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Inhibition of therapeutic protein aggregation by cyclodextrins

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Erklärung

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List of Abbreviations

µDSC	Microcalorimetry
2DUV	Second derivative ultra violet spectroscopy
AF4	Asymmetrical flow field flow fractionation
CDs	Cyclodextrin derivatives
CMC	Critical micelle concentration
DLS	Dynamic light scattering
DSC	Differential scanning calorimetry
EDC/NHS	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride / *N*-hydroxysuccinimide
ESI-MS	Electrospray ionization mass spectrometry
FDA	U.S. Food and Drug Administration
FNU	Formazine nephelometric units
FTIR	Fourier transformed infrared spectroscopy
Hmw	High molecular weight
HPβCD	Hydroxypropyl-β-cyclodextrin
HPγCD	Hydroxypropyl-γ-cyclodextrin
HP-SEC	High performance size exclusion chromatography
IgG	Immunoglobulin class G
ISR	Interfacial shear rheology
ITC	Isothermal titration calorimetry
mAb	Monoclonal antibody
MALDI	Matrix assisted laser desorption ionization
MBPM	Maximum bubble pressure method
MβCD	Randomly methylated β-cyclodextrin
PAGE	Polyacrylamide gel electrophoresis
PAT	(Drop) Profile analysis technology
Phe	Phenylalanine
Ph.Eur.	European Pharmacopoiea
PS 80	Polysorbate 80
PVDF	Polyvinylidene fluoride
Rh-GCSF	Recombinant human granulocyte colony stimulating factor
Rh-GH	Recombinant human growth hormone
rpm	Rounds per minute

RU	Response units
SBEβCD	Sulphobutylether-β-cyclodextrin
sc.	Sub-cutaneous
SDS	Sodium dodecyl sulfate
SPR	Surface plasmon resonance spectroscopy
Tm	Melting temperature
Trp	Tryptophan
Tyr	Tyrosine
V_{ss}	Steady state distribution volume

CHAPTER 1

GENERAL INTRODUCTION

1 CHALLENGES IN THE FORMULATION OF PROTEIN DRUGS

Advances in molecular genetics and recombinant protein (rDNA) technology lead to an increasing availability of protein-based biomolecules[1]. In contrast to conventional low molecular weight synthetic chemical drugs protein drugs provide a relatively high specifity and activity at low concentrations[2]. Therefore, today protein drugs represent a fast-growing class of therapeutic molecules[1,3]. Protein-based drugs offer a multitude of new therapeutic options, mainly for the treatment of severe and chronic diseases such as autoimmune or cancer diseases[2]. However, with the increasing number of "new biologic entities" passing through development and manufacturing and finally reaching patients, also unprecedented challenges for the design of stable, safe and convenient formulations are encountered[4].

The first obvious formulation challenge to be faced concerns the delivery of protein drugs. Oral delivery – which is generally the preferred and most widely applied route of drug administration – is not feasible with protein drugs. Two major obstacles would have to be evaded in order to render oral protein administration possible: the protein's susceptibility to hydrolytic and enzymatic degradation in the gastrointestinal tract and the protein's inability to pass the biological membranes for sufficient resorption due to its hydrophilic surface characteristics and large molecular weight[4]. Therefore, proteins have to be administered parenterally and with few exceptions (e.g. pulmonary or nasal delivery, parenteral depot systems) they are brought to patients via the i.v., i.m. or s.c.-route as aqueous solutions or suspensions.

Maintaining formulation stability throughout the life cycle of a therapeutic protein (typical shelf-life is 24 months at 2-8°C) is a very demanding task considering the only marginal thermodynamic stability of a protein and the multitude of possible degradation pathways proteins can undergo[5]. Therefore maintaining protein stability is a second large formulation challenge. Generally protein stability is divided into chemical and physical stability. However, this distinction does not always apply, since these two major groups of protein degradation pathways are mutually dependent: for example the chemical reaction of reducing sugars with proteins (Maillard reaction) may also result in increased levels of aggregates[6]. Chemical degradation of a protein is understood as a change on the protein that involves the formation or loss of covalent bonds[7]. One of the most frequently encountered chemical instability reactions of proteins appears to be deamidation[2,6-7]. But also disulfide bond breakage and formation[8], hydrolysis[6,9-11], oxidation[9-10], isomerisation[11-13], glycation[17], fragmentation[18] and many more are often reported[2,6,14]. In contrast physical protein instability refers to unfolding

and misfolding of the protein as well as to – most importantly - aggregation[6]. Protein aggregation is considered most problematic in protein formulation as it can occur at almost any stage of production, processing, storage and shipping of the protein drug. It can severely influence the pharmacokinetics as well as the safety of the protein drug, because the phenomenon of protein aggregation is closely linked to another challenge in the formulation of protein drugs: unwanted immunogenicity[15].

The key to controlling the “classical” type of immune reactions, which are directed against neo-antigens, is the production of proteins that are identical or almost identical to the respective endogenous proteins[4]. This type of immune reaction is mainly determined by chemical nature of the protein and not by formulation parameters. However, for the second type of immune reactions encountered with recombinantly produced protein drugs, a break-down of immune-tolerance, the formulation (and the occurrence of aggregates) and the route of administration of the drug are determining factors[3,21-22]. Considering the severe consequences of unwanted immunogenicity, such as loss of efficacy or formation of antibodies against endogenous proteins (as observed with the pure red cell aplasia incident of patients receiving a certain erythropoietin-formulation[16]) it becomes evident that understanding and controlling protein aggregation is the major challenge for the development of safe and efficient protein formulations.

An important way of inhibiting protein aggregation in formulations is the addition of appropriate excipients to the formulations[6,17-18]. However, many of the commonly employed excipients suffer from certain short-comings. For instance, non-ionic surfactants well stabilize therapeutic proteins against surface-induced aggregation, as encountered during agitation of protein solutions, but lead to increased aggregation rates during quiescent long-term storage of the formulations[19-20]. Therefore there is a need for new excipients for the inhibition of protein aggregation, either as alternative to currently used excipients or to complement them. Cyclodextrins (CDs) are reported to be a promising class of excipients for the inhibition of protein aggregation[4,21]. However, to date they have not yet been used in approved parenteral products and little systematic investigations have been carried out elucidating their influence on aggregation of therapeutically relevant proteins. Therefore, the focus of the current work will be on the effects and mechanisms of cyclodextrins as inhibitors of therapeutic protein aggregation.

2 PROTEIN AGGREGATION IN BULK SOLUTION AND AT INTERFACES

Due to its potentially serious consequences protein aggregation has drawn major attention in recent years and has even been identified as a major obstacle to overcome in the development of protein formulations, for instance in the case of highly concentrated monoclonal antibody solutions[22]. As already denoted above, protein aggregation can occur at almost any stage of manufacturing, processing, storage, shipment and administration to the patient. During each of these steps, aggregation is governed by different influencing factors and critical parameters. For understanding and controlling aggregation during any of these steps it is necessary to experimentally isolate the factors triggering aggregation and studying them separately. Therefore, in the present work protein aggregation is not classified by structural characteristics as done in earlier works[23] such as type of bond, reversibility, size or conformation, but classified into the induction factors, that are causing protein aggregation. Since it is well-known that the aggregation behavior in bulk solution is fundamentally different from protein aggregation that involves adsorption to a bulk surface[24] these two phenomena are investigated separately throughout this thesis.

2.1 PROTEIN AGGREGATION IN BULK SOLUTION

Since protein aggregation is a critical phenomenon for the safety and efficacy of protein drugs, extensive research work has been dedicated to elucidate the factors controlling protein aggregation[5-6,15,17,23,25-27]. Although different proteins and a variety of influencing factors were studied there is some common ground between most of the investigations. The most common idea of how protein aggregation in solution proceeds is that partially unfolded states (also referred to as molten globule state or “A” states if the protein is acid-denatured) with reduced (but still substantial) secondary structure and clearly reduced tertiary structure expose hydrophobic surfaces and subsequently aggregate[25]. In order to suppress aggregation in bulk solution, it is necessary to maximize two physical protein properties: conformational and colloidal stability.

Increasing conformational stability means that the population of highly aggregation-prone partially unfolded intermediates has to be kept as low as possible. The relative degree of unfolding of the aggregation-prone intermediates is often very small (at most a few percent[24]) and spectroscopic techniques observing the overall, average conformation of a population of protein molecules might not be able to detect the subtle changes on the molecules. Nevertheless, aggregation in such solutions may rapidly proceed[5]. Oftentimes conditions that allow for a maximum conformational stability do not also provide the best conditions for maintaining the second parameter that should be maximized in order to reduce protein aggregation, colloidal stability. Therefore, often a compromise has to be struck in the

selection of the protein formulation. This compromise is still most conveniently achieved by empirical formulation studies.

Conformational stability can be increased by selecting favorable solution conditions. An important factor leading to favorable solution conditions is an appropriate solution pH. Many proteins tend to slightly unfold on a tertiary structural level when acidic conditions are chosen, such as IgG-antibodies or rh-GH, two proteins that are examined in this thesis. For example, a rhu-mAb anti-CD 20 antibody was found to loose its tertiary structure below pH 3[14]. Generally, weakly acidic conditions (pH 5-6) seem to be optimal for the formulation of mAbs[6]. Also rh-GH partially unfolds at low pH-values. For example, rh-GH is reported to populate a partially unfolded "A-state" at pH 2.5 that, in the presence of NaCl, leads to rapid aggregation of the protein[28]. In contrast, rh-GCSF maintains its conformational stability as determined by urea unfolding even at a low pH of 3.5 [29].

Another important factor that compromises conformational stability of proteins and therefore accelerates aggregation in bulk solution is temperature. High temperatures perturb the native protein conformation to a degree that accelerates aggregation[17]. Often aggregation starts well below the temperature that is experimentally determined as the melting temperature of the protein (the temperature at which 50 % of protein molecules are unfolded during a thermal transition[23]), validating the assumption that aggregates are not formed from fully unfolded monomers but that a certain fraction of partially unfolded monomer is sufficient to promote aggregation[29]. The thermal stability of proteins strongly varies. Compared to other proteins antibodies seem to be less sensitive to high temperatures taking into account their melting temperatures of above 70°C[30] whereas most other proteins already completely unfold below 70°C [2]. Increased aggregation rates upon temperature increase are also the basis of accelerated stability studies at elevated temperature carried out for the prediction of aggregation rates during the shelf-life of a protein. However, the assumption of a simple Arrhenius behavior allowing for the extrapolation of the accelerated stability (e.g. at 50°C) data to shelf-life data (e.g. at 4°C) can be seriously misleading[31] and was reported to potentially lead to the underestimation of the rate coefficient for monomer loss and hence to an overestimation of the shelf life of a therapeutic protein[24,32]. Nevertheless there is little alternative to that kind of studies since multi-year stability data at the target storage condition would not be available until late stages of clinical development at which any changes in the formulations would be very costly and difficult from a regulatory perspective.

Conformational stability can also be influenced by ligand binding. This is reflected by the Wyman linkage function which states that preferential binding of ligands to the native state of a protein is expected to shift the folding equilibrium towards a larger population of native protein molecules. Consequently the protein's propensity to aggregate will be reduced[31,33-34]. In contrast, preferential interaction with the unfolded or partially unfolded state of a protein

will result in a decrease of the thermal stability of the protein, e.g. as observed with the preservative benzyl alcohol when binding to interleukin 1 or rh-GCSF[35-36]. Ligand binding will be of special importance throughout this work since cyclodextrins are reported to preferentially bind to the unfolded state of proteins[21], thereby potentially influencing conformational stability of the proteins under investigation. As discussed in further detail below, nonspecific stabilizing compounds like sucrose also influence conformational stability by being preferentially excluded from the protein surface.

Partial unfolding of protein molecules to highly aggregation-prone intermediates, as expressed by the conformational stability, is often the determining step in the formation of aggregates. However, also tendency of small aggregate nuclei to grow to larger aggregates can be a rate-limiting step in protein aggregation, generally referred to as colloidal stability. A global measure taking into account all sorts of intermolecular interactions between the protein molecules (van der Waals, electrostatic, hard-sphere) is given by the second virial coefficient (B_{22}). Positive B_{22}-values indicate overall repulsive forces between the protein molecules in solution: protein-solvent interactions are favored over protein-protein interactions. In contrast negative B_{22}-values indicate attractive forces between protein molecules when protein-protein interaction is favored over protein-solvent interaction[25]. Since the B_{22}-value greatly depends on protein charge, alterations of the solution pH can have dramatic effects on the colloidal stability of a system. For rh-GCSF, one of the proteins that are investigated in this thesis, the role of colloidal stability is very well understood. At low pH (e.g. 3) the rh-GCSF molecules are positively charged and repulsive forces dominate. However, at neutral pH (between pH 5 and 7), aggregation rapidly proceeds, although conformational stability remains nearly unaltered, because the repulsive forces are no longer dominating. In addition to shifting the solution pH in a way that reduces repulsive forces between proteins, colloidal stability can also be lowered by the addition of salts leading to a shielding of repulsive forces[25,29].

2.2 AGGREGATION AT THE AIR-WATER INTERFACE

In comparison to aggregation in bulk solution, the situation in the presence of large hydrophobic interfaces is fundamentally different, since new reaction pathways for protein aggregation are opened up. When partial unfolding of a protein is the rate limiting step for aggregation, the presence of an interface can massively increase aggregation rates. The reason for this phenomenon is that proteins are amphiphilic molecules and this property leads to their strong tendency to accumulate at interfaces. Most proteins exhibit a remarkable adsorption to hydrophobic surfaces, the air–water interface not only being among the most hydrophobic but also most frequently encountered interfaces, e.g. during mechanical agitation and mixing, spray-drying or filtration[37]. Layer thickness of the air-water interface is reported to be in the order of magnitude of about 2 nm which is about the same size as a

protein molecule[5] or 3.1 nm with a secondary layer below of about 5-7 nm thickness[38]. Other interfaces that therapeutic proteins are typically exposed to during their lifecycle may include the glass–water (in vials) or ice–water–interface (during freezing and thawing) which are discussed in the following section.

Generally protein adsorption to the air–water–interface can be divided into three steps[39]. First, diffusion of the protein–molecules into a subsurface has to take place. Proteins then have to overcome energy barriers (caused by surface pressure and an electrical bilayer) and adsorb to the surface. Finally proteins have to rearrange at the surface which involves partial unfolding of the adsorbed protein segments. By exposing parts of the hydrophobic protein core, contacts with the interface are maximized on both sides of the interface and the molecule regains conformational entropy[39]. A protein that is adsorbed to the air-water-interface experiences forces that are dramatically different from the forces in the bulk solution: it has been estimated that the tension forces perpendicular to the interface are as high as 140 pN and therefore large enough to unfold a protein[5]. The altered protein structure along with high local concentrations at the interface often lead to aggregation processes[17]. In addition, in agitated solutions a new air-water interface is continuously created thereby producing an amount of unfolded proteins that is no longer negligible compared to the amount of protein in the bulk and substantial aggregation often results. As discussed above, it is well-known that protein aggregation may have serious implications for the safety and efficacy of protein drugs[6,40]. Hence for a new protein formulation surface–induced aggregation during processing and storage has to be circumvented.

Agitation-induced aggregation has been reported[17,41] for a variety of proteins[42-47], and it is a serious concern for the formulation of mAbs[27,48-52] and fusion proteins containing parts of immunoglobulins[53]. Aggregates formed by agitation have been determined to be very different in nature from aggregates of the same IgG-antibody formed during storage at elevated temperature[50]. Whereas insoluble heat-induced aggregates showed strong alterations of their secondary structure and did not redissolve into soluble aggregate components upon storage, insoluble aggregates formed by agitation-stress were demonstrated to maintain a very native-like conformation and to exist in equilibrium with other small aggregate types[50]. The degree of mAb-aggregation after agitation is influenced by a variety of parameters. The first parameter is the structure of the mAb itself since some IgG antibodies are reported to significantly aggregate within hours of agitation[27,52] whereas others are reported to exhibit a remarkable resistance to aggregation at the air-water interface, after two weeks of agitation at 200rpm[54] or even after two weeks of shaking in vials[51]. It has been suggested that for the successful development of monoclonal antibodies the surface activity of the potential drug candidate should be taken into account, since a positive correlation of susceptibility to shaking-induced aggregation and surface-activity was

reported[55]. However, it seems that also significantly surface-active mAbs can be very resistant to agitation-induced aggregation[51]. Furthermore the degree of IgG-aggregation during agitation-studies is strongly influenced by the filling volume and the existence of a head space in the shaken container vial[27]. In the absence of a head-space (exchange of the air-water-interface by a glass-water interface) the IgG-antibody remains stable whereas the existence of a head space causes significant aggregation. Finally also the concentration as well as the type of ions is found to have an influence on mAb-aggregation during agitation[52]. With increasing ionic strength agitation-induced aggregation is increased. The nature of the examined cations does not influence aggregation, however the selection of anions has a strong influence on shaking-induced aggregation[52].

Also for recombinant human growth hormone (rh-GH) extensive investigations were carried out regarding the behavior after mechanical stressing and exposure to the air–water–interface. Rh-GH was found to aggregate after vortexing or when being shaken in glass vials[43,47]. In addition investigations were carried out that concluded that shear forces alone cannot be made responsible for rh-GH–aggregation after mechanical stressing but that the presence of an air–water–interface is a necessary prerequisite[56]. This behavior was demonstrated by investigations using a rotor–stator–device and a nitrogen–bubbling–method[57]. Furthermore rh-GH tends to aggregate in the presence of other hydrophobic surfaces such as PTFE whereas the behavior under thermally denaturing conditions cannot be correlated to denaturation at hydrophobic surfaces[58]. Similar findings are reported for lysozyme and insulin inactivation in the presence of the hydrophobic surfaces PTFE and air whereas the presence of less hydrophobic glass material caused a smaller degree of inactivation[46,59].

Little studies are available on the behavior of the third model protein of this thesis, rh-GCSF, during agitation. In studies on PEG-GCSF it was found that there is an inverse relationship between concentration of the protein and susceptibility to agitation-induced aggregation[53]. Since a later work has found that the aggregation mechanism of PEG-GCSF is very similar to that of rh-GCSF it can be assumed that the findings for PEG-GCSF apply to rh-GCSF in a similar manner[60].

2.3 AGGREGATION DURING FREEZE-THAWING AND IN THE PRESENCE OF MICROPARTICLES/VARIOUS SURFACES

As a third major induction factor for protein aggregation, freezing and thawing (F/T) processes are discussed. F/T processes occur at multiple stages during manufacturing, processing, storage and analytics of protein pharmaceuticals[17]. For instance, protein bulk solutions are routinely stored at -70°C as an intermediate step during commercial protein pharmaceuticals production, assuming increased long-term stability as compared to storage in the liquid state. For subsequent processing bulk solutions have to be thawed again.

Protein solutions may also be unintentionally frozen due to inappropriate handling of the final parenteral protein products and finally protein samples may also be frozen and thawed later for analytical purposes when analytics cannot be carried out immediately. All of the mentioned processes may also occur repeatedly, thereby exposing the proteins to significant stress that has to be overcome[17].

Numerous studies identifying factors influencing protein stability during freezing and thawing and characterizing resulting protein instability are available. Obviously, the factors controlling protein stability in solution - conformational and colloidal stability - also influence a protein's susceptibility to freeze-thawing-induced degradation with pH and ionic strength being the key parameters[29,61-62]. In addition, some further factors specific for freeze-thawing-induced stress also influence the extent and the characteristics of protein instability. It was found that freezing by itself can perturb a protein's native conformation: cold denaturation[63]. Freezing processes can also lead to freeze-concentration-processes with locally increased protein-concentration that can result in elevated aggregation rates as already discussed above along with the section dealing with aggregation at the air-water interface. In addition, exposure to the ice-water-interface is reported to induce protein unfolding and subsequent aggregation processes, rendering freeze-thawing-stress a further surface-induced protein instability[58]. Since exposure to the ice-water-interface triggers protein instability, it has to be assumed, that protein-concentration is of importance, because a more favorable protein-surface-ratio can be achieved at high protein concentrations thereby decreasing the rate of aggregation. A lower fraction of protein exposed to the surface also explains why there are several reports on decreased protein aggregation despite increasing protein concentration[62], which usually leads to accelerated aggregation rates in solution as experienced with highly concentrated antibody formulations[22,64]. However, it is reported that this rule does not necessarily always hold true for antibodies, because it is reported that the increase of the concentration of a chimeric antibody (L6) did not inhibit F/T-induced aggregation[14,54].

Exposure of the protein to the ice-water-interface also explains why the freeze-thawing-rates can have an influence on protein stability. One would expect that very fast freezing- and thawing-rates minimize damage of the proteins because that way the time of exposure to the harmful ice-water interface is as short as possible. However, several reports state that even very fast freezing and thawing, for instance achieved by immersion into liquid nitrogen, did not stabilize the proteins under investigation compared to slower freezing and thawing rates[58]. In contrast, too slow freezing rates may foster crystallization of solution components thereby leading to accelerated aggregation rates[6,65]. Finally, also the container material and geometry as well as its size can be critical for protein stability, since they also alter warming and cooling rates and the extent of exposure to the ice-water-interfaces as well as to the container-liquid interface[62]. Consequently the prediction of freeze-thawing-induced

aggregation of large bulk quantities from small-scale stress testing poses a major challenge and if availability of protein material allows it, freeze-thawing-induced damage of the protein should always be evaluated at scale[23]. Also, since during thawing of large bulk quantities of protein containers are usually shaken, thawing steps include mechanical stress of the protein.

For the sake of completeness it should be mentioned that also further surfaces are capable of accelerating protein aggregation. For instance, silica microparticles can be shed from glass vials during the autoclaving procedure[5] and such microparticles were shown to induce heterogeneous nucleation processes in recombinant human platelet-activating factor acetylhydrolase, which lead to significant aggregation[26]. Removing the exogenous particles by filtration suppressed aggregation processes. Another study did not observe increased mAb-aggregation in the presence of glass-microparticles but nevertheless suggested using a testing protocol to routinely examine the potential effects of micro- and nanoparticles that could be shed form wetted surfaces[66]. Further solid-liquid interfaces that therapeutic proteins can be exposed to during their lifecycle and that were demonstrated to potentially compromise protein stability include the Teflon™-water interface (aggregation of insulin)[67], stainless steel particles shed from a filling piston pump causing mAb-aggregation at their interface with the mAb-solution[68]. Also leachates from tungsten as well as silicone oil syringe lubricant were already reported to cause protein precipitation[69-70].

3 EXCIPIENTS FOR THE INHIBITION OF PROTEIN AGGREGATION AND THEIR LIMITATIONS

Protein aggregation is arguably the biggest challenge in protein formulation. The present work investigates ways to influence protein aggregation by the addition of a novel group of excipients for this purpose, cyclodextrins. In order to be able to classify the effects of cyclodextrins, comparisons to formulations with other excipients, which are routinely employed in protein formulation and which could serve as a "benchmark", are important. Therefore a brief overview on commonly applied excipients will be given here beforehand. The application of the excipients and their mechanisms of action as well as associated risks with special regard to aggregation will be discussed.

