250 µM HES-NTA samples (Figure VI.8A), addition of CuCl₂ resulted in a similar result as seen for the titration of hGH without presence of HES-NTA (Figure VI.6C). Again, the presence of higher CuCl₂-amounts (> 3 x 10⁵ nM) leads to protein aggregation in the sample capillaries which renders analysis of those concentrations unusable and explains the lack of data points in Figure VI.8A. In general, aggregated protein leads to an irregular behavior in the measurement signal and deviations from a proper fluorescence change as seen in Figure VI.5B which renders an accurate data analysis unfeasible. The relatively slight increase of the thermophoresis signal in Figure VI.8A indicates that an association between hGH and HES-NTA did not occur. By contrast, no aggregation was observed in the 2.5 mM HES-NTA-formulations after addition of high Cu²⁺-concentrations (Figure VI.8B) and therefore, the whole CuCl₂-concentration series could be successfully measured. This can be seen as further hint for the protective effect of HES-NTA against metal-induced aggregation (see also Figure VI.4). The large amplitude in the thermophoresis-signal - compared to the pure binding between Cu²⁺ and hGH - indicates that the association between HES-NTA and hGH indeed took place.
For verification of the three-component complex, a control experiment was performed where HES-NTA was replaced by linker-free HES (Figure VI.9). Also here, a protective effect of HES against aggregation was observed since higher CuCl$_2$-concentrations were tolerated compared to the HES-free titration curve (Figure VI.6C). The magnitude of thermophoresis-difference is much lower compared to the identical titration with HES-NTA which further confirms the presence of a three-component-complex in Figure VI.8B. Compared to the titration of hGH with CuCl$_2$ alone (Figure VI.6C), presence of HES leads to a slight right-shift of the titration curve which is probably attributed to a slower movement of the protein molecules due to the increased viscosity.

Figure VI.8: Thermophoresis signal for titration series of (A) a mixture of 5 µmol/L hGH and 250 µmol/L HES-NTA with increasing concentrations of CuCl$_2$, and (B) a mixture of 5 µmol/L hGH and 2.5 mmol/L HES-NTA with increasing concentrations of CuCl$_2$.

Figure VI.9: Thermophoresis signal for titration series of a mixture of 5 µmol/L hGH and 2.5 mmol/L linkerfree-HES with increasing concentrations of CuCl$_2$.  

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Summing up the binding experiments by microscale thermophoresis, competition for the metal-ion hampers Cu\(^{2+}\)-mediated linkage between hGH and HES-NTA, and requires high amounts of polymer and copper to facilitate the three-component complex. However, by using sufficient amount of HES-NTA and Cu\(^{2+}\) (approximately 500-fold excess compared to hGH), successful complexation could be demonstrated.