In general, it should be distinguished between the stabilization of liquid formulations and the stabilization of freeze–dried formulations. Since freeze-dried formulations are not subject of this work, excipients and mechanisms that are specific for freeze-drying are beyond the scope of this overview. Generally, the creation of environmental conditions that favor the native state and reduce attractive forces between the protein molecules taking into account the above mentioned parameters such as pH, temperature, ionic strength and protein concentration should be the aim of appropriate excipient addition.

3.1 SUGARS AND POLYOLS

Sugars and polyols form a group of additives that is often referred to as "preferentially excluded cosolvents" or "cosolutes". This designation originates back to the Wyman linkage function and other derived theories, such as that by Thimasheff[34]. Relatively high concentrations of sugars and polyols, but also some amino acids and certain salts, stabilize the native protein state by preferential exclusion. Sucrose is probably the most studied excipient of this group and serves as a good model to explain the mechanism of stabilization by which proteins can be protected by cosolutes. The interaction between sucrose and the protein is thermodynamically unfavored, because of strong repulsion between the protein backbone and sucrose. Thus the greater the surface area of the protein, the more sucrose will be excluded from the protein surface. As during unfolding surface area increases, the amount of "negative binding" between sucrose and the protein increases. Consequently, in the presence of high amounts of sucrose, the native protein state is favored. Sucrose drives proteins towards a compact native state[18,25]. Consequently the population of partially-unfolded aggregation-prone molecules is decreased and aggregation becomes less likely. Besides sucrose, further excipients that are also studied in this thesis, such as trehalose, sorbitol and mannitol, can be counted to this group of excipients.

A potential limitation of preferentially excluded excipients arises upon adsorption of proteins to surfaces, such as the air-water interface. Since preferentially excluded excipients such as sucrose increase the water-surface interfacial tension as well as the protein-water interfacial

tension and leave the protein-surface interfacial tension unaltered, it can be theoretically demonstrated that the free energy of unfolding of a protein molecule adsorbed to a surface is decreased in the presence of preferentially excluded excipients[5,71]. Therefore preferentially excluded excipients inherently lead to a decreased stability of proteins against aggregation induced by the presence of surfaces that possibly has to be overcome by the addition of further excipients.

3.2 BUFFERS AND SALTS

Selecting the appropriate pH range is fundamental for the successful formulation of therapeutic proteins, as already discussed above. However, it is not sufficient to only choose a buffer having an appropriate pK_a at an appropriate concentration. Buffers with comparable pK_as may very well have very different influences on protein stability due to their different ways of interaction with proteins18. In many cases stabilization of proteins through a preferential exclusion mechanism is possible. The Hofmeister series for anions ranks the effectiveness of stabilization by anions: citrate^{3-}/citrate $^{2-}$ > PO_4^{3-} / HPO_4^{2-} / SO_4^{2-} > OAc^-, F^- > Cl^- > Br^- > I^- > ClO_4^-. Attention has to be paid to the concentration of salts of this category because in high concentrations they remarkably decrease protein solubility. In turn this can lead to significant salting-out effects[18].

As already discussed in the colloidal stability section, salts can have an effect on the strength of electrostatic interactions between protein molecules and within one protein molecule[25]. By shielding of charges electrostatic repulsions can be decreased leading to increased aggregation rates at higher salt concentrations.

Additionally binding of salts to proteins may – in the case of multivalent ions – lead to cross-linking of charged residues thereby stabilizing the protein native state whereas the interaction with the peptide backbone potentially destabilizes the protein native state[72-73]. An overview of typical salts and buffers used in protein formulation is given in Table 1.1.

Table 1.1: Salts and buffers commonly used in protein formulation. Taken from [18].

Excipient class	Choices
Buffers	Histidin, Succinate, Acetate, Citrate, Phosphate, Tris, Carbonate
Salts	Sodium Chloride, Calcium Chloride, Magnesium Chloride

3.3 SURFACTANTS

Currently, non-ionic surfactants are the excipients most commonly used to inhibit surface–induced protein aggregation[17,19]. For example, polysorbate 80 (Tween® 80), polysorbate 20 (Tween® 20), Brij® 35 (polyoxyethylene alkyl ether), Pluronic® F 68 and Pluronic® F 88 (polyoxyethylene polyoxypropylene block polymer) were demonstrated to stabilize rhGH

against aggregation during vortexing[47,57]. MAbs as well were demonstrated to be stabilized by polysorbate against mechanical stress[27,48-49] and so were a number of additional proteins[58].

Different mechanisms of stabilization, which appear to depend on the protein being studied, are described. The most obvious mechanism is a competition for adsorption at the air-water-interface, which likely occurs in all cases even if additional routes of polysorbate-induced protein stabilization are operative[17]. In the case of inhibition of aggregation of recombinant Human Factor XIII during agitation,[74] a saturation of the protective effect of polysorbate 20 was reached at a surfactant concentration near the critical micelle concentration – regardless of which protein concentration, i.e. which surfactant–protein ratio, was employed. In addition no evidence for direct binding to the native or the partially unfolded species could be obtained using spectroscopic methods[74].

However, in other cases direct binding between the native protein and the non-ionic surfactant could be observed and also clearly related to the mechanism of aggregation inhibition[43,45]. For example, polysorbate 20 and polysorbate 80 stabilize the fusion protein albutropin against agitation–induced aggregation. By spectroscopic and calorimetric means the two surfactants were shown to bind to this fusion protein in a defined and saturable ratio at concentrations below their respective CMCs. These ratios were also applicable for effective stabilization against aggregation at the air–water–interface. In addition it could be demonstrated that the presence of polysorbate increases the thermodynamic stability of albutropin, thereby reducing the propensity for denaturation and subsequent aggregation[45]. In another study, weak direct binding of polysorbate to hydrophobic patches on native rhGH was demonstrated suggesting the blocking of aggregation–prone sites on the protein surface[43]. Finally, non–ionic surfactants may also act as “molecular chaperones” assisting refolding of partially denatured protein. For example, by adding non-ionic surfactants to denatured carbonic anhydrase II (CAII), refolding of the enzyme could be assisted and aggregation was suppressed[75]. Similar findings were reported for rhGH where a non-ionic surfactant reduced the extent of aggregation during refolding of a molten globule intermediate[42]. Neither direct binding of polysorbate 20 to anti-L-selecting nor thermodynamic stabilization of the protein by polysorbate 20 could be identified as mechanism for fostering the recovery upon reconstitution of freeze-dried anti-L-selectin[76].

Unfortunately, nonionic surfactants and most notably the members of the frequently used polysorbate family are also associated with a number of disadvantages. The use of polysorbate in protein formulations raises major concerns due to autooxidation of the ethylene oxide subunits, which may be followed by the formation of peroxides that in turn can lead to oxidation of the protein[19]. The level of peroxides in formulations of recombinant human granulocyte colony-stimulating factor (rhGCSF) could be correlated to the extent of

protein oxidation[77]. Dual effects of polysorbate 80 on the stability of the model protein IL-2 were reported by Wang et al.[20]: inhibition of shaking-induced aggregation on the one hand but also increased protein oxidation and aggregation during long-term storage in non-agitated solution. These findings are in agreement with a study on pegylated granulocyte colony-stimulating factor (PEG-GCSF) in which polysorbate 20 suppressed protein aggregation induced by agitation, but during quiescent storage increasing polysorbate concentrations lead to increasing amounts of aggregates[53]. Increased levels of aggregation after long-term storage despite good stabilization against surface-induced damage were also reported in the case of recombinant hemoglobin[78]. In addition, the presence of surfactants in protein solutions may also decrease the protein's native state stability, which can be explained by a preferential binding to the unfolded state[79]. Finally polysorbate 80 is also suspected to form mixed micelles with proteins that simulate a viral structure which has been suggested could possibly trigger immune reactions in patients[40].

Because of these shortcomings, there is a need for alternatives (either new kinds of surfactants or even a different class of excipients) to the traditional polyoxyethylene-based surfactants. A disadvantage of new surfactants is that they are not used in approved parenteral products. An effective strategy would be to choose excipients that are already used in approved parenteral products and that may serve the same role as surfactants to inhibit agitation-induced aggregation of therapeutic proteins.

Since cyclodextrins are already used in high concentrations in marketed formulations of low molecular weight drugs they have demonstrated their toxicological suitability for parenteral administration[80]. In addition hints are available that they could be effective at inhibiting aggregation of therapeutic proteins[21,28,81-82]. Therefore one of the aims of this thesis was to test the hypothesis that CDs could be used as alternatives to nonionic surfactants to inhibit surface-induced aggregation of therapeutic proteins.

4 CYCLODEXTRINS

4.1 GENERAL CHARACTERISTICS

4.1.1 STRUCTURES OF NATIVE CYCLODEXTRINS, THEIR DERIVATIVES AND CD POLYMERS

Cyclodextrins (CDs) comprise three major classes of industrially produced, cyclic oligosaccharides, α-, β- and γ-CDs[21,83]. The three native CDs are crystalline, homogeneous, nonhygroscopic substances which have a torus- or truncated cone-like shape. Since CDs are manufactured from starch they are built up by α-glucopyranose units. The enzyme forming CDs is glucosyltransferase, which splits starch chains and rearranges molecules[83].

The system of nomenclature of CDs is based on the number of glucose residues in their structure. α-cyclodextrin comprises six glucopyranose units, β-CD is a heptamer and γ-CD is constituted of eight glucose molecules[83]. CDs containing fewer than six glucose-units can sterically not be formed whereas of the higher homologues only a nine-membered ring has been characterized, but is not industrially produced and did not have any practical importance so far[21,83-84]. Figure 1.1 shows a schematic representation of the three major naturally occurring CDs.

So–called branched CDs result when a section of the amylopectin molecule containing a branching point is incorporated into the cyclic structure thereby attaching one or two glucosyl or maltosyl side chains to the ring. In the 1950s these branched cyclodextrins have also been described as ε-CDs[83]. A number of papers indicate that the use of branched cyclodextrins is increasing again[82,85].

Crystal structures reveal that α-, β- and γ–CDs have a doughnut-like, annular structure with a wide and a narrow hydrophilic end[86]. The narrow end is defined by O(2)H and O(3)H secondary hydroxyl groups and the wide end is marked by O(6)H primary hydroxyl groups. The hydrophobic cavity consists of H(3), H(5) and H(6) hydrogens and O(4) ether oxygens. Glucose molecules are arranged rather rigidly in a $^4C_1$ chair conformation[86]. Figure 1.2 gives an idea of β-CDs' geometry and the localization of the hydroxyl groups that line the narrow and the wide end of the "doughnut – structure".

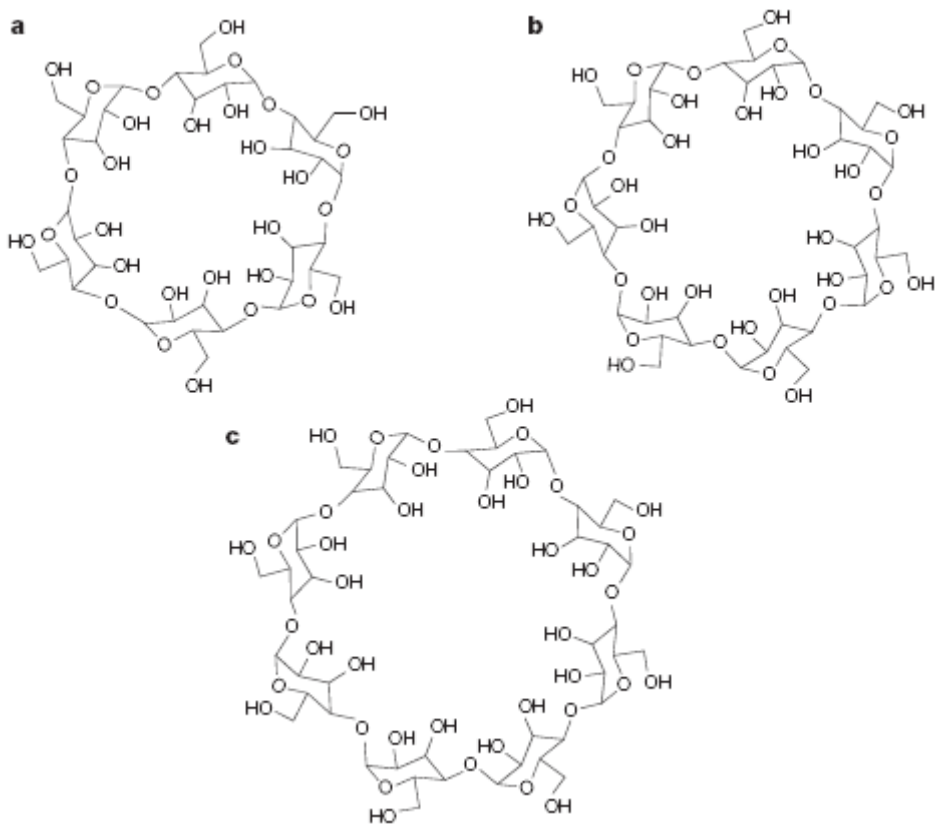


Figure 1.1: Schematic representations of the three major native cyclodextrins: α-CD (a), β-CD (b), γ-CD (c) containing 6, 7 and 8 glucopyranose units, respectively. Taken from [21].

Using neutron diffraction it could be demonstrated that the CD - structure is stabilized in the solid state by intramolecular hydrogen bonding between the secondary hydroxyl groups of neighbored glucose units[86]. Especially in β-CD, a complete secondary belt is formed by these hydrogen bonds. This in turn explains the remarkably low solubility of β-CD in water compared to α-CD (belt is incomplete) and γ – CD (more flexible, non-coplanar structure)[86]. The water solubility (w/w) of the three parent CDs at ambient conditions varies non linearly from approximately 13% to 2% to 26% for α-CD, β-CD and γ – CD, respectively[21]. Substitution of any of the intramolecular hydrogen bond forming hydroxyl groups results in a dramatic increase of aqueous solubility even if the substitutes are hydrophobic moieties such as methoxy and ethoxy functions because hydrogen bond formation of the unsubstituted hydroxyl groups with surrounding water molecules will be possible[87].

The annular volumes and diameters increase remarkably from α-CD to γ–CD which can be seen in Table 1.2. Table 1.2 also summarizes other important characteristics of the three major native CDs.

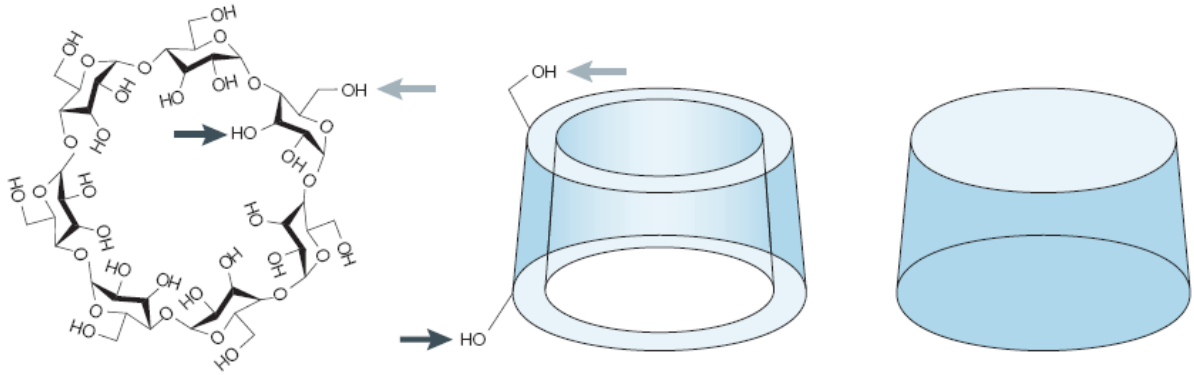


Figure 1.2: Schematic representation of the geometry of β-CD. Taken from [21].

Besides the enhanced possibility to form hydrogen bonds with water, another mechanism that increases the solubility of CDs by substitution is the prevention of their crystallization. By derivatization a statistically substituted material that is made up of many isomeric components is created and leads to the formation of an amorphous product[21]. Furthermore derivatization not only improves solubility but can also reduce the toxicological potential and hemolytic activity of many CDs, especially of ß-CD[88].

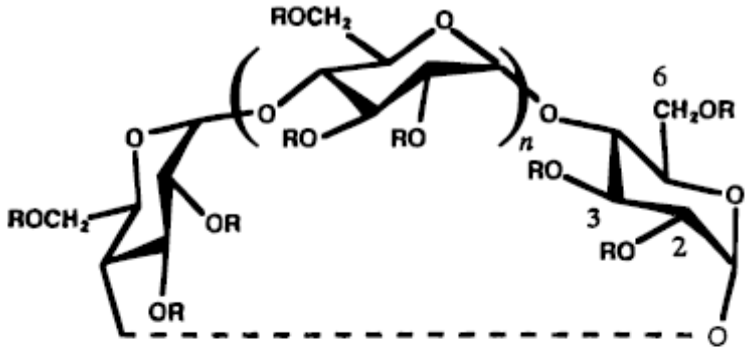
Table 1.2: Key characteristics of native CDs. Adapted from [86].

CD	α-CD	β-CD	γ-CD
Number of glucopyranose units	6	7	8
Molecular weight (anhydrous)	972.85	1134.99	1297.14
Solubility per dm^3 H_2O at 298.2 K	14.5	1.85	23.2
Annular diameter from the C (5) hydrogens [Å]	4.7	6.0	7.5
Annular volume [$Å^3$]	174	262	472

On the surface of the CDs 18 (for α-CD), 21 (β-CD) or 24 (γ-CD) hydroxyl residues can be found and modified chemically. The highest reactivity was observed for the C_6 – OH groups whereas the C_3 – residue was found to be least reactive[83]. However the difference in reactivity is not large. Thus the preparation of selectively derivatized CDs is a rather complex task that is not accomplished on a routine industrial level. The complexity of a statistically substituted mixture of CD – derivatives can well be studied looking at the example of β-CD. As already mentioned there are 21 hydroxyl functional groups and thus 2^{21} – 1 substitutions without even considering optically active centers are possible[21].

Because of its cavity diameter dimensions and its good inclusion properties (see below) β-CD – derivatives have gained a lot of attention. The first derivates reported were the heptakis (2,6-di-O-methyl)-β-CDs and the group of dihydroxypropyl-derivatives which can be prepared by a reaction of epichlorhydrin with natural CDs in alkaline aqueous solution[83]. A third group that has been specified in terms of molecular weight distribution, is the group of hydroxyalkylated CDs, such as hydroxyethyl-, 2-hydroxypropyl and 3-hydroxypropyl –CDs[83].

For 2-hydroxypropyl-β-CD (HPβCD) both a European Pharmacopoeial and a United States Pharmacopoeial monograph exists specifying a molar substitution between 0.4 and 1.5[89]. It is also specified that not more than 1.5% unsubstituted β-CD should be present[89]. Another hydroxypropylated CD derivative, HPγCD, has also gained a certain but not as widespread importance as HPβCD[21].



Cyclodextrin	Abbreviation	R	*n*
α-Cyclodextrin	α-CD	H	4
β-Cyclodextrin	*β*-CD	H	5
γ-Cyclodextrin	*γ*-CD	H	6
Carboxymethyl-*β*-cyclodextrin	CM-*β*-CD	CH_2CO_2H or H	5
Carboxymethyl-ethyl-*β*-cyclodextrin	CME-*β*-CD	CH_2CO_2H, CH_2CH_3 or H	5
Diethyl-*β*-cyclodextrin	DE-*β*-CD	CH_2CH_3 or H	5
Dimethyl-*β*-cyclodextrin	DM-*β*-CD	CH_3 or H	5
Methyl-*β*-cyclodextrin	M-*β*-CD	CH_3 or H	5
Random methyl-*β*-cyclodextrin	RM-*β*-CD	CH_3 or H	5
Glucosyl-*β*-cyclodextrin	G_1-*β*-CD	Glucosyl or H	5
Maltosyl-*β*-cyclodextrin	G_2-*β*-CD	Maltosyl or H	5
Hydroxyethyl-*β*-cyclodextrin	HE-*β*-CD	CH_2CH_2OH or H	5
Hydroxypropyl-*β*-cyclodextrin	HP-*β*-CD	$CH_2CHOHCH_3$ or H	5
Sulfobutylether-*β*-cyclodextrin	SBE-*β*-CD	$(CH_2)_4SO_3Na$ or H	5

Figure 1.3: Selection of CD-derivatives that are frequently used in pharmaceutics. Taken from [90].

Another commonly applied derivatization is methylation. When increasing the degree of methylation, the solubility of β-CD increases until about 2/3 of all hydroxyl groups on the molecule are methylated[83]. Especially randomly methylated β-CD (RMβCD) has been closely examined for potential applications, as it provides good biocompatibility as well as useful complexing efficiencies[21]. Sulfobutylether derivates belong to the group of CDs that has most recently been applied in FDA-approved parenteral products, notably the 2–Sulphobutylether-β-CD (SBEβCD) which is contained in several commercially available preparations[21]. Figure 1.3 gives an overview on the structures of some of the pharmaceutically relevant chemical derivatives of β-CD.

4.1.2 MECHANISM OF COMPLEX FORMATION

It has been described earlier that the central cavities of the CD-molecules are lined with skeletal carbons and etheral oxygens of the glucose residues, which render them lipophilic. It could be demonstrated that the polarity of the central cavity is comparable to the polarity of an aqueous ethanolic solution[87]. Therefore a hydrophobic micro-environment is created in the interior while the cavity exterior of cyclodextrins is hydrophilic due to the presence of hydroxyl functions. This amphiphilic property of CDs is responsible for the aqueous solubility of the CD-molecules on the one hand and for the ability to encapsulate hydrophobic moieties on the other hand.

The incorporation of suitably sized drug molecules is the reason for most pharmaceutical applications of CDs[86]. During the drug-CD complexation process no covalent bonds are formed or broken. In an aqueous solution there is a constant equilibrium between molecules that are included in the hydrophobic interior of the host CD and free molecules[21]. During the inclusion process either the entire guest molecule may be included or as it is the case for proteins only a certain part of the guest molecule is incorporated into the hydrophobic cavity. The included molecules are normally oriented in the host in a position that allows for maximum contact between the hydrophobic part of the guest and the apolar CD cavity, thereby leaving as much as possible of the hydrophilic part of the guest molecule at the outer face of the complex[86]. That way maximum contact with both the solvent and the hydroxyl groups of the host-CD is ensured.

A number of different thermodynamic effects occurring at the same time can explain the complex formation. In most cases a rather large negative ΔH and a ΔS that can be either positive or negative are observed upon complex formation[87]. “Classical” hydrophobic interactions, which are associated with a positive ΔH and also a large positive ΔS (i.e. an entropy driven reaction), can therefore be excluded as single driving force for complex formation. Evidence is available that van der Waals forces are also involved in the complexation process[87]. A term named “compensation” describes the fact that for many inclusion complexes between drugs and CDs a linear relationship between ΔH and ΔS is observed. This observation is often taken as a hint that the release of enthalpy-rich water from the CD – cavity is the major driving force of the complexation process[91]. In addition, other effects like a release of ring-strain seem to play a role as well[87].

4.1.3 TOXICOLOGICAL AND PHARMACOKINETIC SUMMARY

Safety is a major concern when new excipients for pharmaceutical formulations are introduced. Therefore also CDs need to exhibit good biocompatibility in order to be pharmaceutically considered. In the case of CDs toxicity is strongly dependent on the route of administration[88]. Since in this thesis CDs are examined for their potential as excipients in protein formulations only parenteral toxicity is discussed in this paragraph.

An important aspect regarding safety of drug excipients is their level of cytotoxicity. Studies with CDs and isolated erythrocytes showed that CDs are capable of interacting with components of biomembranes[92]. The hemolytic activity was found to be in the order β-CD > α-CD > γ-CD reflecting the different solubilization rates of membrane components by each cyclodextrin[92]. The extraction of membrane cholesterol by CDs results in an increased membrane fluidity, which then induces membrane invagination and can consequently – using high doses of CDs – lead to lysis of the cell. Besides cholesterol, CDs can also extract phospholipids and for β – CD it was even shown that it can remove proteins from erythrocyte membranes[88]. The mechanism of interaction with membranes is different from the one that can be observed with surfactants because CDs do not incorporate into the lipid bilayer but form new lipid containing compartments outside the membrane[85,88].

For parenteral administration toxicity on the kidneys is the most critical constraint. For α – CDs and β – CDs nephrotoxicity is manifested through alterations in vacuolar organelles of the proximal tubule and further cellular changes that were irreversible and ultimately toxic to the cells[88,93]. For β-CD additional toxicity arises from its bad aqueous solubility which leads to microcrystalline precipitation in the kidney. Furthermore, the complexes formed by β-CD and cholesterol can accumulate in the kidney and produce renal tubule damage as well. The LD_{50} values of α -, β - and γ – CDs for intravenous administration are approximately 1.0 g per kg, 0.79 g per kg and more than 4.0 g per kg, respectively. Many of the described problems can be attenuated by functionalizing the parent CDs[21]. As described earlier, improving aqueous solubility can be achieved by almost any substitution on the native CD-molecules[83]. However, an increase in solubility does not necessarily also solve systemic toxicity problems. For example, the improved solubility of methylated CDs did not lead to reduced toxicity[88]. On the other hand, conversion of crystalline β-CDs into amorphous HPβCD by hydroxyalkylation yielded highly water soluble molecules with very low systemic toxicity[94]. Furthermore, also sulfoalkylethers of β-CDs are reported to be well tolerated [95]. Sulfobutylether-β-cyclodextrin can be found in FDA-approved products as well as HPβCD[80]. Typical concentrations

Intravenously administered CDs are rapidly eliminated from systemic circulation. CDs are mainly excreted via the kidneys in an unmetabolized form[88]. Furthermore no deep compartment storage occurs and the V_{ss} is therefore comparable to the extra cellular fluid volume. In addition, it can be observed that the plasma clearance rates are in the same order of magnitude as for inulin which is known to be eliminated at the rate of glomerular filtration (about 110 – 130 ml/min in humans)[88].

4.2 PHARMACEUTICAL APPLICATIONS OF CYCLODEXTRINS

In oral drug delivery the main mechanism of action of cyclodextrins is explained by an increase of drug bioavailability which can be achieved by an increase in the apparent rate

and extent of drug dissolution upon CD complexation, provided that dissociation of the drug is the limiting step of the overall resorption process[21]. This application may become increasingly important as retrospective studies have proved that more than 40 % of failures during drug development are due to poor biopharmaceutical properties[21]. The reasons for these bad biopharmaceutical properties are notably poor dissolution, as drugs are becoming increasingly lipophilic, and also poor permeability due to a tendency towards increasing molecular weights[96-97].

Other applications of CDs in oral drug delivery include a possible increase in drug stability or release time during gastrointestinal (GI) transit which can be accomplished through a modification of the drug release site and time profile[90]. Furthermore, a decrease in local tissue irritation can be achieved by the use of cyclodextrins which could be demonstrated e.g. for β – cyclodextrin in piroxicam-formulations[90,98]. Furthermore masking of poor taste has been reported to be possible with CDs. For example α-CD was able to mask fenbufen bitterness[99].

In parenteral drug delivery the main reason to apply CDs is their capability to solubilize drugs[86]. That way the administration of poorly water-soluble drugs for intravenous and intramuscular dosing can be made feasible. As explained earlier the main critical factor limiting the potential use of CDs as solubilizing agents is their systemic toxicity. In addition also the question whether a linear relationship between drug solubility and the concentration of added CDs exists can have an effect on the acceptability of CDs in parenteral formulations. This linear relationship is necessary for dilution steps because a non – linear relation will possibly result in precipitation of the drug upon dilution[90]. Marketed intravenous formulations containing CDs, include HPβCD for the formulation of the antifungal agent itraconazol (Sporanox®). Another formulation containing HPβCD was approved by the FDA for the formulation of Mitomycin C (MitoExtra®). Moreover, SBEβCD was approved in formulations of voriconazole for i.v. application (Vfend®) and in formulations of ziprazidone for i.m. application (Zeldox®)[21].

Besides the application of CDs with the purpose to improve drug solubility, decreasing the irritation level at the site of administration seems to be possible, too. Other applications of CDs in parenteral formulations comprise the improvement of stability of drugs in an aqueous environment[90], such as for example in the commercial preparation of prostavasin together with α-CD.

Apart from the mentioned pharmaceutical applications of CDs many other possibilities to exploit the unique physico-chemical properties of CDs are either under investigation or already accomplished and readily available on the market. These applications include the conversions of liquids into powders, the reduction of evaporation and thus for example the

stabilization of flavors[21]. Finally, hemolysis can potentially be inhibited and also admixture incompatibilities may be prevented by CDs[21].

4.3 CYCLODEXTRINS AS EXCIPIENTS FOR PROTEIN FORMULATION

A number of drug-CD complexes have already been marketed for parenteral use. However, these products exclusively contain low-molecular-weight drugs[21,100]. In the following section results of studies examining the potential use of cyclodextrins in protein formulations are discussed. Mainly due to their ability to bind to solvent-exposed hydrophobic residues CDs have been found to suppress aggregation of several therapeutically relevant proteins[101]. In turn this can also lead to stabilization of the unfolded state[102-103].

CDs' ability to suppress aggregation has also been exploited for the use in simple artificial chaperone systems based on the ability of CDs to interact with denatured/aggregated proteins in a way that allows for natural refolding. These artificial chaperone systems frequently comprise a combination of a detergent and a CD[104-107].

4.3.1 CYCLODEXTRIN-PROTEIN INTERACTIONS: EXAMPLES AND STRUCTURAL BACKGROUND

Many studies have already been dedicated to elucidate the structural basis of the interaction between CDs and proteins from different perspectives. Almost all studies have identified aromatic amino acid residues as the main site of interaction with CDs. Notably β-CD-derivatives whose cavity diameter allows a fit of Phe, Tyr, His and Trp into the hydrophobic moiety shows this kind of interaction[28,101,108-112]. However, according to Otzen et al., interaction should not generally be limited to aromatic amino acids[28]. Linear chains, for example of Ile, also allow a good fit into the α-CD cavity. However, binding affinities of aliphatic amino acids towards β-CDs are several fold lower than binding affinities of aromatic amino acids towards β-CDs[28].

A wide range of techniques to identify the precise sites of interaction has been applied. The interaction between human growth hormone (rh-GH) Phe- and Tyr-residues and H-3 and H-5 on the interior of the hydrophobic cavity of β-CD has been proven on an atomic level using NMR[28]. In another study NMR spectroscopy was also chosen for the investigations because it is capable of providing atomic level information about complex supramolecular systems[101]. Circular dichroism in contrast was not sensitive enough to measure the rather weak interactions between rh-GH and β-CD. Aachmann et al. detected a multitude of interactions between aromatic residues in insulin and β-CD, but no interactions at all between aliphatic residues and β-CD[101]. Furthermore, the same study proved that for the interaction of two other proteins (CI2 and S6) with β-CD also aromatic residues were responsible (Phe, Tyr). In contrast, ubiquitin could not be complexed at all, which is probably due the fact that it has no solvent exposed aromatic amino acids – an important observation that has to be considered throughout this entire work, too[101].

For studying the interaction of cyclodextrins with peptides containing aromatic amino acids Horsky and Pitha used competitive spectrophotometry[109]. In their study oligopeptides containing aromatic amino acids served as models for unfolded protein structures and it could be demonstrated that the affinity of interaction between CDs and the oligopeptides was at least as high as for the interaction of CDs and the isolated amino acids. An observed slight increase in affinity compared to studies with the amino acids themselves was explained by potential hydrogen bonding between the peptide backbone and secondary hydroxyls on the proteins.

A further study[110] applied steady-state and time-resolved fluorescence spectroscopy as well as fluorescence polarization to study the interaction of a Trp-residue in melittin with HPβCD. Cryogenic measurements of the melittin fluorescence spectra showed Trp to be in a low water environment after addition of HPβCD. Therefore interaction between the lone Trp residue and HPβCD is very probable. Again association of the polypeptide with the CD showed a dissociation constant (K_d) that is rather low and that lies within the range of the association of HPβCD with the single amino acid.

Finally another study[111] combines a number of different methods for the examination of the interaction of HPβCD with [D-Trp^6, Des-Gly^{10}] LHRH. Using UV and fluorescence spectroscopy a change in polarity of the environment of the chromophores occurred. Circular dichroism and ITC further supported the view that aromatic amino acids of deslorelin were included in the hydrophobic cavity of HPβCD.

In addition, a number of crystal structures of carbohydrate-binding proteins in complex with cyclodextrins can be obtained from the PDB database. For example the crystal structure of cyclodextrin glycosyl transferase with β- CD can be found there[113]. Most of the interactions between CDs and carbohydrate binding proteins also seem to take place between aromatic amino acids and the hydrophobic interior of the CDs[101].

4.3.2 STOICHIOMETRY AND AFFINITY OF INTERACTION; THERMODYNAMIC STUDIES

In most studies no clear stoichiometry of the binding of CDs to proteins could be determined. In the cases of peptides containing only one aromatic amino acid residue, such as for melittin[110], the site and stoichiometry of interaction is obvious. However, for rh-GH matters are already more complicated. Electrospray ionization mass spectrometry (ESI-MS) studies could show that rh-GH has at least two binding sites for the interaction with maltosyl-β-CD[85]. Further two-dimensional NOESY-spectra also indicate that rh-GH provides at least two different sites of interaction with β-CD in the native state[28]. For steric reasons probably only a fraction of potential interaction sites is populated by CDs at a given point of time. Naturally in the denatured state (for instance in the acid-denatured state as demonstrated for rh-GH[28]) an even larger number of amino-acids will be solvent accessible and therefore potentially be able to interact with cyclodextrins. A thermodynamic study of thermal unfolding of proteins

using DSC and assuming identical and independent binding sites on the unfolded state of the proteins came to the result that Lysozyme, RNAse and Ubiquitin should contain 12, 5.4 and 3.7 binding sites, respectively[102]. These estimates are rather consistent with aromatic amino acid contents of the three proteins (Lysozyme 6 Trp, 3 Tyr and 3 Phe; RNAse 0 Trp, 6 Tyr, 3 Phe and Ubiquitin 0 Trp, 1 Tyr, 2 Phe).

For insulin Aachmann et al. have determined four sites of interaction on the monomeric form of insulin and one per subunit on the dimeric form of insulin[101]. In that study it was not possible to determine single dissociation constants for interaction sites. The data of Aachmann et al. is at least in part consistent with data from thermodynamic studies[114] in which by using calorimetric dilution data it could be determined that insulin monomer contains at least two binding sites.

Table 1.3 gives an overview on the binding affinities between different CD-derivatives and proteins as well as model peptides that have been determined in the different studies. The global dissociation constants are listed if not indicated otherwise.

Table 1.3: Binding affinities of different CD-derivatives to therapeutic proteins.

CD-derivative	Protein / Peptide	K_d [mol/L]	Analytical method of determination	Reference
HP-βCD	Rh-GH	$4.6 * 10^{-3}$	Fluorescence titration	Otzen et al.[28]
Methyl-β-CD	Insulin at 55°C	$2.1 * 10^{-3}$	ESI-MS and 1 H NMR	Dotsikas and Loukas[108]
HP-βCD	Trp-Gly	$2.0 * 10^{-2}$	Competitive spectrophotometry	Horsky and Pitha[109]
β-CD	Aspartame	$6.7 * 10^{-3}$	Competitive spectrophotometry	Horsky and Pitha[109]
HP-βCD	Melittin	$5 * 10^{-2}$	Fluorescence (time – resolved and steady -state)	Khajehpour et al.[110]
HP-βCD	[D-Trp^6, Des-Gly^{10}] LHRH	$8 * 10^{-3}$	Isothermal titration calorimetry	Koushik et al.[111]
Methyl-β-CD	Insulin	$K_{d1} = 5 * 10^{-2}$ $K_{d2} = 1.5 * 10^{-1}$	Dilution microcalorimetry	Lovatt et al.[114]
G_2-β-CyD (branched)	Rh-GH	$5.8 * 10^{-3}$	Fluorescence method	Tavornvipas et al.[82]
HPβCD	Rh-GH	$1.3 * 10^{-2}$	Fluorescence method	Tavornvipas et al.[82]

Thermodynamic studies do not only yield information on binding affinities and the number of interaction sites, but also demonstrated reduction of the thermal stability of proteins in the majority of cases. For globular proteins this has first been demonstrated by Cooper et al[102]. They found that the binding of CD-derivatives to exposed side chains destabilized the native folded form of globular proteins (lysozyme, RNase A, ubiquitin, phosphoglycerate kinase) as evidenced by a decrease of T_m observed in DSC – studies. This observation has been confirmed later on for example by Tavornvipas et al.[81] who found that the addition of a variety of CD-derivatives to lysozyme formulations leads to a decrease in $T_{m.}$ Interestingly in an earlier study Tavornvipas et al. reported an increase in T_m upon the addition of branched CDs to formulations of rhGH[82]. Surprisingly, their interpretation of these results was that the interactions of branched CDs with accessible hydrophobic side chains in the rhGH molecule lead to a less compact conformation of the protein.

4.3.3 CYCLODEXTRINS AS INHIBITORS OF PROTEIN AGGREGATION

As a number of the presented studies have shown, CDs can lead to a preferential stabilization of the unfolded state. On the other hand interaction with hydrophobic groups on protein oligomers can lead to dissociation of protein aggregates, notably when the interaction occurs at sites in the protein-protein interface. For example, this behavior was observed for the enhanced dissociation of bovine insulin dimers in the presence of different cyclodextrins[103,114]. Furthermore, not only the dissociation of existing aggregates by CDs has been reported but also the inhibition of protein aggregate formation *a priori* is demonstrated in a number of cases. Examples are available for insulin[108], rh-GH[28,82] and several other proteins[115-116]. Protein aggregation represents a major drawback in the development of stable and safe protein formulations and therefore the significant potential of CD-derivatives to suppress aggregation has attracted a lot of interest and a veritable number of studies examining the effect of different classes of CDs on protein aggregation can be found in literature. Table 1.4 gives an overview on studies and results.

Interestingly, in the study of Tavornvipas et al.[82] a correlation between the extent of reduction of aggregation and binding constants between CD-derivatives and rh-GH was found. In their study branched CDs turned out to be most efficient in the prevention of chemically and of thermally induced unfolding and these CD-derivatives also showed the highest stability constants of all CDs tested in the study. HPβCD, which proved useful only in the prevention

Table 1.4: Effect of different CD-derivatives on aggregation of a number of therapeutic proteins.

Protein	CDs	Molar ratio CD:protein	Accelerated stability model	Effect on inhibition of aggregation	References
Lysozyme	12 different CD-derivatives	10-40	Chemical denaturation with GdmHCl	Branched β-CDs and DMβCD most effective	Tavornvipas et al. [81]
bFGF	9 different CDs	> 10000	Chemical denaturation with GdmHCl	DMβCD most effective	Tavornvipas et al. [81]
Lysozyme	5 different CDs	666	Thermal denaturation by DSC	Branched β-CDs and DMβCD most effective	Tavornvipas et al. [81]
bFGF	6 different CDs	830	Thermal denaturation by DSC	SBEβCDs most effective - because protein charged?	Tavornvipas et al. [81]
bFGF	7 different CDs	850	Acid inactivation	SBEβCDs most effective – because protein charged?	Tavornvipas et al. [81]
Salmon calcitonin	β-CD and derivatives	5	Thermal challenge at 55°C	Native CDs no effect, HPβCD and RMβCD increase stability	Sigurjonsdottir et al. [116]
Bovine carbonic anhydrase	16 derivatives of α-, β- and γ-CDs	1500	Denaturation in 6M GdmHCl	Acetyl-CDs most effective,	Sharma, Sharma [115]
O-GH	HPβCD	0 – 400	pH = 7.4 (O-GH stable at pH =11)	Clear solution obtained	Brewster et al. [117]
IL-2	HPβCD	0.0 - 250	Lyophilization	Clear solutions beginning at 2-fold concentration obtained	Brewster et al. [117]
Bovine insulin	HPβCD	400	Long – term stability	Good prevention of precipitation	Brewster et al. [117]
MN12 (Mouse IgG_{2a} Monoclonal Antibody)	HPβCD	50	Lyophilization and storage	More effective than sucrose or dextran as lyoprotectant	Ressing et al. [118]

Protein	CDs	Molar ratio CD:protein	Accelerated stability model	Effect on inhibition of aggregation	References
Porcine Growth Hormone	HPβCD	0 - 400	Thermal (63°C for 1h)	Significant reduction of precipitation	Charman et al. [119]
Porcine Growth Hormone	HPβCD	0 – 400	Guanidine dilution	Ineffective	Charman et al. [119]
Porcine Growth Hormone	HPβCD	0 – 400	Interfacial denaturation (vortexing for 60s)	Significant reduction of precipitation	Charman et al. [119]
Rh-GH	β-CD, HPβCD and various branch-ed CDs	233 (50mM CD and 4.73mg/ml protein)	Chemical denaturation (4.5M GdmHCl)	Branched CDs significantly inhibit aggregation, better than α-, γ- and HPβCDs	Tavornvipas et al. [82]
Recombinant Rh-GH	β-CD, HPβCD and various branch-ed CDs	233 (50mM CD and 4.73mg/ml protein)	Thermal denaturation (DSC)	Branched CDs significantly inhibit aggregation, better than α-, γ- and HPβ-CDs	Tavornvipas et al. [82]
Rh-GH	β-CD, HPβCD and various branch-ed CDs	233 (50mM CD and 4.73mg/ml protein)	Interfacial denaturation (vortexing)	HPβCD superior to other CDs due to its surface activity	Tavornvipas et al. [82]
Rh-GH	α- , γ – CD, various β-CD derivatives	Up to 70 mM of CDs used	pH = 2.5; 1M NaCl	HPβCD, Glucosyl-β-CD, SBEβCD many fold more effective than α- and γ-CD, sulfated CD and monoacetyl-CD not effective at all	Otzen et al. [28]
Recombinant mink and porcine growth hormone	α-, β- and γ-CDs, varying degrees of substitution	Up to 150 mM of CDs, Up to 4.9 mg/ml of protein (ratio up to 670)	Renaturation after urea unfolding	Increased renaturation yield, best results with HPβCD and MβCD; Increased onset of unfolding temp.	Bajorunaite et al.[120]
Mink growth hormone	HPβCD, MβCD, Acetyl-β-CD, γ-CD	0.2 mg/ml mGH, up to 45 mM CD (ratio 4950)	Incubation at 60°C for 5h	HPβCD and MβCD most effective	Bajorunaite et al.[120]

of interfacial aggregation of rh-GH, showed a weaker stability constant and was determined to be less effective in reducing aggregation in most cases. It was suggested that HPβCD acts in a manner similar to non-ionic surfactants by displacing the protein from the interface and thereby preventing surface-induced unfolding and subsequent aggregation. In contrast, for the stabilization against chemically and thermally induced aggregation by branched cyclodextrins, efficient binding was identified as a prerequisite for stabilization.

Looking at the results of the studies presented in Table 1.4 it is difficult to identify general patterns that could lead to a more rational use of certain types of CDs in protein formulations. What might be useful for one therapeutic protein can be without any significant effect for another protein or even compromise protein stability. CD-derivatives inhibiting aggregation arising from a certain stress condition can be incapable of inhibiting aggregation under another stress condition. To solve this problem and in order to provide a rational for using CD-derivatives in protein formulation, Aachmann et al. suggest paying attention to highly solvent accessible exposed hydrophobic residues on the proteins, which could be an important but not compelling prerequisite for the interaction of proteins and CDs[101].

Another systematic approach could comprise a more detailed investigation of the effects of substitution of the CD-ring as the type of substitution seems to be of great influence on the capability to inhibit aggregation (Table 1.4). Further approaches towards a more rational application of cyclodextrin-derivatives in protein formulation should link the binding affinity between CDs and proteins to the effect on aggregation, as already reported once by Tavornvipas et al.[82] Finally, since for HPβCD surface-activity was proposed as a reason for protein-stabilization it is suggested here that the composition of surface layers of mixed CD-protein solutions should be studied.

4.3.4 CYCLODEXTRINS AS FOLDING AIDES / ARTIFICIAL CHAPERONES

It is reported that CDs can be used in vitro as folding assistants. An example is the application of CDs as protein folding aids for carbonic anhydrase B[121]. This involves a one-step technique with the CD introduced in the solution of the denatured protein. The CD transiently interacts with the non-natively folded protein thereby supposedly shifting the equilibrium from intermolecular interaction between the peptide segments towards intramolecular association that favors natural refolding. This technique is often referred to as “dilution additive mode”[107,122].

In other studies CDs have even been found to behave as artificial chaperones. The endogenous GroE chaperone system served as a model for the development of an artificial chaperone system. Like the endogenous GroE chaperone system the artificial chaperone acts by a two step mechanism. In the first step binding of a detergent that captures the non-native protein prevents aggregation but on the other hand also renaturation. In a second step the detergent is stripped away from the protein by the addition of a suitable stripping agent.

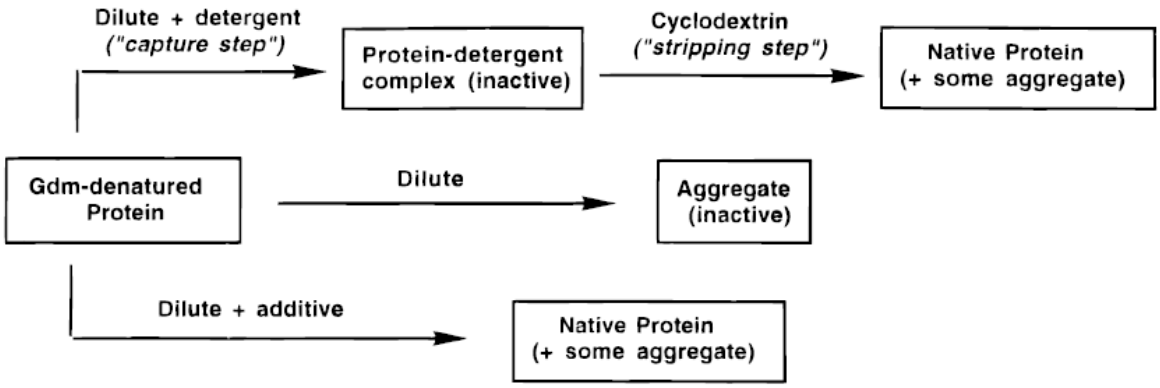


Figure 1.4: Artificial refolding of denatured proteins. Taken from [106].