3.3.2 Stability against shaking stress

The basic concept of protein stabilization due to metal coordination was tested by shaking-studies, since hGH is susceptible for aggregation during agitation. [31, 41, 42] Stability of pure hGH-solution was compared against a mixture of hGH, Cu\(^{2+}\), and HES-NTA, which were used in the same concentrations where binding had been shown in the previous thermophoresis-section. As control, unmodified HES in the same molecular weight as HES-NTA was added to hGH to evaluate the sole effect of polymer, and in a second control, hGH-stability was investigated in presence of Cu\(^{2+}\) and NTA to see the influence of complexed copper. The results in Figure VI.10 show a gradual decrease in monomer for the pure hGH-formulation with increasing duration of agitation, and after 5 minutes shaking, remaining monomer decreased to 40%. Interestingly, after the first minute of shaking, a considerable decrease in monomer (from 100 to 60%) was observed, but the rate of destabilization decreased with further shaking time. By contrast, the formulation with Cu\(^{2+}\) and HES-NTA, and the formulation with unmodified HES showed a comparable initial monomer decrease as the pure hGH-solution, but different further aggregation progress. HES alone leads to a increase in further protein aggregation, whereas the presence of Cu\(^{2+}\) and HES-NTA decelerates the aggregation-rate. These differences can be explained by the general theory of protein aggregation, which is classified in several sub-processes. [43] By this, the first steps in the aggregation process seem unaffected by the presence of Cu\(^{2+}\) and HES-NTA, or HES alone, but the presence of HES leads to a greater destabilization in the further process. In general, different effects of crowding agents (such as HES in the present case) were described regarding protein aggregation. [12] On the one side, crowded environments were considered to decelerate the rate of protein unfolding which often represents the first step of further aggregation. [43] But on the other side, crowding agents push the unfolded proteins towards association due to the lack of intermolecular space. [12] The present data (Figure VI.10) show no deceleration due to HES in the first aggregation steps, but rather an acceleration in further protein aggregation. Contrary to that, the negative effect of HES was counterbalanced by Cu\(^{2+}\) and HES-NTA, leading to similar monomer-recoveries as the pure hGH-solution. It might be speculated that the interaction between hGH and Cu-HES-NTA is affecting - and probably shielding - the responsible molecule-parts on the protein which
trigger aggregate formation. The protective effect of Cu$^{2+}$ is even more pronounced by exchanging HES-NTA with an equimolar amount of NTA, since adverse effects of the polymer were eliminated.

Figure VI.10: Remaining monomer [%] after 0, 1, 2, and 5 minutes shaking stress of 5 µmol/L hGH-formulations in varied combination with CuCl$_2$ (2.5 mmol/l), HES-NTA (2.5 mmol/l), HES (2.5 mmol/l), and NTA (2.5 mmol/L).
4. Conclusions

In the present chapter, a novel concept of protein stabilization was investigated which is based on metal-mediated complexation between proteins and hydroxyethyl starch. By this, HES with a molecular weight from 23 to 35 kDa was modified with two chelating linkers, namely DTPA and NTA. Binding between DTPA and HES was facilitated by using HMDA as linker-molecule, and the success of the synthesis was verified by conductometric titration. However, binding experiments by HP-SEC showed no interaction between the model protein hGH and DTPA-modified HES in the presence of Zn$^{2+}$ which was ascribed to the multi-denticity and high metal-affinity of the DTPA-molecule, and an insufficient complexation of Zn$^{2+}$ by hGH.

As a second linker, NTA - which possesses lower metal-affinity compared to DTPA - was covalently attached to the HES-molecule, and success of coupling was demonstrated by NMR-spectroscopy. Metal coordination was investigated by using GCSF and hGH as model proteins. For GCSF, a low affinity between HES-NTA and protein in the presence of Cu$^{2+}$ was observed by HP-SEC, but shaking-stress experiments showed no benefit in protein stabilization by presence of the polymer.

Microscale thermophoresis was applied to investigate the metal-mediated binding-behavior between hGH and HES-NTA. By this, Cu$^{2+}$ was identified as suitable multivalent cation for further binding studies due to sufficient affinity to the protein. However, formation of the three-component complex between hGH, Cu$^{2+}$, and HES-NTA was impeded since protein and polymer compete against binding to the cation, and sufficient metal-coordinated complexation required 500-fold excess of HES-NTA and Cu$^{2+}$ compared to the hGH-concentration. Stability of hGH after non-covalent HESylation was investigated during shaking-stress, but no significant stabilization of hGH was observed compared to the pure hGH-solution.