Cyclodextrins have proved to be useful as stripping agents in the cases of CAB[122], citrate synthase[106] and Lysozyme[107]. As these three proteins are structurally very different from each other a general applicability of the system can be assumed. Figure 1.4 gives an overview on the different ways of artificial refolding of non-native proteins.

5 SUMMARY OF INTRODUCTION

Protein aggregation is one of the biggest challenges in protein formulation development, since it can severely influence safety and efficacy of protein drugs. Therefore it is essential to understand and also control the driving forces of protein aggregation. Besides carefully maintaining appropriate manufacturing, processing, shipping and storage conditions as well as selecting optimal solution conditions the use of excipients to inhibit aggregate formation is without alternative. However, all major groups of excipients that are commonly used in protein formulation suffer from certain short-comings. Sugars and polyols, which are preferentially excluded from the protein-surface, destabilize proteins after adsorption to interfaces. Therefore they are usually combined with non-ionic surfactants, which reduce the extent of surface-induced aggregation. Polysorbates are by far the most commonly employed non-ionic surfactants in protein formulation. However, due to residual as well as in-situ forming peroxides, addition of polysorbate might also lead to increased protein degradation rates. Furthermore, mixed polysorbate-protein micelles have been associated with severe immunogenic events in patients. Hence there is a need for new excipients that could either complement or even substitute common excipients such as non-ionic surfactants.

Cyclodextrins comprise a family of cyclic oligosaccharides that exist in a great variety of chemical derivatives. Two cyclodextrins are currently administered to patients in approved parenteral products: hydroxypropyl-β-cyclodextrin and sulfobutylether-β-cyclodextrin. To date cyclodextrins are exclusively used for the formulation of small molecular entities, mainly in an attempt to increase aqueous solubility and hence bioavailability of poorly soluble drugs. However, there are promising reports in literature that indicate a potential use of cyclodextrin-derivatives in protein formulation since they were found to inhibit protein-aggregation under a variety of accelerated stability conditions. Often the potency of cyclodextrins to suppress protein aggregation is ascribed to their ability to accommodate suitably sized, hydrophobic, solvent-exposed amino acid residues into their hydrophobic core. To date, little systematic and mechanistic investigations allowing for a rational application of cyclodextrins in protein formulation are available. Also, the number of relevant therapeutic proteins for which aggregation inhibition by cyclodextrins was demonstrated, is small. Most notably, no investigations for the currently most widespread class of therapeutic proteins, monoclonal antibodies, are available.

6 OBJECTIVES OF THE THESIS

The overall aim of this thesis was to investigate the role of cyclodextrins in the inhibition of aggregation of therapeutic proteins.

It was thus a first major objective to investigate the effects of various cyclodextrin-derivatives on the aggregation of structurally different model proteins under pharmaceutically relevant stress conditions. As a first model protein monoclonal antibodies as the currently most important class of protein pharmaceuticals were examined (Chapter 3).

The studies on the effect of CDs on mAb-aggregation were complemented by comparison to two further model proteins, rh-GCSF and rh-GH. Thereby, it was planned to distinguish between general effects of cyclodextrins on protein aggregation and effects that depend on the structural properties of the protein being studied, in that way allowing for a more rational application of cyclodextrins in protein formulation.

Since there is a need for alternatives to non-ionic surfactants in protein formulation it was a further objective to evaluate the potential of CD-derivatives to serve as a substitute to non-ionic surfactants in protein formulation. Non-ionic surfactants are most problematic during quiescent long-term storage of proteins. Therefore it was investigated whether the use of cyclodextrins instead of non-ionic surfactants can circumvent increased aggregation rates after quiescent storage.

The second major aim of this thesis was the detailed investigation of the underlying mechanisms that contribute to protein aggregation inhibition by cyclodextrins.

The basis for the first set of investigations was the literature assumption that the shielding of hydrophobic interaction between proteins is the major reason for the inhibition of protein aggregation by CDs. Thus in Chapter 5 it was intended to assess binding between cyclodextrins and proteins in bulk solution and to correlate the results to the effects on protein aggregation.

The experimental approach discussed in Chapter 6 was intended to identify or exclude the potential mechanisms by which CDs stabilize the investigated therapeutic proteins against aggregation at the air-water interface. More precisely, the hypothesis that cyclodextrins act like non-ionic surfactants at the air-water interface, i.e. by displacing proteins from the air-water interface thereby protecting the protein from unfolding and subsequent aggregation, was to be tested.

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CHAPTER 2

MATERIALS AND METHODS

1 MATERIALS

1.1 PROTEINS

1.1.1 MONOCLONAL ANTIBODY

A monoclonal antibody (mAb) of the IgG4 class was kindly donated by Roche Diagnostics GmbH, Penzberg, Germany.

The large majority of therapeutically used antibodies belongs to the IgG class[1]. IgG-antibodies consist of two identical light and heavy chains and each light and heavy chain consists of a constant and a variable region. The variable domain on an antibody molecule contains the complementary determining regions which form an antigen binding pocket on the IgG molecule[2]. There are several subclasses of IgG-molecules (IgG1, IgG2, IgG3 and IgG4) depending on the number and location of interchain disulfide bonds and the length of the hinge region[3]. The antibody used in this thesis belongs to the IgG4 subtype which means that a number of potential instability reactions, that are not encountered in the same frequency with other IgG-subtypes such as disulfide scrambling in the hinge region and subsequent formation of half-antibodies, have to be expected[3]. The total molecular weight of this particular antibody is 146.3 kDa as determined by MALDI mass spectrometry. The major type of secondary structure in IgGs is β-sheet-structure and the content of β-sheet in the molecule is estimated to be about 70 %[2]. Compared to other therapeutic proteins an IgG's thermal resistance is comparably high since the melting point is above 70°C[2].

The IgG bulk material provided for this work was formulated in a 20 mM histidin buffer at a pH of 5.8. Bulk concentration was 2.4 mg/ml. Protein solutions were filtered through Acrodisc® 0.2µm PVDF syringe filter units (Pall GmbH, Dreieich, Germany) prior to usage in formulations. The IgG bulk material consisted of 91.70 % monomer and 8.30 % soluble aggregates of which 5.77 % can presumably be attributed to dimers and the rest to higher order oligomers as determined by HP-SEC. The concentration of the bulk solution was determined by UV-spectroscopy (on an Agilent 8453 UV-VIS diode array spectrophotometer) using an extinction coefficient $\varepsilon = 1.40\ ml*mg^{-1}*cm^{-1}$ at an absorption wavelength $\lambda = 280$ nm. If not stated otherwise the concentration of the mAb during all the accelerated stability assays and storage studies was 1.8 mg/ml.

A second batch of the same IgG4 antibody was also formulated in a histidin 20 mM buffer at a pH of 5.8 but had a bulk concentration of 9.22 mg/ml and contained much lower levels of soluble aggregates (1.37 %). This batch was only used for the so-called "high-concentration"

experiments. Prior to usage in formulations the solution was concentrated by centrifugation at 6000 g in Vivaspin® 20 (Sartorius Stedim Biotech GmbH, Goettingen, Germany) tubes with a molecular weight cut-off of 10,000 g/mol at 4°C to a concentration of roughly 75 mg/ml. The concentrated solution was then adjusted to a concentration of 66.67 mg/ml, then serving as stock solution for the production of the highly concentrated formulations at 50 mg/ml. Again the mAb solution was filtered through Acrodisc® 0.2µm PVDF syringe filter units (Pall GmbH, Dreieich, Germany) prior to usage in formulations.

For a freeze-thaw experiment discussed in Chapter 3 the mAb was dialyzed to a 10 mM potassium phosphate buffer with a pH of 7.4. Dialysis was carried out in Slide-a-Lyzer® (Thermo Fisher Scientific, Rockford, Il, USA) cassettes (size 12-30 mL) made of low-binding regenerated cellulose membranes, against a large excess of buffer (5 L). The procedure was repeated two more times with the last buffer exchange step carried out overnight. After removal of the mAb-solution from the cassettes the concentration was determined spectrophotometrically as described above and the solution was filtered through Acrodisc® 0.2µm PVDF syringe filter units.

In order to overcome problems due to a too low concentration of the mAb bulk material (c = 2.4mg/ml) for the IR-experiments carried out on the Bomem series instrument, the native mAb was concentrated to 17.5 mg/ml using Centricon® Tubes.

The exact composition of the formulations and solutions containing the monoclonal antibody can be found in the respective data chapters (Chapter 3, 4, 5 and 6).

1.1.2 RECOMBINANT HUMAN GRANULOCYTE COLONY STIMULATING FACTOR

Recombinant human granulocyte-colony stimulating factor (rh-GCSF) was a gift from Wacker Biotech GmbH, Jena, Germany. Rh-GCSF is physiologically stimulating the maturation, proliferation and differentiation of stem cell progeny to form neutrophils[4]. Therefore it can be used to treat neutropenia. Structurally the protein belongs to the family of the four-helix bundle cytokines with four α-helices ranged in an up-up and down-down topology[4-6]. Rh-GCSF contains two Trp-moieties, one at residue 58 and one at residue 118. Rh-GCSF is derived from E. coli and the protein is therefore not glycosilated. The molecular weight of this particular rh-GCSF batch was determined to be 18,816 g/mol by ESI-MS.

The bulk drug substance as provided by Wacker Biotech GmbH contained rh-GCSF at a concentration of 4.04 mg/mL. It was formulated in a 10 mM sodium acetate buffer at pH 4. Also the bulk solution contained 0.004 % polysorbate 20. Prior to the usage of the bulk solution in any formulation containing rh-GCSF, the bulk solution was excessively dialyzed against 20 mM acetate buffer at pH 4 in order to remove any traces of polysorbate 20 which would otherwise interfere with the analysis of excipient effects in the formulation studies. Dialysis was carried out in Slide-a-Lyzer® cassettes (size 12-30 mL) according to the protocol described above for the monoclonal antibody. No traces of polysorbate 20 could be detected

by ESI-MS after dialysis to 20 mM ammonium acetate buffer confirming the successful and complete removal of polysorbate 20 from the bulk solution. Rh-GCSF concentration was determined spectrophotometrically using a UV extinction coefficient ε = 0.815 ml*mg^{-1}*cm^{-1} (λ = 280 nm) on an Agilent 8453 UV-VIS diode array spectrophotometer. If not stated otherwise the concentration of rh-GCSF during the accelerated stability assays and storage studies was 1.5 mg/ml.

1.1.3 RECOMBINANT HUMAN GROWTH HORMONE

Recombinant human growth hormone (rh-GH) was provided by the group of Prof. Randolph at the University of Colorado Center for Pharmaceutical Biotechnology (Boulder, CO, USA). Rh-GH also belongs to the family of four-helix bundle proteins[7]. The single-domain protein consists of 191 residues and even in its native state it exposes an unusually high amount of aromatic amino acids making it prone to misfolding and aggregation reactions[8]. The molecular weight of rh-GH as determined by ESI-MS is 22,126.8 Da on average[9]. Rh-GH replacement therapies are frequently applied and rh-GH is also subject to abuse such as for athlete doping[9-10].

Cloning, sequence analysis and expression plasmid construction were completed at BaroFold Inc. (Boulder, CO, USA). After fermentation, purification and refolding rh-GH was lyophilized from a 20 mM Tris buffer at a pH 7.5 with 1% sucrose. After reconstitution in purified water rh-GH was dialyzed against a large excess (same protocol as for the mAb and rh-GCSF) of 1.13 mg/mL histidin buffer (pH 6.5) containing 19.3 mg/ml mannitol in order to match as closely as possible the buffer used by Fradkin et al.[11] for potential later immunogenicity studies. For concentration determination by UV-spectroscopy (carried out on an Agilent 8453 UV-VIS diode array spectrophotometer) an extinction coefficient of ε = 0.859 ml*mg^{-1}*cm^{-1} at λ = 280 nm was used. If not stated otherwise the concentration of rh-GH during the accelerated stability assays and storage studies was 1.0 mg/ml.

1.1.4 RECOMBINANT INTERFERON α-2A

Recombinant interferon α-2a (rh-IFN α-2a) was exclusively employed for mass-spectrometric investigations in this thesis. Rh-IFN α-2a was provided by Roche Diagnostics GmbH, Penzberg, Germany. The protein is not glycosilated and consists of 166 amino acids[12]. It has a molecular weight of 19,241 g/mol as determined by ESI-MS. The bulk formulation had a concentration of 1.5 mg/ml and contained 25 mM acetate buffer (pH 5.0) and 120 mM sodium chloride at pH 5. Prior to mass spectrometric analysis the protein was dialyzed into 20 mM ammonium acetate buffer (pH 5.0) according to the procedure that was described for the other proteins above.

1.1.5 HEN EGG WHITE LYSOZYME

Hen egg white Lysozyme (from here on referred to as “lysozyme”) was purchased from Sigma Aldrich (Steinheim, Germany) as powder. The molecular mass of this lysozyme batch is determined as 14.310 g/mol by ESI-MS. The protein was also exclusively used for mass spectrometric experiments.

1.2 EXCIPIENTS, REAGENTS, CHEMICALS, PACKAGING MATERIALS

1.2.1 FORMULATION EXCIPIENTS

The following table gives an overview on all the excipients used in formulations throughout this work.

Table 2.1: Excipients used in protein formulations throughout this work.

Excipient	Purity / Description	Source
6-O-Maltosyl-β-cyclodextrin	> 97 %	Cyclolab Ltd. (Budapest, Hungary)
Dipotassium hydrogen phosphate	p.a.	VWR International GmbH (Darmstadt, Germany)
Disodium hydrogen phosphate	p.a.	VWR International GmbH (Darmstadt, Germany)
D-Mannitol	Ph.Eur.	Cerestar (Cargill Europe BVBA, Mechelen, Belgium)
D-sorbitol		Merck KGaA (Darmstadt, Germany)
Glacial Acetic Acid (100 %)	p.a.	VWR International GmbH (Darmstadt, Germany)
Hydrochloric Acid	p.a.	VWR International GmbH (Darmstadt, Germany)
Hydroxypropyl-β-cyclodextrin	Pharmaceutical Grade	Wacker Chemie AG (Burghausen, Germany)
Hydroxypropyl-γ-cyclodextrin	Pharmaceutical Grade	Wacker Chemie AG (Burghausen, Germany
L-Histidin	EMPROVE® exp Ph.Eur., USP	Merck KGaA (Darmstadt, Germany)
Maltoheptaose	> 90 %	Cyclolab Ltd. (Budapest, Hungary)
Methyl-β-cyclodextrin	Pharmaceutical Grade	Wacker Chemie AG (Burghausen, Germany)

Excipient	Purity / Description	Source
Polysorbate 20	Super Refined®	Croda Inc. (Edison, NJ, USA)
Polysorbate 80	Super Refined®	Croda Inc. (Edison, NJ, USA)
Potassium dihydrogen phosphate monohydrate	p.a.	VWR International GmbH (Darmstadt, Germany)
Sodium dihydrogen phosphate monohydrate	p.a.	VWR International GmbH (Darmstadt, Germany)
Sodium Hydroxide	p.a.	VWR International GmbH (Darmstadt, Germany)
Sucrose	> 99.5 %	Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany)
Sulfobutylether-β-cyclodextrin	Captisol® D.S. (6.7) Pharmaceutical grade	CyDex Inc. (Lenexa, KS, USA)
Sulfobutylether-β-cyclodextrin	D.S. (4.1)	CyDex Inc. (Lenexa, KS, USA)
Sulfobutylether-γ-cyclodextrin	D.S. (4.3)	CyDex Inc. (Lenexa, KS, USA)
Sulfobutylether-γ-cyclodextrin	D.S. (5.2)	CyDex Inc. (Lenexa, KS, USA)
Trehalose dihydrate	High purity, low endotoxin	Ferro Pfanstiehl Laboratories Inc. (Waukegan, IL, USA)
α-Cyclodextrin	Pharmaceutical Grade	Wacker Chemie AG (Burghausen, Germany)
β-Cyclodextrin	Pharmaceutical Grade	Wacker Chemie AG (Burghausen, Germany
β-Cyclodextrin-sulphate	~ 18 mol sulfate per mol cyclodextrin	Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany)
γ-Cyclodextrin	Pharmaceutical Grade	Wacker Chemie AG (Burghausen, Germany)

1.2.2 FURTHER REAGENTS AND CHEMICALS

In the following table all further chemicals and reagents are listed that have been used throughout this work and were not employed in formulations but mainly for analytical purposes only.

Table 2.2: Further chemicals and reagents used for analytical purposes.

Chemical	Purity / Description	Source
Ammonium acetate	p.a.	VWR International GmbH (Darmstadt, Germany)
Ethanolamine	> 99 %	Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany)
L-Tryptophan	reagent grade (≥ 98 %)	Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany)
L-Tyrosine	reagent grade (≥ 98 %)	Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany)
Maltopentaose	> 95 %	Sigma-Aldrich Handels GmbH (Vienna, Austria)
N-Acetyl-L-tryptophanamide	> 98 %	Sigma-Aldrich Handels GmbH (Vienna, Austria)
N-Acetyl-L-tyrosinamide	> 98 %	Sigma-Aldrich Handels GmbH (Vienna, Austria)
N-ethyl-N9-(dimethylaminopropyl) carbodiimide		Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany)
N-hydroxy-succinimide		Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany)
Potassium Chloride	p.a.	VWR International GmbH (Darmstadt, Germany)
Sodium Chloride	p.a.	VWR International GmbH (Darmstadt, Germany)
Urea	Sigma Ultra	Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany

2 METHODS

2.1 PREPARATION OF FORMULATIONS

All formulations were prepared from stock solutions containing the respective excipient in a higher concentration than in the formulation, protein bulk solutions and the formulation buffer itself. For instance trehalose dihydrate, sucrose, D-mannitol and D-sorbitol were prepared as 1M stock solutions in histidin buffer for the mAb-experiments. Cyclodextrin-derivatives (CDs) and polysorbates were always dissolved in the respective formulation buffer to yield 100 mM stock solutions. The pH of the formulation buffers was adjusted either by using hydrochloric acid (Histidin buffer) or sodium hydroxide (Sodium acetate buffer). All protein formulations were filtered through Acrodisc® 0.2µm PVDF syringe filter units (Pall GmbH, Dreieich, Germany) before the beginning of the accelerated stability study. For all accelerated stability studies, samples were tested in triplicate, and triplicate samples were left unstressed (meaning quiescent at 20°C in the same primary packaging material as the stressed samples) as control samples.

2.2 ACCELERATED STABILITY TESTING AND STORAGE

2.2.1 AGITATION

Agitation experiments of the mAb were carried out on a “Thermomixer” or on an “Orbit 300” shaking device. Polypropylene centrifugal tubes (1.5 ml) were placed vertically onto these devices which were then shaken at a speed of 1200 rpm or 800 rpm, respectively. Rh-GCSF and rh-GH were also agitated on a Thermomixer but at 1100 rpm and 1000 rpm, respectively. By agitation the air-water interface within in the tubes was greatly increased and constantly renewed with substantial entrainment of air bubbles into the solution. The temperature was 20°C throughout all agitation experiments. The tubes were initially filled with 1ml of the respective formulation, leaving enough headspace for the formation of a large air-water interface. At certain intervals (depending on the experiment) 100 µl aliquots of the samples were drawn, centrifuged at 12100 g to remove potential precipitates before subjecting the supernatants to further analysis for remaining monomer and soluble aggregates. In addition tubes were filled with 1.5 ml formulation (leaving no headspace) as a reference in order to evaluate the effect of the absence of an air-water interface.

For the experiments at high mAb-concentration (50 mg/ml) 2R vials (Glass type I, Schott AG, Mainz, Germany) were used instead of polypropylene centrifugal tubes and the vials were fixed horizontally on a shaking device where they moved horizontally at 200 rpm. By filling the vials with 2 mL sufficient headspace for bubble entrainment and constant renewal of the air-water interface was left. After certain intervals aliquots of 100 µL were drawn from the vials which were then closed again with a stopper (FluroTec®-coating, West Pharmaceutical

Services, Eschweiler, Germany) and crimped again until the next time point of analysis. At the start and at the end of the experiment the centrifuged supernatant was also analyzed for alterations in the mAb's secondary and tertiary structure by IR-spectroscopy and UV second derivative spectroscopy.

2.2.2 STIRRING

The stirring stress onto the mAb-formulations was exerted at a constant stirring rate of 200 rpm by placing 6R vials vertically onto a multi-position magnetic stirring device (Variomag™ Magnetic Stirrer, Thermo Electron GmbH, Langenselbold, Germany). Washed and sterilized 6 mm × 3 mm Teflon® coated stirrer bars (VWR International GmbH, Darmstadt, Germany) were put into the vials and the vials were filled with 3 mL mAb-solution each. Temperature was kept constant at 20°C and the samples were protected from direct light. Vials were analyzed at the intervals shown in Chapter 3 by removing aliquots of 100 µL from the vials, centrifuging the aliquots at 12,000 g and subjecting the supernatants to HP-SEC analysis for remaining monomer and soluble aggregates. As usual formulations were tested in triplicates and control samples without a Teflon® stirrer bar were analyzed as well to be sure that the observed effects are due to the stirring-stress.

2.2.3 AGITATION WITH GLASS BEADS

The agitation experiment described above for the highly concentrated mAb-formulations was also carried out at the lower mAb-concentration (1.8 mg/ml) in the presence of glass beads (size 0.25-0.50 mm Carl Roth GmbH + Co. KG, Karlsruhe, Germany). The addition of 1.4 g of glass beads to each vial (filled with no headspace at all, roughly 4 mL per vial) was carried out in order to create an extensive glass-water interface to which the mAb can potentially adsorb. The vials were agitated so that constant renewal of the interface was guaranteed and to create an accelerated stability model in which desorbed and potentially structurally altered and aggregated mAb can subsequently be detected in solution.

2.2.4 FREEZE-THAW EXPERIMENTS

Samples were freeze-thawed (referred to as "FT") by filling 1.0 mL of the respective formulation aliquot into 1.5 mL polypropylenes tubes. The tubes were then immersed into liquid nitrogen for 5 min to ensure complete freezing of the samples. To thaw the samples, the tubes were kept in a Thermomixer™ (without agitation) for 15 min at 25°C. The freeze-thaw cycles were repeated 15 times and after gentle homogenization aliquots of 100µL were drawn from the tubes after every five cycles and analyzed according to the procedure described for the agitation experiment above. The procedure was the same for all three model proteins studied in this thesis.

2.2.5 INCUBATION AT ELEVATED TEMPERATURE AND LONG-TERM STORAGE

MONOCLONAL ANTIBODY

For the evaluation of mAb stability at elevated temperature, 1 ml samples were incubated in polypropylene centrifugal tubes for 8 days at 60°C. Prior to analysis, all samples were first gently homogenized and then centrifuged at 12100 g. The supernatants were analyzed for monomer and soluble aggregates by size exclusion chromatography.

For the mAb-long term incubation study the formulations were stored in cleaned and sterilized 2R-vials (Glass type I, Schott AG, Mainz, Germany) that were sealed with Teflon®-coated rubber stoppers (FluroTec®-coating, West Pharmaceutical Services, Eschweiler, Germany) under a nitrogen atmosphere and subsequently crimped. Each vial was filled with 2 mL of the respective formulation and all samples were prepared in triplicates. The samples were analyzed after 0 months, 3 months and 6 months of storage. Instead of removing aliquots for analysis separate vials were prepared for each time point of analysis in order to avoid extrinsic particle contamination. Storage was carried out at 4°C, 25°C and 40°C.

For the evaluation of mAb temperature stability at higher concentrations (50 mg/ml) a storage study at 50°C was carried out. After certain intervals aliquots of 100 µL were drawn from the vials which were then again closed with a stopper (FluroTec®-coating, West Pharmaceutical Services, Eschweiler, Germany) and crimped again until the next time point of analysis. At the start and at the end of the experiment the centrifuged supernatant was also analyzed for alterations in the mAb's secondary and tertiary structure by IR-spectroscopy and UV second derivative spectroscopy.

RECOMBINANT GRANULOCYTE-COLONY STIMULATING FACTOR

For the evaluation of rh-GCSF stability at elevated temperature, 1ml samples were incubated in polypropylene centrifugal tubes for 230 h at 50°C. After certain intervals 100 µL aliquots were removed from the incubated samples which were then centrifuged at 12000g. The supernatants were analyzed for monomer and soluble aggregates by size exclusion chromatography and at the beginning and at the end of the study for conformational changes by IR-spectroscopy and second-derivative UV-spectroscopy.

RECOMBINANT HUMAN GROWTH HORMONE

For the evaluation of rh-GH stability at elevated temperature, 1ml samples were incubated in polypropylene centrifugal tubes for one month at 50°C. After certain intervals 100 µL aliquots were removed from the incubated samples which were then centrifuged at 12000g. The supernatants were analyzed for monomer and soluble aggregates by size exclusion chromatography. In addition to that the uncentrifuged samples were analyzed for high molecular weight soluble and insoluble aggregates by asymmetric flow field flow fractionation.

2.3 ANALYTICAL METHODS

2.4 PROTEIN AGGREGATION AND CONFORMATIONAL STABILITY

2.4.1 SIZE-EXCLUSION CHROMATOGRAPHY

MONOCLONAL ANTIBODY

After removing insoluble aggregates by centrifuging, the supernatants were analyzed by size exclusion high performance liquid chromatography (SE-HPLC) on an Ultimate® 3000-system (Dionex Softron GmbH, Germering, Germany). 20 µl of the sample were injected onto a Tosoh TSK Gel 3000SW_{xl}–column. Detection was carried out on a UV-Vis Variable Wavelength Detector at 280 nm. The mobile phase consisted of 250 mM potassium phosphate and 200 mM potassium chloride at pH 7, and a flow rate of 0.5 ml/min was used. Peak areas of monomer, dimer, soluble oligomers and fragments were integrated and monitored throughout the incubation experiments. For the initial studies at low mAb-concentration the amount of remaining monomer was calculated in relation to the amount of soluble monomer of the respective formulation at T=0. The amount of remaining monomer and soluble aggregates in all the other experiments was obtained by dividing the respective peak areas of obtained from incubated samples by the total protein peak area of unincubated control samples in the same formulation buffer. The amount of insoluble aggregates can be calculated indirectly as the difference between the total amount of protein at T = 0 and the total amount of protein of an incubated sample after centrifugation and removal of insoluble protein aggregates. For the SE-HPLC analysis of the mAb-samples at 50 mg/ml the aliquots were diluted by a factor of 20 in order avoid a protein overload of the column.

RECOMBINANT GRANULOCYTE-COLONY STIMULATING FACTOR

The same equipment as for the mAb was used with the following differences: injection of 40 µL onto the column and as running buffer 100 mM sodium phosphate at pH 7.0 was chosen. The amount of remaining monomer and soluble aggregates in all the experiments was obtained by dividing the respective peak areas of the incubated samples by the total protein peak area of an unincubated control samples in the same formulation buffer.

RECOMBINANT HUMAN GROWTH HORMONE

The procedure was identical as for rh-GCSF with the exception of the running buffer: 10 mM sodium phosphate with 50 mM sodium chloride at a pH of 7.2 was chosen.

2.4.2 ASYMMETRIC FIELD-FLOW FRACTIONATION

Aggregation of rh-GH was also monitored by asymmetrical field flow fractionation (AF4). The method was applied in order to provide complementary information to the results of the HP-SEC analysis notably concerning the occurrence of high molecular weight soluble and insoluble aggregates in the submicron range that potentially cause immune reactions[11, 13].

The amount of drug substance was unfortunately too limited in order to allow for further analytical techniques to characterize particle formation.

The separation principles by AF4 are reviewed elsewhere[14] and for the analysis here a Wyatt Eclipse 2 system (Wyatt Technology Europe GmbH, Dernbach, Germany) attached to an Agilent 1100 HPLC system (pump running in isocratic mode, autosampler, degasser, UV- and RI-detector, Agilent Technologies, Böblingen, Germany) was used. For the separation of the stressed rh-GH samples an 18 cm channel equipped with a 490 µm spacer and a regenerated cellulose membrane with a 5 kDa cut-off was employed. The running buffer exactly matched the one used for the HP-SEC experiments. However, the aliquots subjected to AF4 analysis were not centrifuged prior analysis in contrast to the samples for HP-SEC analysis. The channel flow was set to 0.6 ml/min and the injection flow was 0.4 ml/min. 5 µl of the rh-GH formulations were injected. The total focusing period was 2 min at a focus flow of 1.6 ml/min. For the separation, an initial cross-flow of 1.6 ml/min was applied for 10 min, before lowering it to 0.0 ml/min within 8 min using a linear gradient and subsequently eluting without cross-flow for another 5 minutes.

2.4.3 TURBIDITY

The turbidity of mAb formulations was determined using a NEPHLA turbidimeter (Dr. Lange, Düsseldorf, Germany). The turbidity was measured in formazine nephelometric units (FNU) by 90° light scattering at a wavelength of λ = 860 nm, a procedure that is described in the European Pharmacopoeia (method 2.2.1).

The turbidity of the highly concentrated mAb-solutions was determined by measuring the UV-absorption at 350 nm. 300 µL of each sample were filled into one well of the 96-well quartz plate and the absorption was measured in the Fluostar Omega (BMG Labtech, Offenburg, Germany) microplate absorbance reader.

2.4.4 LIGHT OBSCURATION

The size and the amount of particles in the range of 1 – 200 µm were determined by light obscuration on a SVSS-C40 apparatus (PAMAS GmbH, Rutesheim, Germany). The system was cleaned with purified water, which was essentially free of particles. Cleaning was performed until less than 100 particles greater than 1 µm could be detected in 1 mL of purified water. The cleanness was checked in regular intervals between the measuring of the samples and after each sample the system was rinsed with 5 mL of purified water.

About 1.5 mL of formulation were removed from a vial and filled into a cleaned glass tube. The system was then flushed with 0.3 mL of sample liquid and subsequently 3 aliquots of 0.3 mL of each sample were analyzed for particles. The average value of the 3 aliquots was calculated from each measurement and this mean amount of particles (for the sizes ≥ 1 µm, ≥ 4.1 µm, ≥ 10 µm, ≥ 25 µm) was related to a sample volume of 1 mL.

2.4.5 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

In order to obtain further information on the nature of mAb-aggregates SDS-PAGE was carried out under non-reducing conditions. NuPAGE® pre-cast 7 % tris-acetate gels 1mm were put into an XCell II Mini cell system (Novex, San Diego, CA, USA) after the 10 wells were previously loaded with diluted mAb-samples. For sample preparation the mAb-samples were diluted to an approximate final concentration of 0.025 mg/mL in a pH 6.8 tris-buffer. In this buffer they were subsequently denatured at 95°C for 20 minutes. 20 µL of the sample preparation were loaded into each well. Separation was carried out at a voltage of 150 V and the running time was approximately 40 min.

The gels were stained with the SilverXPress® Silver Staining Kit (Invitrogen, Karlsruhe, Germany). A standard (Mark 12 Unstained Standard, Invitrogen, Karlsruhe, Germany) was also loaded onto each gel in order to allow for a rough estimation of the molecular weight of the separated samples fractions.

2.4.6 ISOELECTRIC FOCUSING

In order to evaluate whether the isoelectric point of the mAb is altered in its different formulations after storage, isoelectric focussing was conducted. Isoelectric focusing of the IgG was performed by using a Multiphor II Electrophoresis system (GE Healthcare Europe GmbH, Freiburg, Germany) and reagents from Serva Electrophoresis GmbH (Heidelberg, Germany). Samples were loaded onto precast gels (Servalyt® Precotes® pH 6-9) and were run against standards with pIs ranging from 3.5 – 10.7 (IEF Markers 3-10, Serva Electrophoresis, Heidelberg, Germany). Gels were stained by using Serva Violet 17 Staining Kit (Serva Electrophoresis GmbH, Heidelberg, Germany).

2.4.7 FOURIER-TRANSFORM INFRARED SPECTROSCOPY

In order to study secondary structural changes within the precipitates of agitated samples, precipitates were collected as described above and analyzed by infrared (IR) spectroscopy. These IR spectra were then compared to those of the native mAb in solution. To overcome problems due to a too low concentration of the mAb bulk material (c = 2.4 mg/ml) the native mAb was concentrated to 17.5 mg/ml using Centricon® Tubes. For analysis of the precipitates' secondary structure, precipitates of samples containing the mAb without CD were collected after a 24 h and a 1 week incubation period.

IR measurements were performed on a Bomem MB series Fourier transform infrared spectrometer (ABB Bomem, Quebec, Canada). Approximately 20 µl of the aqueous precipitate samples were placed into a cell with CaF_2–windows and a 7.5 µm spacer. Each spectrum was collected as a 256-scan interferogram with a 4 cm^{-1} resolution in single-beam mode. Spectra were recorded in presence of buffer only, buffer and CD as well as in the presence of all three; protein, CD and buffer. Protein spectra were obtained by subtraction

procedures and criteria that were established earlier[15-16]. The spectra were area-normalized and calculation of the second-derivative-spectra was performed using a seven-point Savitsky-Golay derivative function.

All other IR-measurements (mAb-long term storage and high concentration studies, all IR measurements with rh-GCSF) described in this thesis were conducted on a Tensor 27 FTIR spectrometer (Bruker Optics, Ettlingen, Germany) using the calcium fluoride window flow through cell (Aquaspec 1110 M, Bruker Optics, Ettlingen, Germany) with a path length of 6.5 µm and a nitrogen-cooled photovoltaic MCT (mercury-cadmium-telluride) detector. Approximately 50 µL of each sample were injected into the cell for each measurement thereby first rinsing the cell with a sufficient amount of sample liquid. The protein concentrations for the measurements can be obtained in the respective data chapters. The temperature was set to a constant 20°C and the average of 240-scans was used in order to record a spectrum. Spectra were obtained from 4000 cm^{-1} to 850 cm^{-1} with a resolution of 4cm^{-1} and the exactly respective placebo solution was always recorded and subtracted from the protein spectrum. The background-subtracted spectrum was area-normalized and the second-derivative was calculated using a 17-point Savitzky-Golay derivative function. All spectra processing procedures were carried out using OPUS-software (Bruker Optics, Ettlingen, Germany).

2.4.8 UV-Spectroscopy (protein content and 2nd derivative and absorbance 350)

Protein concentrations were determined by UV absorbance measurement at 280 nm using the extinction coefficients that are indicated above in the description of the protein material. All UV-measurements were carried out on an Agilent 8453 UV-VIS spectrophotometer (Agilent Technologies Deutschland GmbH, Böblingen, Germany) equipped with a Peltier temperature controller (Agilent Technologies Deutschland GmbH, Böblingen, Germany). For studies on protein tertiary structure, spectra were collected from 190 to 500 nm with an integration time of 15 s in a 1 cm path length quartz cuvette. Sample concentration depended on the protein being studied and if necessary dilution was carried out in the respective sample buffer in order to remain in the linear range of the instrument ($A \leq 1.5$). All spectra were recorded against the matching placebo formulation.

Processing of the obtained spectra was carried out with the UV-VIS Chemstation software (Agilent Technologies Deutschland GmbH, Böblingen, Germany). Second derivative spectra were calculated using a nine point data filter and a third degree Savitzky-Golay polynomial as well as fitting to a cubic function. The obtained spectrum was interpolated with 99 data points per raw data point. Hence the resolution of the interpolated spectrum was 0.01 nm. Minima and maxima of the exported spectra were calculated using Excel® software.

2.4.9 FLUORESCENCE SPECTROSCOPY AND UNFOLDING

The effect of CDs on thermal stability of the mAb was monitored using intrinsic steady-state fluorescence spectroscopy. A 0.24 mg/ml solution of the mAb in presence of 25 mM CD in a 1cm Quartz cuvette was excited at 280nm and the shift of the emission wavelength maximum during heating was monitored. Scans were collected at 2.5°C increments as the solution was heated from 10 - 90°C with a 5 min equilibration time after each heating step. The measurements were performed on a PTI QuantaMaster (Photon Technology International, Inc., Birmingham, NJ) fluorescence spectrometer. Slits for both excitation and emission were set at 3 nm.

Fluorescence spectroscopy was also used in order to study urea-unfolding of the mAb. An excitation wavelength of λ = 280 nm and an emission wavelength of 327 nm were used to follow the unfolding transition on a Varian Cary Eclipse fluorescence spectrometer (Varian Inc., Darmstadt, Germany). Excitation and emission slits were set to 5 nm. MAb-concentration was 30 µg/mL and solutions with increasing urea-concentrations were produced from a 10 M urea-stock solution.

2.4.10 MICROCALORIMETRY

Thermal stability of the mAb in different formulations was also monitored by high sensitivity differential scanning calorimetry (µDSC) on a MicroCal differential scanning calorimeter (MicroCal Inc., MA, USA). The degassed samples and references were loaded into the cells with a Hamilton syringe and subsequently heated from 30-100°C with a scan rate of 60°C/h. Reversibility of unfolding was tested by performing two consecutive upscans (immediate cooling of the sample after the first upscan).

The samples consisted of mAb (1.8 mg/ml) in His buffer and either HPβCD, MβCD or SBEβCD (all CD-derivatives in a concentration of 2.5 mM) or polysorbate 80 (0.04 % or 0.004 %) or no excipient. The respective reference samples consisted of the buffer only, and the thermograms for the buffer background were subtracted from the mAb-thermograms using Origin 7.0 software. All thermograms were normalized to the concentration of the mAb. Origin 7.0 software was also employed for all data analysis and data deconvolution processes. The melting temperatures (midpoint of unfolding, T_m) of the transitions as well as the free energy of unfolding ($\Delta H_{unfolding}$) were determined.

The thermal stability of rh-GCSF in selected formulations was also assessed by microcalorimetry using the identical protocol as for the mAb except that the rh-GCSF-concentration was 0.5 mg/ml.

2.5 BINDING BETWEEN CYCLODEXTRINS AND PROTEINS

2.5.1 SURFACE PLASMON RESONANCE SPECTROSCOPY

Measurements were performed on a Biacore X-instrument (GE Healthcare Europe GmbH, Freiburg, Germany). Immobilization of rh-GCSF was carried out on a CM-5 research chip following standard immobilization procedures described by Biacore Life Sciences. The surfaces of research grade CM5 chips were activated by a 6-min injection of a solution containing 0.2 M *N*-ethyl-*N*9-(dimethylaminopropyl) carbodiimide and 0.05 M *N*-hydroxy-succinimide. After immobilization of the protein and deactivation of the reference cell with ethanolamine approximately 2000 RU remained on the chip (difference between the steady state response before and after immobilization of the protein). Therefore, taking into account the CDs' molecular weights a theoretical maximal response of 229 RU for SBEβCD and 104 RU for α CD can be calculated.

As running buffer 20mM Acetate (pH = 4) was used. Cyclodextrins were dissolved in the exact same kind of buffer. In order to examine the effect on binding of pH in later measurements 20 mM Phosphate buffer (pH = 7) was applied to dissolve cyclodextrins. If not stated otherwise measurements were performed at a temperature of 25°C.

The flow rate was set to 30 µL/min and 60 µL of cyclodextrin solution were injected in various concentrations. This means that the contact between immobilized protein and cyclodextrin solution lasted for 2 minutes. The response was monitored as difference of the responses of the cell containing the immobilized protein and the reference cell in order to avoid measuring a simple bulk effect.

For the determination of the steady state - affinity between cyclodextrins and proteins cyclodextrin solutions of various concentrations showing a response in this experimental setup were injected consecutively. Using the software tool Biaevaluation® the average maximum response was calculated for every cyclodextrin solution injected. These maximum responses were then plotted against the concentration of each CD-solution. From the best fit the average steady state affinity could then be calculated. From ESI-MS experiment hints were available that binding occurs in a 1:1-stochiometry and therefore a simple 1:1 (Langmuir)-binding model was assumed for the calculation of the steady-state affinity.

For the mAb and rh-GH the immobilization procedure and materials as well as the monitoring of CD-binding were exactly the same, only the amount of protein attached to the chip varied, as indicated in the respective data chapter.

2.5.2 FLUORESCENCE SPECTROSCOPY

MAB

Concentrated stock solutions of CD-derivatives were titrated into 2 ml of a 0.24 mg/ml solution of the mAb in His 20 mM buffer and changes in intrinsic steady-state fluorescence

spectra were monitored. Each titration was carried out three times and after recording the spectra were corrected for dilution. The rest of the experimental conditions were identical to those described above for the assessment of changes in the apparent melting temperature by fluorescence spectroscopy.

RH-GCSF

Fluorescence titration was carried out at an excitation wavelength of 280 nm and at an emission wavelength of 337 nm on a Varian Cary Eclipse fluorescence spectrometer (Varian Inc., Darmstadt, Germany). Protein concentration was 1 µg/ml and the protein was buffered in 20 mM sodium phosphate buffer at pH = 4. SBEβCD was titrated to the solution to yield a final concentration of 8 mM.

2.5.3 SURFACE ACOUSTIC WAVE SENSOR

Surface acoustic wave sensors use piezoelectric materials to generate an acoustic wave. The amplitude and/or the velocity of the surface acoustic wave is strongly influenced by coupling to any medium contacting the surface. In contrast to SPR, SAW sensors are not sensitive to changes in the bulk refractive index thereby providing useful complementary information to the SPR results.

The experiments were carried out on the commercially available S-sens® K5 (Biosensor GmbH, Bonn, Germany) instrument. The central measurement unit consists of a read-out system into which the gold-coated quartz sensor is placed and the detected signals of the five measurement cells are recorded independently in real-time. Changes in phase and amplitude of the surface acoustic wave (in this a case a Love-wave) are triggered by changes in the bound mass and viscosity, respectively.

The gold-coated sensor chip was incubated overnight in a solution of mercaptoundecanoic acid thereby allowing for later coupling of proteins to carboxylic groups on the chip. After activation of the carboxylic groups with a mixture of EDC/NHS, rh-GCSF (dissolved in phosphate buffer) was immobilized to the surface of the chip. Unsaturated carboxylic functions were afterwards deactivated by Ethanolamine.

In order to be able to discriminate between phase shifts due to changes in bound mass and shifts due to changes in viscosity, 80 µL of an aqueous solution of glycerol (5 % m/m) were injected. The subsequent change in the binding signal can be solely attributed to a change in viscosity and using this information the Biosens K12 software can later correct the phase shift of the protein immobilization for changes in viscosity.

Increasing concentrations of cyclodextrins were injected onto the immobilized rh-GCSF and the binding signals were recorded. Using the Biosens K12 software and assuming a simple 1:1 binding model a kinetic analysis of the binding events was carried out. The association

constant k_a and the dissociation constant k_d were fitted to the binding curves and from the ratio of k_d and k_a the equilibrium binding constant was finally calculated.

2.5.4 ELECTROSPRAY-IONIZATION MASS SPECTROMETRY

All measurements were performed on a Bruker Daltonics Esquire 3000^{plus} 3D-ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) fitted with an orthogonal electrospray (ESI) ion source under the following conditions: capillary voltage, 4.0 kV (positive ions) and -4 kV (negative ions; in the case of the Captisol®-sample) and curtain gas temperature 300°C.

Pure protein samples (rh-IFNα-2a, rh-GCSF, rh-GH, Lysozyme) were initially analyzed by infusion of a 1 pmol/μl solution of methanol : water = 1 : 1 containing 0.1 % glacial acetic acid (Merck, Darmstadt, Germany) at a flow rate of 3 μl/min with a Cole-Parmer syringe pump (Core-Parmer, Vernon Hills, IL, USA).

Pure cyclodextrins and derivatives thereof were analyzed by infusion of a 1 mg/ml solution of methanol : water = 1 : 1 at a flow rate of 3 μl/min.

In order to detect complexes between the proteins and cyclodextrins and its derivatives a purely aqueous solution (without adding acetic acid) of a molar ratio of protein : carbohydrate = 1 : 10 was chosen according to S. Cao et al.[17]. Additionally, similar experiments were run after adding 10 mM ammonium acetate to these aqueous solutions (pH 4 for rh-GCSF and pH 5 for rh-GH). Furthermore by lowering in steps the molar amount of carbohydrate to a molar ratio of protein : carbohydrate to 1 : 1 and even to 10 : 1 the selectivity of the complex formation was tested. Control experiments were conducted using the linear carbohydrates maltoheptaose, maltopentaose, sucrose and trehalose in order to evaluate whether the existence of the CD-cavity is a necessary prerequisite for binding. Furthermore, in order to evaluate the influence of basicity control experiments with the amino acids L-tryptophan and L-tyrosine as well as their derivatives N-Acetyl-L-tryptophanamide and N-Acetyl-L-tyrosinamide were conducted. Further information regarding molar ratios of the solution-components as well as absolute concentrations can be taken from the respective figures in the data chapter.

2.6 INTERFACIAL INVESTIGATIONS

2.6.1 MAXIMUM BUBBLE PRESSURE MEASUREMENTS

The dynamic surface tension of solutions of the 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 bubble pressure of a bubble that is growing at the end of 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 the studies discussed in this thesis was the BPA-1P (Sinterface Technologies, Berlin, Germany).