By analyzing the effect of Cu$^{2+}$ and NTA on protein stability during shaking, reduced aggregation was observed for hGH, and increased aggregation in the case of GCSF. Protein stability after metal-complexation is therefore protein-specific and dependent on the respective metal-binding site. A possible explanation could be that the presence of metal-ions induces protein-dependent changes or partial unfolding processes in the native protein conformation to facilitate better access for complexation, and these changes could be responsible for further aggregation kinetics. For both proteins, the stabilizing or destabilizing effect of Cu$^{2+}$ and NTA was compensated by exchanging NTA with an equimolar amount of HES-NTA. This indicates, that the presence of the polymer is primary responsible to neutralize the effect of the metal-ion on protein stability. A significant contribution of HES to stabilize the protein itself - which was the initial intention of the project - did not appear.
As an overall summary, the novel approach of non-covalent HESylation did not result in the desired effect for protein stabilization. Also, the need for inappropriate high Cu$^{2+}$-concentrations renders the construct unsuitable for marketable protein pharmaceuticals.
5. References


Chapter VII

Summary of the thesis

The present thesis is predominantly focused on the stabilization of liquid therapeutic antibody formulations by cyclodextrins, and the investigation of the underlying interaction between monoclonal antibodies and hydroxypropyl-beta-cyclodextrin (HPβCD). In the first part (chapter 2), the effect of different β-cyclodextrin derivatives on a 100 mg/mL IgG formulation was studied. By this, HPβCD and MβCD showed a promising protection against mechanically-induced aggregation but, cyclodextrins could not prevent the IgG from aggregation during thermal stress, and polysorbate 80 was superior over each cyclodextrin in preventing the protein from particle formation. SBEβCD was effective in the viscosity-reduction of a highly concentrated IgG solution which is a hint for the chaotropic nature of the SBEβCD-anion. However, SBEβCD leads to a slight destabilization under thermal stress. The effect of HPβCD on antibody stability was investigated in detail by using two different IgG antibodies in respective low (1.8 mg/mL) and high (100 mg/mL) concentrations. Here, increasing HPβCD-concentrations lead to a stepwise increase in the stabilization against mechanical stress which was seen in an increased monomer-recovery by HP-SEC and a decrease in formulation turbidity and particle count. However, formulations containing higher concentrations of HPβCD resulted in a lower resistance against protein aggregation during storage at elevated temperature. This effect was additionally mirrored in a decrease of the respective protein melting temperature and points towards an interaction between HPβCD and the IgGs.

In chapter 3, the behavior of polysorbate 80 and HPβCD at the air-water interface of an IgG solution was studied. Aim of the study was to determine if HPβCD exhibits surfactant-like characteristics in the stabilization of the antibody against surface-induced aggregation. To that end, studies by drop profile analysis were performed which give information about surface tension and interfacial rheology of the sample solutions. For one part of the study, a special double capillary setup was used which allows sequential adsorption experiments. The results clearly demonstrate that polysorbate 80 displaces the protein from the surface in both simultaneous and sequential adsorption experiments. By contrast, HPβCD shows differences dependent on the way of adsorption. In simultaneous adsorption experiments, cyclodextrin and antibody is concomitant present at the surface layer, whereas in sequential adsorption experiments, HPβCD is not incorporated in the protein film at the air-water.
interface, and no protein displacement occurs. The data clearly demonstrate that HPβCD acts in a different manner compared to polysorbate 80.

The next section (chapter 4) sheds light upon the question if HPβCD directly interacts with hydrophobic parts on the protein surface. Interaction between HPβCD and two IgGs was studied by using quartz crystal microbalance (QCM) and static/dynamic light scattering. QCM-experiments were performed with native as well as unfolded protein, whereas unfolding was induced by addition of 6 M guanidine-HCl. For the native and unfolded IgG, a reduced adsorption onto the hydrophobic gold surface was observed in the presence of HPβCD, and the reduction was more pronounced for artificially unfolded protein. This can be seen as a hint that HPβCD interacts with hydrophobic parts on the protein surface. A control-experiment with maltoheptaose showed no effect on the protein adsorption rate. Furthermore, the interaction parameters $B_{22}$ and $k_D$ – which were determined by using static and dynamic light scattering – showed a clear increase in repulsive forces between the IgG molecules with increasing HPβCD-concentrations. This can be seen as further confirmation that HPβCD leads to a shielding of hydrophobic parts on the protein surface.