2.6.2 RING TENSIOMETER

Surface tension measurements to evaluate the extent of mAb, CD and polysorbate adsorption to the air-water interface were initially carried out on a Krüss digital tensiometer K 100 (Krüss GmbH, Hamburg, Germany) using a Wilhelmy plate made of roughened platinum which was heated in a Bunsen burner flame prior to each measurement. 3 mL of the respective freshly prepared solution were placed in a circular, thermostatted and thoroughly-cleaned dish. Each measurement was performed for 240 s and the last 20 values determined within this period were averaged. These measurements were repeated at least five times and averaged to yield the reported surface tension value.

2.6.3 DROP PROFILE ANALYSIS AND DILATIONAL SHEAR RHEOLOGY

Drop profile analysis was employed for the detailed characterization of the dynamic surface tension and of rheological parameters of the surface layers of pure mAb and also of mixed mAb-HPβCD as well as of mAb-polysorbate 80 solutions. The instrument used for these investigations was a Profile Analysis Tensiometer (PAT 1, Sinterface Technologies, Berlin, Germany).

As indicated in Figure 2.1 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 obtaining the dynamic surface tension as the only free variable in the experiment[19]. 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.

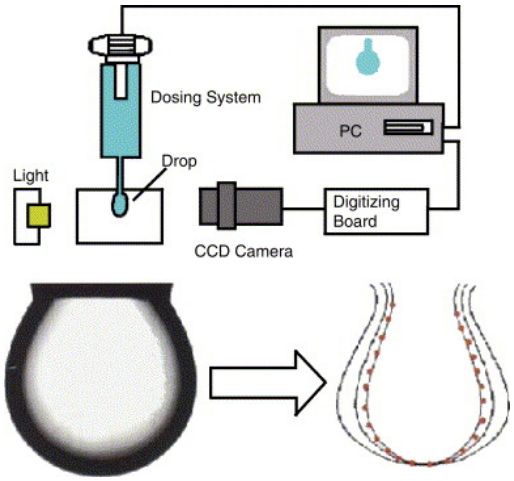

Figure 2.1: Schematic representation of main components of the drop profile tensiometer PAT 1 (Sinterface Technologies, Berlin, Germany) and drop profile analysis with video picture and profile coordinates. Taken from [18].

The very same experiment can also be used to determine dilational rheological properties of the surface layers. For this purpose harmonic area oscillations of the drop at low frequency are performed by the dosing system. 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[20].

For the investigations at the air-water interface all mixed solutions were prepared at a constant mAb-concentration of 10^{-6} mol/L. After equilibrium surface tension is reached the harmonic area oscillations are exerted on the drop. The frequencies chosen for the experiment were: 0.01, 0.2, 0.28, 0.4 Hz. Subsequently, Fourier analysis is performed and the dilational viscosity and dilational elasticity are obtained.

2.6.4 INTERFACIAL SHEAR RHEOLOGY

Interfacial shear rheology experiments were performed in order to determine the shear rheological properties of the surface layers of pure mAb as well as mAb-HPβCD and mAb-polysorbate 80 solutions. The instrument chosen for the investigations was a torsion pendulum rheometer (ISR-1, Sinterface Technologies, Berlin, Germany). The experimental set-up is described in detail elsewhere[21]. In short, the measuring body (a circular steel knife touching the surface of the solution to be characterized) attached to the pendulum is deflected by a certain (0.5-3°) short angle. After deflection the measuring body starts to oscillate and depending on the mechanical properties of the surface layer the oscillation is damped to a certain degree. From the shape of the damped oscillation curve finally the surface shear viscosity and the surface shear elasticity can be calculated.

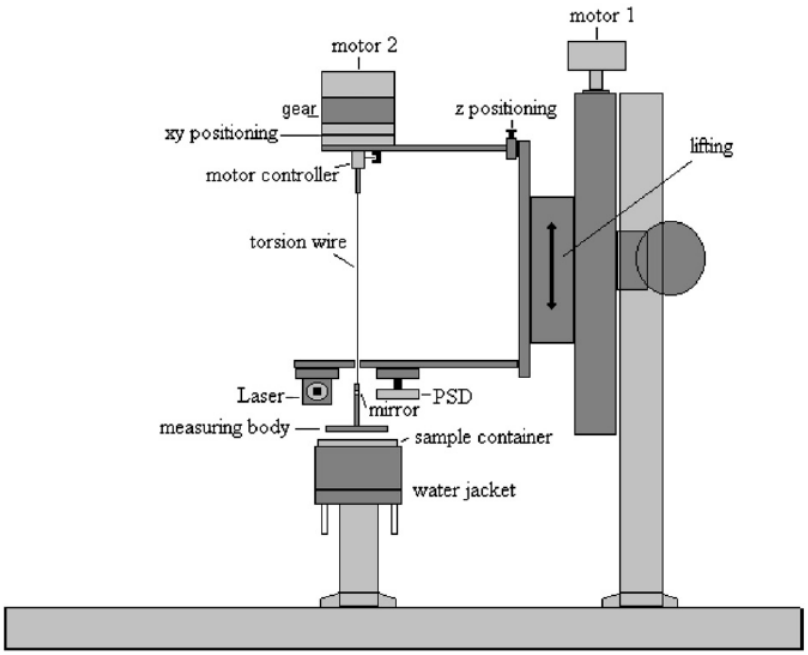

Figure 2.2: Schematic representation of a torsion pendulum Interfacial Shear Rheometer. Taken from [21].

The measuring solution which has been prepared prior to the experiment is placed in a PTFE dish of cylindrical shape. Immediately before beginning the experiment the surface of the measuring solution is cleaned by suction. As soon as mechanical equilibrium is reached the system automatically starts to record the experimental data.

For the experiments a deflection angle of 1° was chosen and the pendulum was first deflected again after 7 min and then every 15 min until the end of the experiment. The duration of the experiment varied depending on the solution measured and the time until adsorption equilibrium was achieved.

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CHAPTER 3

CYCLODEXTRINS AS NOVEL EXCIPIENTS FOR ANTIBODY-STABILIZATION

1 INTRODUCTION

Mabs are exposed to various surfaces during their manufacturing process, storage, shipping and administration to the patient[1]. As described in more detail in Chapter 1 the air-water-interface has to be considered the most frequently encountered interface. Also, since air is among the most hydrophobic surfaces it leads to strong adsorption of proteins.

It is also described in Chapter 1 that the adsorption of mAbs to the air-water-interface and subsequent unfolding are often followed by significant aggregation. There are a number of studies indicating that polysorbates are capable of counteracting surface-induced aggregation of mAbs[2-4]. This property of polysorbates can be considered as the main rationale for these excipients to be included in the majority of mAb-formulations that are available on the market[5-6].

However, there are also many studies available that point out the limitations and disadvantages of polysorbates and non-ionic surfactants in general, most notably occurring during quiescent storage for longer periods of time[7-10]. Increased levels of oxidized degradation products and aggregates have been observed[11] and in addition, the presence of polysorbates was related to the formation of protein-containing micelles that could possibly contribute to an increased immunogenicity of protein formulations[12-13]. Considering these disadvantages, there is a need for alternatives to non-ionic-surfactants in mAb-formulation. Taking into account results from literature (also discussed in detail in Chapter 1) that CD-derivatives may be capable to inhibit agitation-induced aggregation of proteins, the objective of the following studies becomes apparent: it was intended to evaluate the potential of CD-derivatives to serve as an alternative to non-ionic surfactants in mAb-formulation with a focus on aggregation at the air-water-interface. Also, the influences of cyclodextrins on mAb aggregation during other stress conditions, that are associated with the presence of interfaces, such as freeze-thaw stress or stirring with Teflon® bars, are discussed in this chapter.

In addition to the investigation of the potential to stabilize against interface-induced aggregation possible instabilities of CD-containing mAb-formulations during quiescent storage at elevated temperatures for up to six months were also investigated since the use of non-ionic surfactants under these conditions is problematic. CDs should show improvements in this respect in order to fulfill the gap as a formulation alternative to non-ionic surfactants that is strongly needed.

Since CDs are reported to preferentially bind to the unfolded state of proteins thereby suppressing aggregation reactions, a decrease of the proteins' thermal stability can be expected [14] Therefore, from a mechanistic point of view (hints for binding) and also to correlate these results with findings from the long-term quiescent storage study at elevated temperature, a thorough analysis of the CDs' impact on mAb thermal stability is of importance and will be discussed in this chapter. The influence of CDs on the apparent melting temperatures of the mAb in presence of CDs was determined.

Finally, since formulation viscosity is a critical issue for the development of highly concentrated mAb-formulations[15] the influence of CD-addition on solution viscosity was investigated as well.

2 EFFECTS OF CYCLODEXTRIN-ADDITION ON SURFACE-INDUCED AGGREGATION

The results of studies examining the potential of cyclodextrins to inhibit surface-induced aggregation of the model IgG are presented in the following section. The focus of these studies was on aggregation that is induced by the presence of an extensive air-water-interface created by agitation but also other surfaces are investigated. All samples of the agitation-studies were agitated in centrifugal tubes that were placed vertically on a shaking-device. The studies carried out at high protein concentration were conducted in vials that were placed horizontally on a shaking device at 200 rpm. Further interfaces that the CD-mAb-formulations were exposed to include the ice-water-interface (during F/T-studies), the glass-water-interface (agitation in the presence of glass beads) and the Teflon®-water-interface (during stirring studies in the presence of Teflon®-stirrer-bars).

In addition to the comparisons to polysorbate 80 (which was selected as a very common representative of the class of non-ionic surfactants), CDs were also compared to other excipients that are commonly employed in protein formulation: the sugars and sugar alcohols sucrose, trehalose, mannitol and sorbitol. Comparisons to the linear sugar maltoheptaose were carried out in order to evaluate the relevance of the cavity of the CD-molecules in comparison to an equivalent linear sugar.

2.1 CYCLODEXTRINS INHIBIT AGITATION-INDUCED AGGREGATION

A variety of methods can be used to investigate the effect of protein exposure to the air-water interface: air bubbling methods[16], vigorous vortexing for seconds or minutes[17], agitating vials either horizontally or vertically with and without headspace or performing the agitation experiment on shaking devices like a temperature-controlled Thermomixer which was done in this case. The approach used in the current study was chosen because it led to significant loss of monomer of more than 50 % within 3-5 days in preliminary experiments (data not shown).

In Figure 3.1 the results of an agitation study comprising mAb-formulations with four different CD-derivatives (HPβCD, MβCD, SBEβCD and HPγCD; each in three different concentrations) in comparison to a formulation without any CDs are shown. Loss of monomer was almost exclusively due to the formation of insoluble aggregates that were removed by centrifugation prior to analysis of the supernatants for SE-HPLC. It can be seen that after subjecting the samples to agitation for 72 h the formulation without CDs contains less than 10 % of the original amount of soluble monomer. In contrast all the formulations containing MβCD and the formulations containing HPβCD in a concentration higher than or equal to 2.5 mM were found to completely suppress agitation-induced aggregation. However, formulations containing HPγCD were less effectively protected and formulations containing

the ionic CD-derivative SBEβCD exhibited no significant stabilization compared to the formulation without CD.

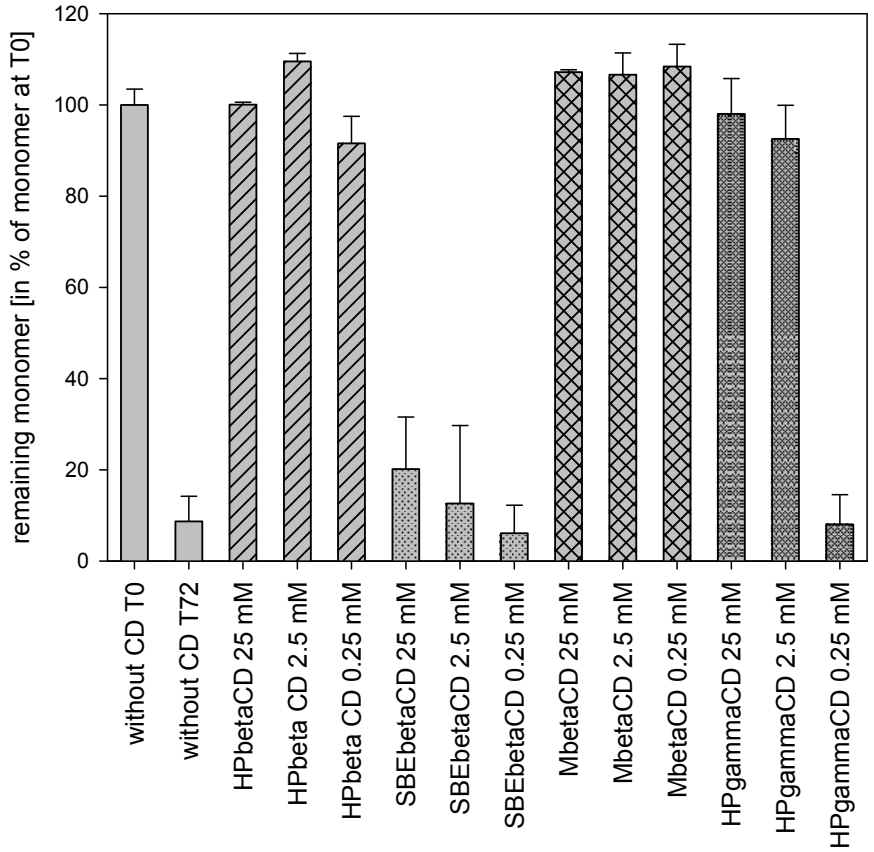

Figure 3.1: IgG-aggregation after 72 h of agitation at 800 rpm. Remaining monomer as followed by size exclusion chromatography. Each value represents the mean of three samples ± standard deviation.

Some samples in Figure 3.1 appear to have a monomer content that exceeds the theoretical maximum of 100 % which would be higher than in the sample before incubation. This phenomenon can be explained by the presence of about 8 % soluble aggregates in the bulk starting material. These aggregates partially or even completely dissolve into monomer throughout the course of the experiment.

Reference samples with no headspace left in the centrifugal tube were subjected to the agitation experiment as well. These samples did not show any aggregation supporting the conclusion that aggregation in the presence of headspace is due to the presence of the extensive air-water-interface. Furthermore samples with regular headspace that were not agitated for the duration of the experiment also did not show any aggregation.

To date this is the first published demonstration that HPβCD prevents aggregation of an IgG during agitation. Compared to studies that were conducted earlier and that investigated the potential of CDs for the stabilization of other proteins the concentrations found in the current study to be sufficient for complete aggregation-suppression were surprisingly low (≤2.5 mM which equals about 0.35 % (w/w). Furthermore considering the relatively high concentrations

of HPβCD employed in commercially available parenteral formulations of LMW-drugs, mAb formulations with 2.5 mM HPβCD should be safe from a toxicological point of view. For comparison, the FDA-approved parenteral formulation of itraconazole contains 40 % (w/v) of HPßCD which is more than a hundred-fold the amount necessary for complete aggregation inhibition in our experiments[18-19].

2.2 EVALUATION OF NOVEL SULFOBUTYETHER-ß- AND Y-CYCLODEXTRINS

In section 2.1 it was described that employing SBEβCD in a mAb-formulation does not lead to a stabilization of the mAb against aggregation induced by the air-water-interface. At this point it is unclear why some of the CD-derivatives stabilize the mAb and SBEßCD does not. Since SBEβCD was the only ionic CD-derivative tested, the detrimental effects on protein stability could be related to its charge. If competition at the air-water-interface plays a role in inhibiting agitation-induced aggregation the comparably low surface activity (Chapter 6) of SBEβCD could also play a role. In order to further test these hypotheses, studies were conducted comprising further SBE-CD-derivatives: ß-CD with two different degrees of substitution and γ-CD with two different degrees of substitution (Table 3.1). Since surface activity of SBECD-derivatives changes with the degree of substitution a possible influence of surface activity would be observable (Table 3.1, surface activity data provided by CyDex™). In addition all four derivatives are ionic so that if this property were the dominating reason for SBEßCD to not stabilize the mAb all four derivatives should influence mAb-aggregation at the air-water-interface in a comparable (negative) fashion.

Table 3.1: Derivatives employed in the investigation of SBE-CD-effects on agitation-induced mAb-aggregation. Data provided by CyDex Inc. (Lenexa, KS, USA).

CD-derivative	Degree of substitution	Molecular weight [g/mol]	Surface Tension [mN/m]
Captisol	6.7	2194.6	71.0
SBEßCD (4.1)	4.1	1783.6	72.0
SBEγCD (4.3)	4.3	1977.2	64.5
SBEγCD (5.2)	5.2	2119.5	68.5

Figure 3.2 shows the amount of mAb-monomer and soluble aggregates after an agitation period of 24 h at 1150 rpm on the Thermomixer™. The amount of monomer in the samples without any further excipients besides buffer decreases to less than 70 % whereas the amount of soluble aggregates only slightly increases from about 9 % to about 15 % which means that large amounts of insoluble aggregates were created (the amount of fragments remains constant throughout the experiment, data not shown). This finding is in agreement

with the visually detectable strong turbidity of the samples. The HPßCD-samples that were included into this study as a benchmark formulation exhibited a complete preservation of monomer after the 24h-agitation-period which confirms the results of the study discussed above.

However, none of the SBE-CDs under investigation led to a clear stabilization of the mAb. Even more, the two formulations containing ß-CD-derivatives exhibited accelerated aggregation as compared to the reference formulation which is indicated by a loss of monomer to about 40-50 % of the original monomer content. The two γ-CD-derivatives performed slightly better with SBEγCD (4.3) yielding slightly more remaining monomer than the reference without excipients. This behavior could be due to their higher surface-activity compared to the SBEβCD-derivatives as summarized in Table 3.1.

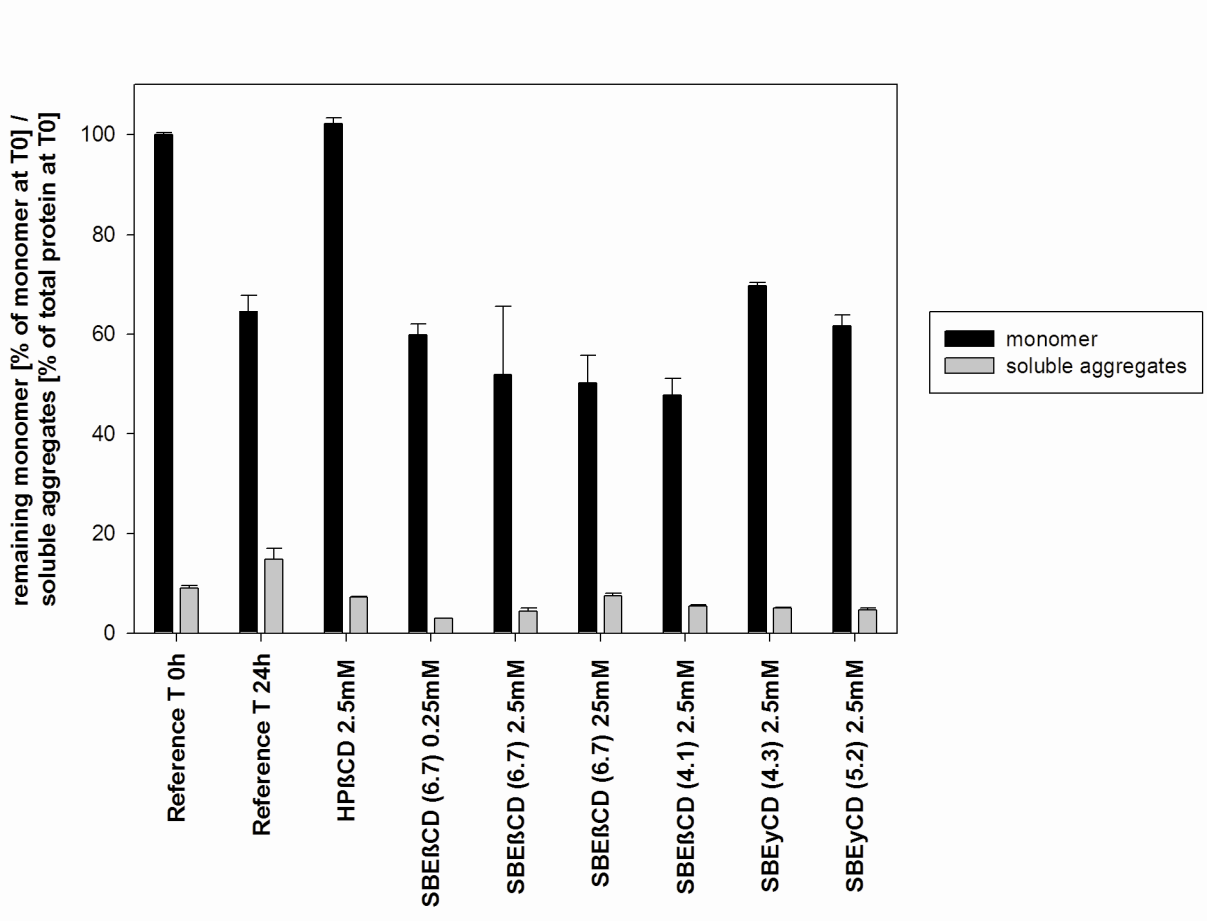

Figure 3.2: Aggregation after 24h of agitation in centrifugal tubes at 1150rpm – comparison of different SBE-CDs to formulations without any excipients besides buffer (reference) and a formulation containing HPßCD. Each value represents the mean of three samples ± standard deviation.

Taking together these results with the results from Fig.1 it appears that Sulfobutylether-CD-derivatives are generally inappropriate for mAb formulation – independent of their ring size or degree of substitution (and therefore also independent of their surface activity). Since other non-charged β- and γ-CD-derivatives showed good or even complete preservation of mAb-stability under the same conditions it can assumed that charge plays a key role for an

explanation of the different behavior of these derivatives. An impact on colloidal stability protein stability due to the shielding of repulsive forces between the IgG-molecules would be a possible explanation[20]. The influence of SBE-CDs on conformational mAb-stability will be discussed later (section 4.4).

2.3 COMPARISON TO POLYSORBATE

As noted in the introduction there is a need for alternatives to the traditional polyoxyethylene-based surfactants like polysorbate 80 which are often added to protein solutions in order to prevent aggregation due to interfacial stresses. In order to compare the behavior of HPβCD to polysorbate 80 an additional agitation experiment was conducted. It compared a formulation containing polysorbate 80 at a rather low concentration (0.004 % = 0.03 mM, molar ratio polysorbate 80 : mAb = 2.5 : 1) close to the CMC of polysorbate 80 (0.012 mM in water[21]) to a formulation containing polysorbate 80 in a high concentration far above the CMC (0.04 % = 0.3 mM, molar ratio 25 : 1) and to a formulation containing 2.5 mM HPβCD.

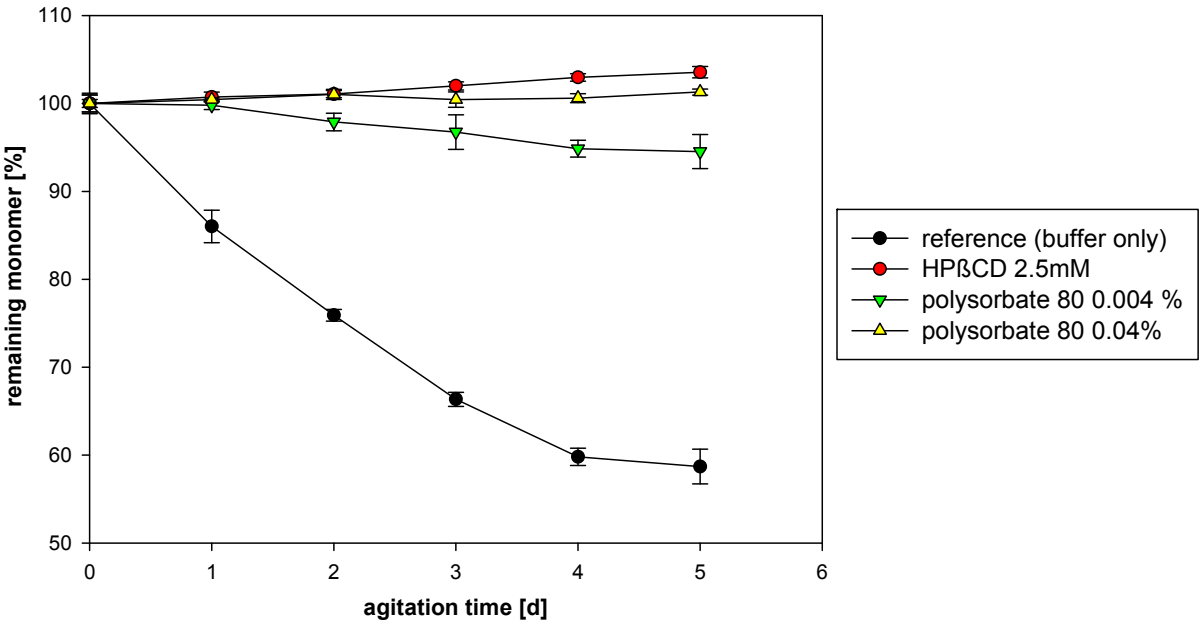

Figure 3.3: Aggregation after agitation for five days in centrifugal tubes at 800 rpm. Comparison between mAb-formulations containing 2.5 mM HPßCD to formulations containing polysorbate 80 at 0.004 % and 0.04 % and a mAb-reference formulation with no excipients besides histidin 20 mM buffer at a pH of 5.8. Each value represents the mean of three samples ± standard deviation.

Polysorbate 80 at 0.3 mM provided the same degree of protection from agitation-induced aggregation as HPβCD did at 2.5 mM (Figure 3.3). However, at the lower polysorbate 80 concentration the formation of soluble aggregates was observed (Figure 3.4), and the HPßCD-formulation had a superior resistance to agitation-induced aggregation.

Since the effects of HPβCD on agitation-induced aggregation described here, qualitatively resemble those of polysorbate 80 one can assume that CDs might prevent protein aggregation at the air-water interface in a similar fashion as nonionic surfactants. As outlined

in Chapter 1 different mechanisms of stabilization of proteins by non-ionic surfactants which appear to depend on the protein being studied must be considered.

The most obvious mechanism is a competition for adsorption at the air-water-interface, which likely occurs in all cases even if additional routes of polysorbate-induced protein stabilization are operative[22]. If this behavior also occurs in the presence of cyclodextrins is discussed in Chapter 6 on the basis of dynamic surface tension studies in combination with dilational and shear rheology.

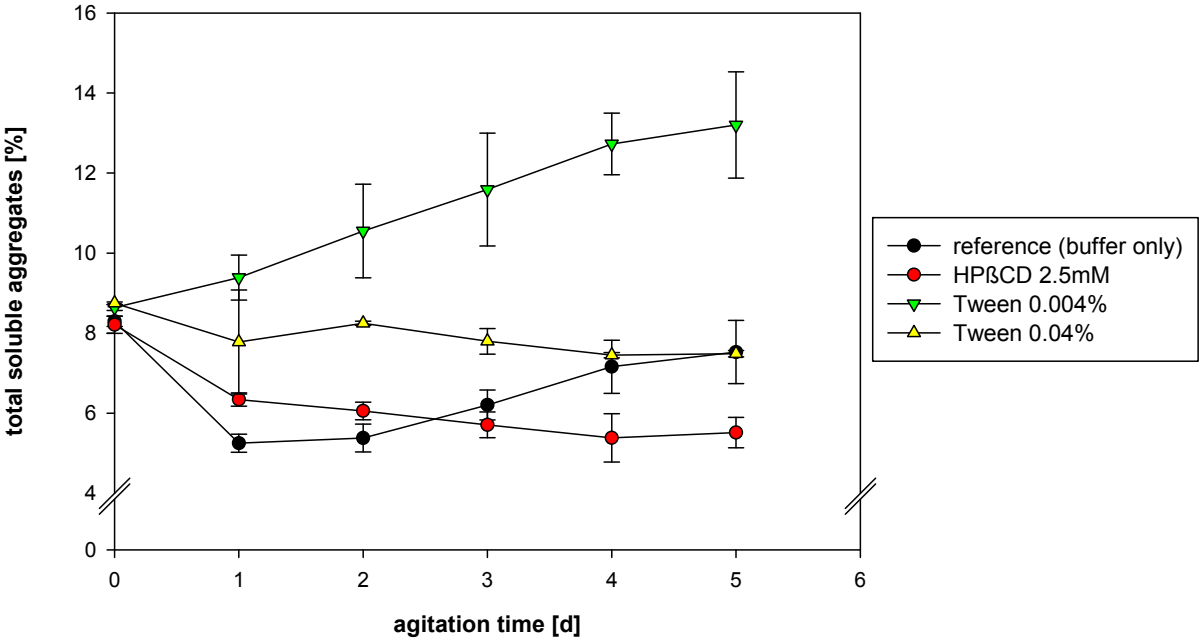

Figure 3.4: Formation of soluble aggregates after agitation for five days in centrifugal tubes. Comparison between mAb-formulations containing 2.5 mM HPßCD to formulations containing polysorbate 80 at a concentration of 0.004 % and 0.04 % and a mAb-reference formulation with no excipients besides buffer. Each value represents the mean of three samples ± standard deviation.

However, in other cases direct binding between the native protein and the non-ionic surfactant in the bulk solution were observed and also clearly related to the mechanism of aggregation inhibition[23-24]. Whether binding in solution between HPßCD and the mAb plays a role in the observed stabilization will be discussed in Chapter 5. Surface Plasmon resonance studies and fluorescence titrations were carried out to analyze binding in solution.

To gain insight into the potential route of aggregation during agitation, and hence mechanism for inhibition, IgG secondary structural changes in the formed precipitates were studied. In contrast to other spectroscopic methods IR spectroscopy is well suited to measure samples in almost any physical state[25].

Therefore it was decided to compare the secondary structure of the protein in precipitates with the native protein in solution by employing IR spectroscopy. Because no significant shifts within the characteristic IR bands are apparent when comparing the spectra (Figure 3.5) it has to be assumed that protein molecules in the precipitates have a very native-like

secondary structure and that they presumably did not expose large hydrophobic regions by unfolding before aggregating. This observation suggests that the prevention of mAb-aggregation at the air-water-interface by CDs does not involve the inhibition of structural alterations on a secondary structural level but it must be due to different mechanisms such as competition at the air-water-interface or prevention of partial unfolding on a tertiary structural level.

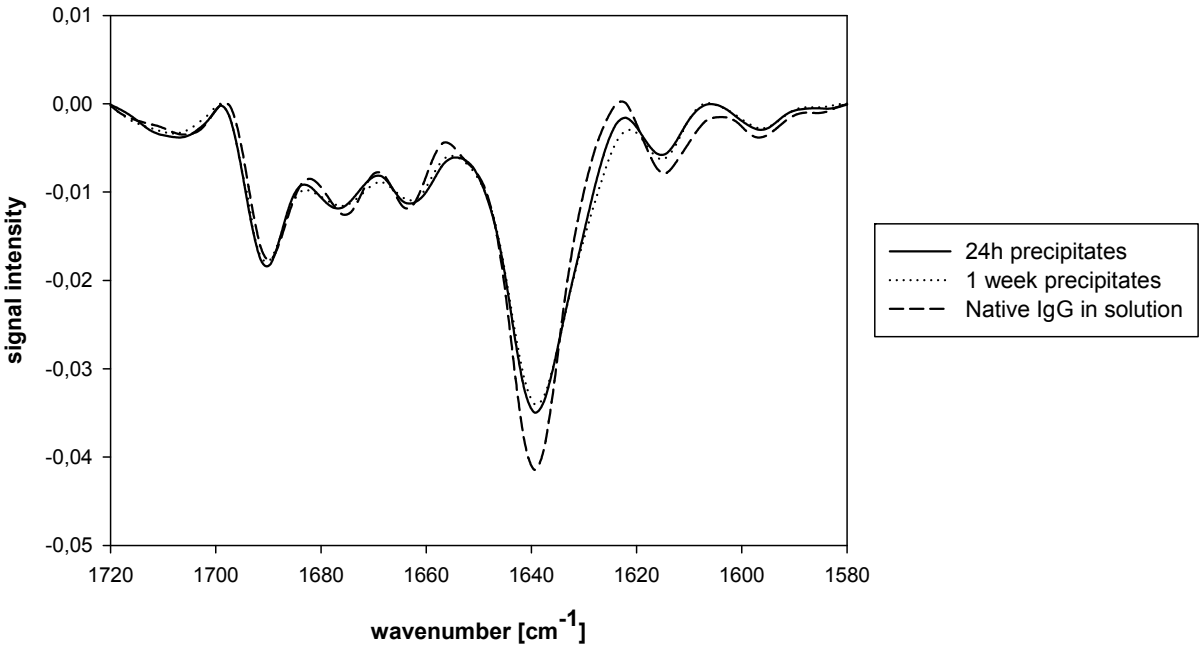

Figure 3.5: 2nd-derivative IR-spectra of IgG in precipitates formed after 24 h and 1 week of agitation in comparison to the spectrum for the native protein in solution

Also these IR spectroscopic results are important from a patient safety viewpoint. This is because large aggregates containing an array of non-denatured protein molecules are thought potentially to be the most immunogenic types of aggregates in therapeutic protein products[26]. Therefore, it is particularly important to inhibit formation of such agitation-induced aggregates in mAb products.

2.4 COMPARISON OF CYCLODEXTRIN-DERIVATIVES TO FURTHER EXCIPIENTS FOR PROTEIN FORMULATION

In addition, the stabilization of the IgG by HPβCD and MβCD was compared to that afforded by sugars and sugar alcohols (sucrose, trehalose, mannitol and sorbitol) at concentrations typically used in protein formulations. These excipients are frequently employed in protein formulation and mainly increase protein stability via the preferential exclusion mechanism[20] as explained in detail in the general introduction (Chapter 1). The results of the agitation study comparing the sugar and sugar alcohol excipients to the two CDs are summarized in Figure 3.6.

As in the previous agitation study, the two formulations containing HPβCD and MβCD completely inhibited aggregation with remaining monomer values even exceeding the theoretical maximum of 100 % which is due to the partial dissolution of soluble aggregates present in the starting material. On the other hand the reference formulation without any CD or sugar excipients had a monomer content of about 40 % after 80h of agitation. Surprisingly, all the sugar- and sugar alcohol-containing formulations exhibited accelerated aggregation during agitation. After 80h of agitation the IgG monomer content decreased to less than 10 % in these samples.

As a consequence it is concluded that CDs – although chemically classified as sugars – act completely differently from these low molecular weight sugars. In addition it can be stated that in contrast to the general opinion in protein formulation[6] sugars may promote IgG aggregation during agitation. A detailed theoretical explanation for this behavior is given in Chapter 6 along with surface-tension data of these formulations. In brief, it can be expected that due to preferential exclusion of the low molecular weight sugars from the protein surface and due to an increase of surface tension of sugar-containing solutions, the mAb will become less stable upon adsorption at the air-water-interface in the presence of sugars[27].

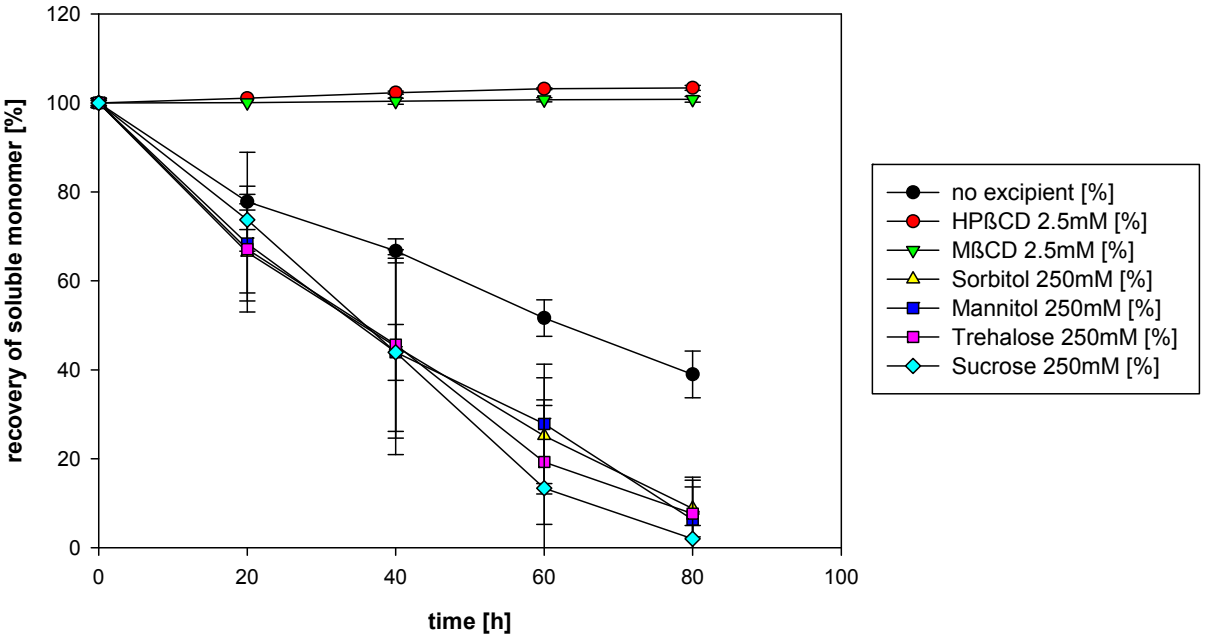

Figure 3.6: Comparison of the extent of IgG-aggregation after agitation at 1200 rpm on a Thermomixer® between CD-formulations and low molecular weight sugar-formulations. Each point represents the mean of three samples ± standard deviation.

In addition to the comparison to LMW-sugars an experiment comparing the influence of HPßCD and MßCD on agitation-induced aggregation to the oligosaccharide maltoheptaose was carried out (Figure 3.7). Maltoheptaose is a linear sugar consisting of seven units of 1a, 4e-linked oligomers of α-D-glucopyranoside, i.e. the same number and linkage of glucose-molecules as in the cyclic β-CD-molecule, except that it does not possess a hydrophobic

cavity like cyclodextrins do[28]. It can be seen that the presence of 2.5 mM maltoheptaose does not offer the same degree of protection from aggregation as HPßCD and MßCD do. This behavior could be taken as a hint, that the existence of the CD-cavity is a necessary prerequisite for aggregation inhibition of the mAb and that the incorporation of exposed hydrophobic residues on the protein into the CD-cavity is important for aggregation inhibition. However, it also has to be taken into account that maltoheptaose is not the exact linear match of the two CD-derivatives tested here since it does not contain hydroxypropyl- or methyl-residues on its surface, which for instance leads to a different hydrogen bonding pattern. Interestingly, also Maltoheptaose slows down mAb-aggregation when compared to the reference without any further excipients besides buffer. This behavior was quite unexpected and its origin remains speculative. One possible explanation for this behavior could be the fact that also linear oligosaccharides are also amphiphilic molecules (like CDs are). As such they are for instance reported to substantially increase the critical micelle concentration (cmc) of surfactants in solution by direct interaction between the dextrin molecules and surfactants[28]. Therefore it is conceivable that stabilization of the IgG by maltoheptaose could occur through mechanisms that can also be assumed for non-ionic surfactants or cyclodextrins (competition at the air-water-interface, direct interaction of hydrophobic parts of the protein with hydrophobic parts on the maltoheptaose-molecule). It has to be concluded that maltoheptaose only to a limited degree serves as a suitable negative control for the existence of the CD-cavity since it possesses physico-chemical properties that strongly resemble those of the amphiphilic CDs.

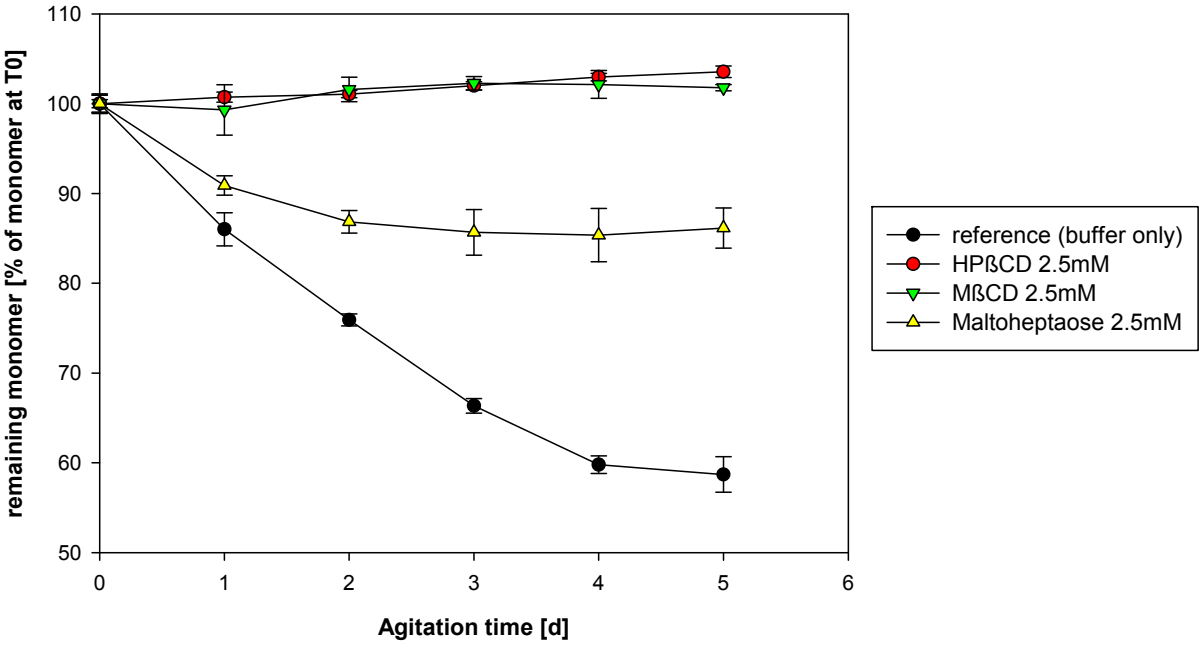

Figure 3.7: Comparison of the extent of IgG-aggregation after agitation between CD-formulations and a maltoheptaose-formulation. Each point represents the mean of three samples ± standard deviation.

2.5 AGITATION AT HIGH PROTEIN CONCENTRATION

The experiments discussed so far were all carried out at a mAb concentration of 1.8 mg/mL which represents a typical concentration for an intravenous mAb-formulation[6]. Recently however, mAb-formulations at higher concentrations have gained importance. The need for stable highly concentrated mAb-formulations is mainly due to the desire to administer mAbs via the subcutaneous-route (sc.) in order to improve patient convenience and to render home application of the drugs possible. With mAb-doses being in the mg/kg-range and a maximum applicable volume of sc.-injections of 1.5 mL it becomes apparent that such formulations need to be highly concentrated, typically in a range of 50 mg/mL up to 150 mg/mL or even more[15,29].

In addition to the usual challenges encountered in protein formulation that are discussed in Chapter 1 the formulation of highly concentrated mAb-formulations accounts for a number of unique challenges like increased, concentration-dependent aggregation rates during storage or reversible self-association[15,30-31]. In addition high solution viscosity (that often results from reversible self-association) and subsequent problems regarding “syringability” can be limiting factors as well[30]. Furthermore opalescence of highly concentrated protein formulations is also often reported which represents a challenge to the development and analytics of highly concentrated mAbs as well since it may be confused with turbid formulations containing high amounts of potentially dangerous particles[32].

However, little has been published so far about agitation-induced aggregation in highly concentrated protein formulations and the control of possible consequences. From an earlier publication on PEG-GCSF it can be expected that increasing the protein-concentration leads to a decreased rate of aggregation induced by the air-water interface since the ratio of interfacial-area to amount of protein was found to be critical for the susceptibility to agitation-induced aggregation[9]. Another study investigated the influence of ions on agitated, highly-concentrated mAb-formulations and also compared mAb-aggregation at 70 mg/mL to aggregation after quiescent storage at 45°C[33]. It was found that agitation resulted in turbid formulations and in contrast to the quiescently stored formulations no increase in the amount of soluble aggregates compared to the control was observed. Hence different kinds of aggregates are formed during agitation and quiescent storage of the highly concentrated mAb-formulations. In contrast a very recent study reports that increasing the mAb-concentration leads to an increased percentage of soluble aggregates as determined by HP-SEC[34]. However, the total loss of protein in terms of recovery (accounting for soluble as well as insoluble aggregates) was not published.

Little studies at all were carried out investigating the influence of excipients on agitation-induced aggregation of highly concentrated mAb-formulations (above 50 mg/mL). Mahler et al. found out that the addition of polysorbate 20 to highly concentrated mAb-formulations

(investigated range from 10 mg/mL to 150 mg/mL) had no effect on soluble aggregates and turbidity, but very low concentrations of polysorbate 20 (0.005 %) were sufficient to inhibit the appearance of visual particles[34].

When analyzing the excipients employed for the stabilization of high concentration protein-formulations it becomes apparent that they very much resemble those used for the formulation of lower concentrations of mAbs[6]. The problems that can be expected are that sugars such as sucrose and trehalose add to the inherently high viscosity of the formulations. Also they do not necessarily protect against aggregation since also protein aggregates may be stabilized by sugars under the solution conditions in highly concentrated mAb-formulations[15]. Non-ionic surfactants must be expected to exhibit the same problems as in low-concentration mAb-formulations: oxidation due to in-situ-forming peroxides and increased levels of aggregates during quiescent storage[7-8].

Since agitation of highly-concentrated protein formulations and its control by excipients has so far not been thoroughly studied and since HPßCD turned out to be a valuable excipient for the stabilization of the mAb at lower concentration, studies were carried out examining the effects of HPßCD on agitation-induced aggregation of the mAb at a concentration of 50 mg/mL. Table 3.2 lists the formulations under investigation. In contrast to earlier studies agitation was carried out in 2R-vials (placed horizontally on a shaking device at 200 rpm) since agitation in centrifugal tubes did not lead to aggregation (data not shown).

Table 3.2: Formulations examined at a mAb-concentration of 50 mg/mL.