The development of highly concentrated protein formulations is often associated with a substantial increase in solution viscosity which hampers the application via thin syringe needles. Therefore, viscosity-screening is one important part in the formulation development of highly concentrated antibody formulations. In chapter 5, two low-volume methods were compared regarding their applicability to measure the viscosity of a highly concentrated IgG solution, namely dynamic light scattering after addition of standard-sized beads and microfluidic viscosimetry. The results indicate that the dynamic light scattering method implicates some hurdles since the added standard beads induced protein aggregation processes which render the method inapplicable. By contrast, microfluidic viscosimetry provided reasonable results and is therefore suggested as alternative for the DLS-method.

In the last part (chapter 6), a potential novel strategy for protein stabilization was investigated which is based on the metal coordination between proteins and hydroxyethyl starch. By this, two complexing linker moieties, namely DTPA and NTA, were covalently attached to the HES-molecule. Binding analysis by HP-SEC showed no Zn$^{2+}$-mediated interaction between hGH and HES which was modified by DTPA, and the effect might be ascribed to the high cation affinity of DTPA. Therefore, NTA was investigated as second linker, which has a less affinity to metal ions. By this, a Cu$^{2+}$-originated interaction between HES-NTA and GCSF was shown in HP-SEC experiments, and the interaction between HES-NTA and hGH was extensively investigated by microscale thermophoresis. It was demonstrated that a 500-fold excess of HES-NTA is required to obtain the complexation of hGH. Afterwards, the capability
of non-covalent HESylation for stabilizing proteins against shaking stress was analyzed but, no protein stabilization was observed during mechanical stress which makes the concept not applicable at first sight.
Publications and presentations associated with this thesis

Publications

The Role of Polysorbate 80 and HPβCD at the Air-Water Interface of IgG Solutions
Tim Serno, Elisabeth Härtl, Ahmed Besheer, Reinhard Miller, Gerhard Winter
Pharmaceutical Research, January 2013, Volume 30, Issue 1, pp 117-130

Weak antibody-cyclodextrin interactions determined by quartz crystal microbalance and dynamic/static light scattering
Elisabeth Härtl, Nitin Dixit, Ahmed Besheer, Devendra Kalonia, Gerhard Winter
European Journal of Pharmaceutics and Biopharmaceutics, 2013, article in press

Influence of Hydroxypropyl-beta-cyclodextrin on the stability of dilute and highly concentrated IgG formulations
Elisabeth Härtl, Gerhard Winter, Ahmed Besheer

A critical eye on methods for the determination of the viscosity of highly concentrated antibody solutions
Elisabeth Härtl, Ahmed Besheer, Gerhard Winter
Pharmaceutical Development and Technology, 2013, article in rebuttal

Oral presentation

Novel techniques for shedding light upon the interaction between IgG and Hydroxypropyl-β-cyclodextrin
Elisabeth Härtl, Nitin Dixit, Ahmed Besheer, Devendra Kalonia, Gerhard Winter
CRS - German Chapter Annual Meeting, Ludwigshafen, Germany (March 2013)
Poster presentations

Cyclodextrins as stabilizing excipients in protein formulation
Raimund Geidobler, Elisabeth Härtl, Tim Serno, Ahmed Besheer, Gerhard Winter
Forum Life Science, Munich, Germany (March 2011)

Influence of HPβCD on stability of highly concentrated antibody formulations during stirring
Elisabeth Härtl, Ahmed Besheer, Gerhard Winter
Bio Production Forum, Ulm, Germany (June 2011)

Comparing the behavior of Hydroxypropyl-β-cyclodextrin and polysorbate 80 at the air-water interface of an IgG solution
Elisabeth Härtl, Ahmed Besheer, Reinhard Miller, Gerhard Winter
8th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Istanbul, Turkey (March 2012)

Influence of Hydroxypropyl-β-cyclodextrin (HPβCD) on the stability of highly concentrated antibody formulations
Elisabeth Härtl, Ahmed Besheer, Gerhard Winter
8th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Istanbul, Turkey (March 2012)