Formulation	***Cyclodextrin [mM / %]***	***Polysorbate 80 [mM / %]***	**Sucrose [mM/%]**
Reference	0	0	0
HPßCD 0.25 mM	0.25 / 0.035	0	0
HPßCD 2.5 mM	2.5 / 0.35	0	0
HPßCD 25 mM	25 / 3.5	0	0
Polysorbate 0.004 %	0	0.03 / 0.004	0
Polysorbate 0.04 %	0	0.3 / 0.04	0
Sucrose 250 mM	0	0	250 / 8.55
Sucrose 250 mM + PS low	0	0.03 / 0.004	250 / 8.55
Sucrose 250 mM + PS high	0	0.3 / 0.04	250 / 8.55
Sucrose 250 mM + HPßCD	2.5 / 0.35	0	250 / 8.55

Figure 3.8 A shows results of the agitation study. It is obvious that all the solutions showed strongly increased turbidity levels after agitation for 240h (10 days). When kept quiescently after agitation a layer of white amorphous precipitate forms at the bottom of the vials and the

supernatant becomes clear again. Therefore clearly, the formation of insoluble aggregates took place. The addition of HPßCD as well as of polysorbate 80 both reduces the degree of insoluble aggregate formation. For HPßCD a minimum of 2.5 mM seems to be necessary to achieve the maximally possible stabilization (no further decrease of turbidity at higher HPβCD-concentration) which is in good agreement with the experiment performed at lower mAb-concentrations described in Chapter 2.1. Therefore it is not the HPßCD-mAb-ratio which is critical for stabilization against aggregation at the air-water-interface but the absolute HPßCD-concentration in solution and consequently the resulting absolute concentration at the interface. However, it has to be noted that in contrast to the experiments at a mAb-concentration of 1.8 mg/mL a complete stabilization against aggregation at the air-water-interface was not achievable keeping in mind the lower concentrated mAb-formulations remained perfectly clear upon agitation in the presence of HPßCD and polysorbate 80. Interestingly, the sucrose formulation shows the highest degree of turbidity after agitation which can be taken as a hint that aggregation was even increased in the presence of sucrose. Also, the amount of soluble aggregates is the highest in the sucrose formulation as shown by Figure 3.9 B. Accelerated mAb-aggregation after agitation in the presence of excipients that are preferentially excluded from the protein surface was also observed in section 2.4 of this Chapter for the lower concentrated mAb-formulations. Therefore regarding the effect of LMW-sugars on mAb-aggregation induced by agitation no qualitative difference is observed between the high and low mAb-concentrations. However, when adding polysorbate 80 or HPßCD to the sucrose-containing formulation the detrimental effect of sucrose on mAb-aggregation during agitation can be counteracted and the level of turbidity of the combined formulations is comparable to that of the HPßCD- and polysorbate 80-formulations in the absence of sucrose. Therefore it will be of interest to evaluate the results from the incubation studies at 50°C in which a positive effect of sucrose as preferentially excluded excipient on temperature-induced-aggregation can be expected (as it was already observed for the lower concentrations in section 4.3).

The relative degree of formation of insoluble aggregates in formulations at 50 mg/mL is much smaller than at the lower concentrations of 1.8 mg/mL. This becomes evident when analyzing the mAb-recovery rates after 240h of agitation as shown in Figure 3.8 B. Recovery never falls below 90 % of the original total protein content whereas in the first agitation experiment discussed in this Chapter (Figure 3.1) recovery rates of less than 10 % were observed after agitation. Figure 3.8 B also reveals that the formulation showing the highest turbidity (Sucrose 250 mM) also contains the largest amount of soluble aggregates. Therefore it can be speculated that the formation of soluble aggregates is a precursor for the formation larger insoluble aggregates and also in terms of soluble aggregates sucrose turns out to promote protein aggregation upon agitation.

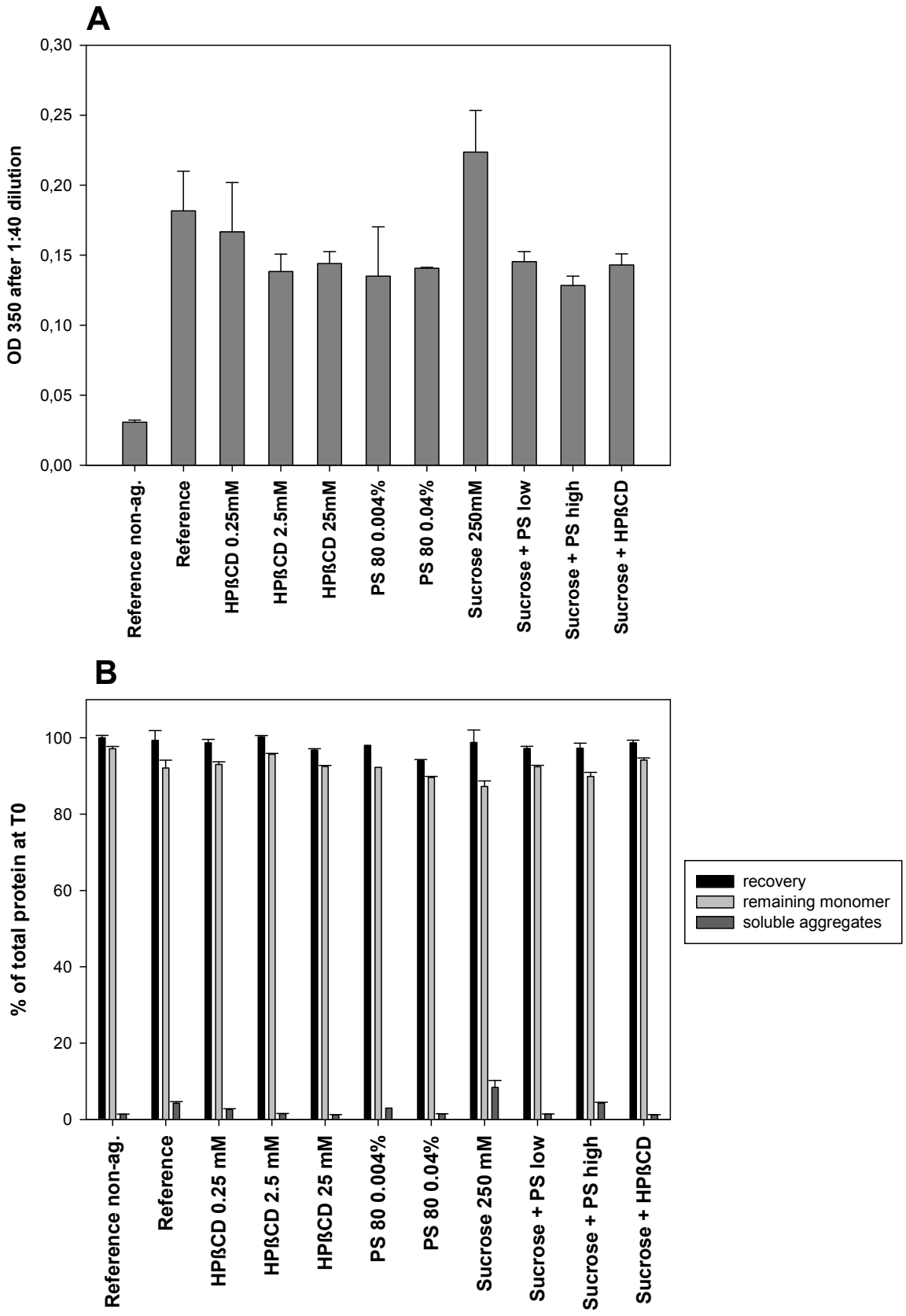

Figure 3.8: UV-Absorbance at 350 nm in different 50 mg/mL mAb – formulations after 240 h of agitation (A) and aggregation in the same formulations after 240h of agitation as determined by HP-SEC (B). Each bar represents the mean of three samples ± standard deviation.

The aggregation results are in agreement with earlier studies on PEG-GCSF[9] in which the relative extent of mAb-aggregation (soluble and insoluble aggregates taken together, readily identifiable as amount of remaining monomer in Figure 3.8 B and Figure 3.9 A) drastically decreases when agitating at 50 mg/mL. This becomes even more apparent when taking into consideration that under the conditions chosen for the agitation studies at 1.8 mg/mL (in centrifugal tubes, vertically) no aggregation at all could be observed for the high mAb-concentration. Not until the change to harsher conditions (horizontal agitation in vials with a large headspace) aggregation could be observed at all. Therefore the hypothesis that the inverse relation of aggregation tendency and concentration can be attributed to the ratio of surface-area to amount of protein in solution[9] seems to be confirmed by our studies.

Concerning the effect of HPßCD on mAb-aggregation as determined by HP-SEC, Figure 3.9 shows that no soluble aggregates are formed in formulations containing HPßCD at a concentration of 2.5 mM – no matter if sucrose is additionally present or not whereas in the reference formulation without excipients almost 4 % soluble aggregates are formed. Since formulations with HPßCD 2.5 mM in absence and presence of sucrose also showed the highest amount of remaining monomer in solution after agitation it can be indirectly concluded that for these two formulations also the amount of insoluble aggregates was the smallest. The formulation containing 25 mM HPßCD behaves very comparably, only the amount of remaining monomer is slightly lower. At 0.25 mM a higher amount of soluble aggregates can be observed throughout the agitation period but the amount of soluble aggregates is still lower than in the formulation without any further excipients besides buffer. Thus it can be reasoned that also at high mAb-concentration HPßCD provides stabilization against agitation-induced aggregation and the maximally possible degree of stabilization is achieved beginning at a concentration of 2.5 mM HPßCD. These findings are in agreement with the turbidity data discussed earlier. Again it can be concluded that rather the absolute concentration of HPßCD than the molar ratio between HPßCD and the mAb is crucial for mAb-stabilization since also at lower mAb-concentration HPßCD 2.5 mM afforded complete aggregation inhibition.

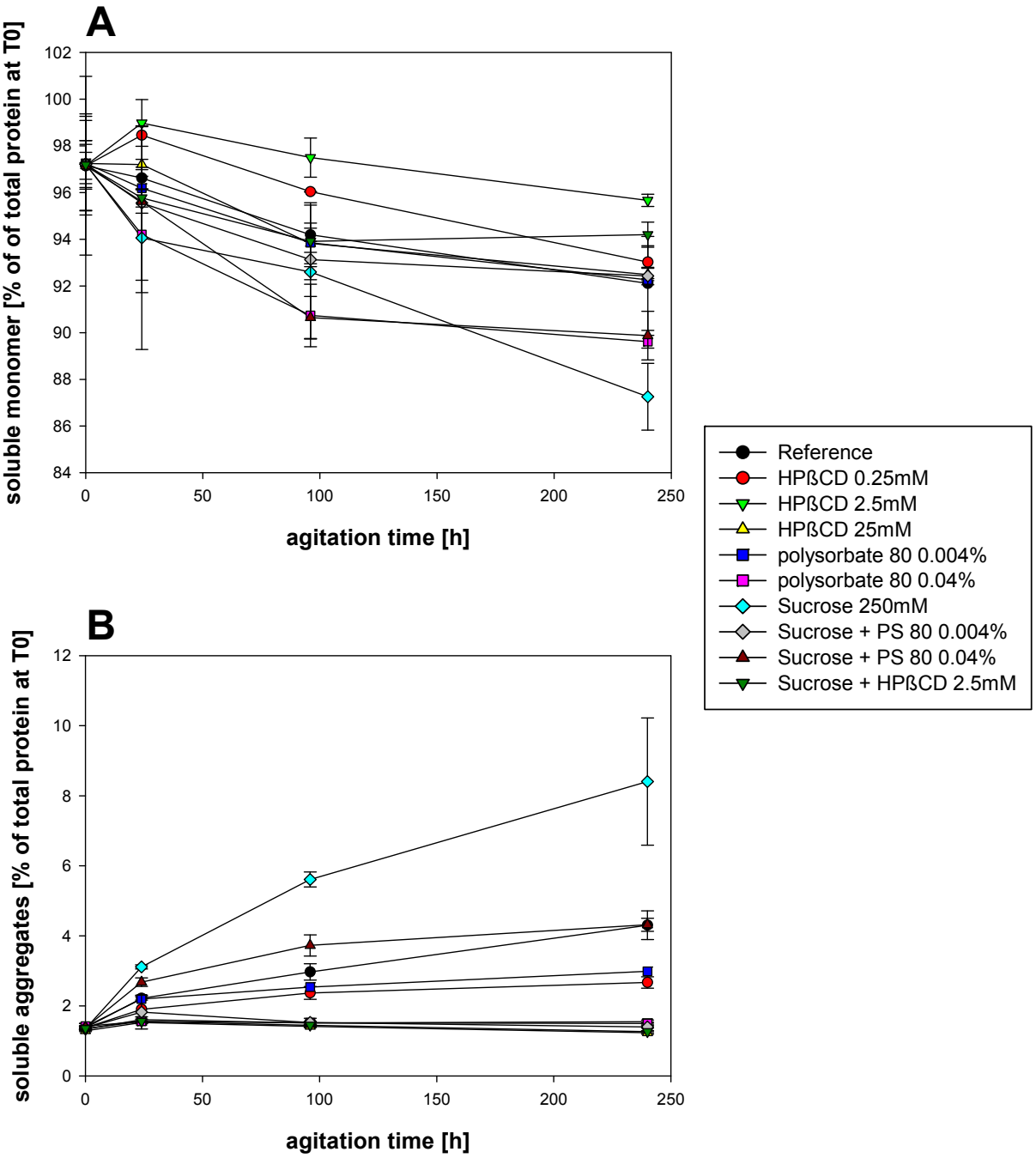

Figure 3.9: Loss of mAb-monomer in different formulations after 240h of agitation in glass vials (A) and resulting soluble aggregates (B). Each point represents the mean of three samples ± standard deviation.

Polysorbate 80 also offers some degree of protection against agitation-induced aggregation as compared to the reference formulation (Figure 3.8 and Figure 3.9). However, in the formulation containing the lower concentration of polysorbate 80 (0.004 %) more soluble aggregates than in the HPßCD-formulation are observed and in the higher concentration (0.04 %) a lower amount of remaining monomer (89.6 % in absence of sucrose and 89.9 % in presence of sucrose vs. 92.5 % for the HPßCD-formulation in absence and presence of sucrose) results. Therefore it can be stated that HPßCD offers a formulation alternative to polysorbate 80 for the inhibition of aggregation at the air-water-interface of highly

concentrated mAb-formulations since it affords at least the same or an even higher degree of aggregation protection as polysorbate 80 does in our studies.

Again it was intended to gain insight into a possible route of aggregation and hence also mechanism of stabilization by spectroscopic analysis of agitated samples for conformational changes. Spectra of the stressed samples were compared to the respective native samples before the exertion of agitation. Since these IR experiments were accomplished using a transmission cell that requires clear solutions all samples were centrifuged prior to analysis and the remaining supernatant of the samples was injected for analysis.

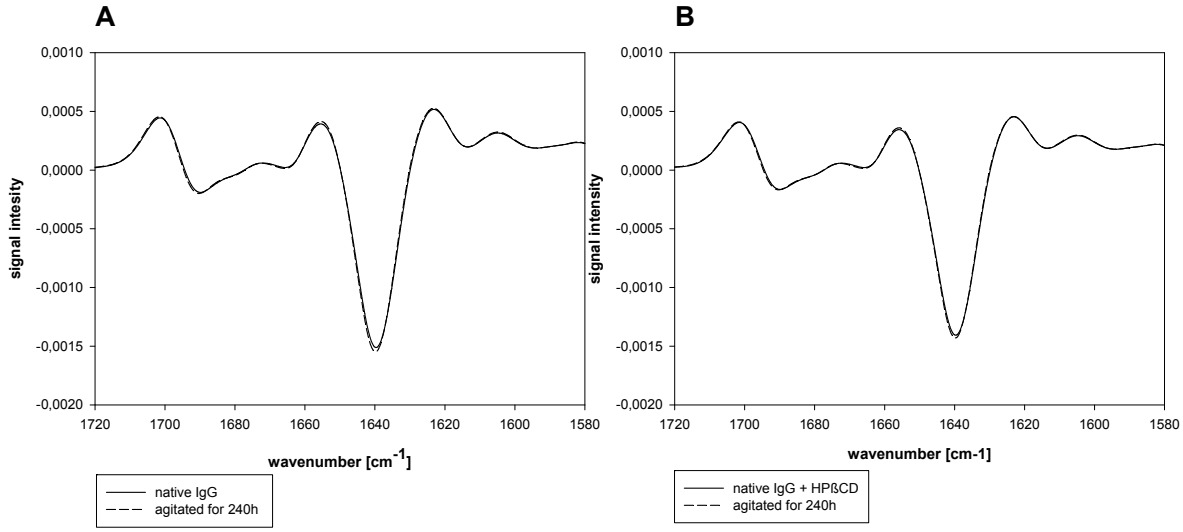

Figure 3.10: Overlaid 2nd-derivative amide I spectra of mAb before and after 240 h of agitation in glass-vials at a concentration of 50 mg/mL in absence (A) and presence of 2.5 mM HPßCD.

Again, no shifts within the characteristic IR bands and not even intensity changes at 1639 cm^{-1} are apparent and all spectra perfectly overlay when comparing the samples before and after agitation (Figure 3.10) It makes no difference whether HPßCD, which protected the mAb from aggregation, is present or not. It is thus concluded that aggregation of the protein molecules occurs from a very native-like secondary structure and proteins presumably did not expose large hydrophobic regions by unfolding before aggregating. This observation suggests that the partial prevention of mAb-aggregation at the air-water-interface by CDs does not involve the inhibition of structural alterations on a secondary structural level but it must be due to different mechanisms such as competition at the air-water-interface or prevention of partial unfolding on a tertiary structural level. Another explanation of these results could be that a very small population of partially unfolded molecules cannot be detected within the large collective of the average conformation of a population of mAb molecules[27].

In summary also at high protein concentration HPßCD was capable of reducing mAb-aggregation at the air-water-interface. However, a complete inhibition of aggregation as observed with the 1.8 mg/mL–concentration was not achievable under the harsher conditions

chosen for these experiments although the relative extent of protein aggregation decreased compared to the earlier experiments. The aggregation-promoting effects of sucrose could be entirely counteracted by the addition of HPßCD to the mAb-formulations which means that also a combination of sucrose and HPßCD can be an option for the development of stable highly-concentrated mAb-formulations. The addition of HPßCD turned out to even slightly better stabilize the mAb against agitation-induced aggregation than polysorbate 80. As with the low protein concentration no conformational changes in the mAb could be identified by IR spectroscopy upon agitation and very native-like aggregates were formed.

2.6 STIRRING STUDIES

In order to evaluate whether CDs are capable of stabilizing the mAb against further stress conditions that occur during the pharmaceutical processing of the protein, stirring studies were carried out. Stirring studies on mAbs have already been carried out using Teflon stirrer bars in vials[2-3]. It was found that in comparison to agitation stress with exposure to the air-water-interface stirring stress can be much more harmful to protein stability and that the formed aggregated species are quite different in terms of amount and size[2]. In addition it seems as if stirring stress can be counteracted by surfactants only to a much smaller extent than agitation for which complete inhibition of aggregation is possible by adding non-ionic surfactants[2-3]. Even concentrations as high as 0.05 % of polysorbate 20 are reported to be insufficient to completely inhibit particle formation and lower concentrations of polysorbate 20 were reported to completely fail to stabilize the mAb against stirring-induced aggregation[2]. It was concluded that polysorbate 20 can inhibit the adsorption of the antibody to the air-water interface (since stabilization against agitation-induced aggregation could be achieved) but that it is incapable to protect against the additional stress conditions provoked by stirring such as cavitation, accelerated mass transport, local heating and shear. Furthermore in an earlier study it was speculated that polysorbate 80 which also failed to stabilize against stirring-induced stress and provoked the formation of large amounts of small aggregates stabilizes small aggregates and inhibits the growth to larger particles[3].

In literature it remains a subject of debate whether stirring stress and the resulting protein instabilities such as aggregation can actually be caused by shear alone or whether only the combined occurrence of shear and surfaces causes the detrimental effects on protein stability. Many of the reports stating that shear by itself could cause the protein damage lack clear evidence for this assumption, because it is difficult to completely remove the influence of solid-liquid interfaces when investigating the influence of shear[35]. In two recent studies it was demonstrated that the isolated exposure to high shear forces alone causes no or only very minor damage to the protein[35-36]. For instance cyctochrome-c did not measurably unfold even upon the exertion of shear rates of 200,000s^{-1}. Bee et al. exposed a mAb to isolated shear stress in an order of magnitude that could well occur during commercial unit operations

and they did not observe antibody unfolding or aggregation[35]. Minor aggregation occurring during shearing in a stainless steel parallel-plate rheometer as well as in a stainless steel chamber was attributed to the synergistic effect of adsorption of the protein to stainless steel *and* shear[35,37]. These findings are consistent with studies conducted earlier on rhGH in which an insignificant effect of shear alone was observed but for which shear in the presence of an air-liquid interface caused the formation of noncovalent aggregates[16,38]. Finally a study investigated lysozyme inactivation and aggregation in stirred reactors in the presence of different surfaces[39]. It was found that the presence of PTFE or air was four times more detrimental to lysozyme stability than the presence of a glass-liquid interface.

Summing up the findings in literature and transferring them to the experimental set-up chosen for the experiments discussed below with mAb-solution in vials being stirred by Teflon™-coated stirrer bars this means that aggregation of the protein can be expected to result as a consequence of the combined occurrence of a hydrophobic surface and shear forces. Since non-ionic surfactants turned out to only insufficiently stabilize mAbs against the harsh conditions created during a stirring experiment and since CDs were demonstrated to stabilize the mAb against aggregation at the hydrophobic air-water-interface it is interesting and important to evaluate the effect of different CD-derivatives on aggregation induced by stirring.

In Figure 3.11 the amount of remaining monomer in different formulations throughout the stirring experiment is depicted. The stirring stress leads to a steady loss of monomer and after 120h the reference formulation without any excipients besides buffer contains less than 60 % of the original amount of monomer. It is obvious from Figure 3.11 that none of the formulations tested is suitable to completely stabilize the mAb against the stirring-induced degradation. Nevertheless some excipients lead to a partial protection of the mAb and others deteriorate protein stability. Polysorbate 80 at a concentration of 0.04 % leads to a remaining monomer content of roughly 70 % after the incubation period. However, due to the relatively large standard deviations the difference to the reference formulation cannot be regarded as statistically significant. The same conclusion holds true for the earlier time points of the experiment for which the monomer-values of the reference and of the polysorbate 80–formulation lie even closer together. This result is qualitatively in agreement with literature sources that report that the complete inhibition of stirring-induced damage on the mAb could not be achieved even by the addition of comparably high amounts of non-ionic surfactant[2-3]. It can thus be concluded that either the stressing conditions chosen for this experiment were too harsh for any excipient to be able to counteract them or other principles than Teflon-surface-induced aggregation govern the degradation of the mAb in this case and the stabilizing principles of polysorbate 80 / CDs during agitation such as competition at the interface are insufficient to protect the mAb.

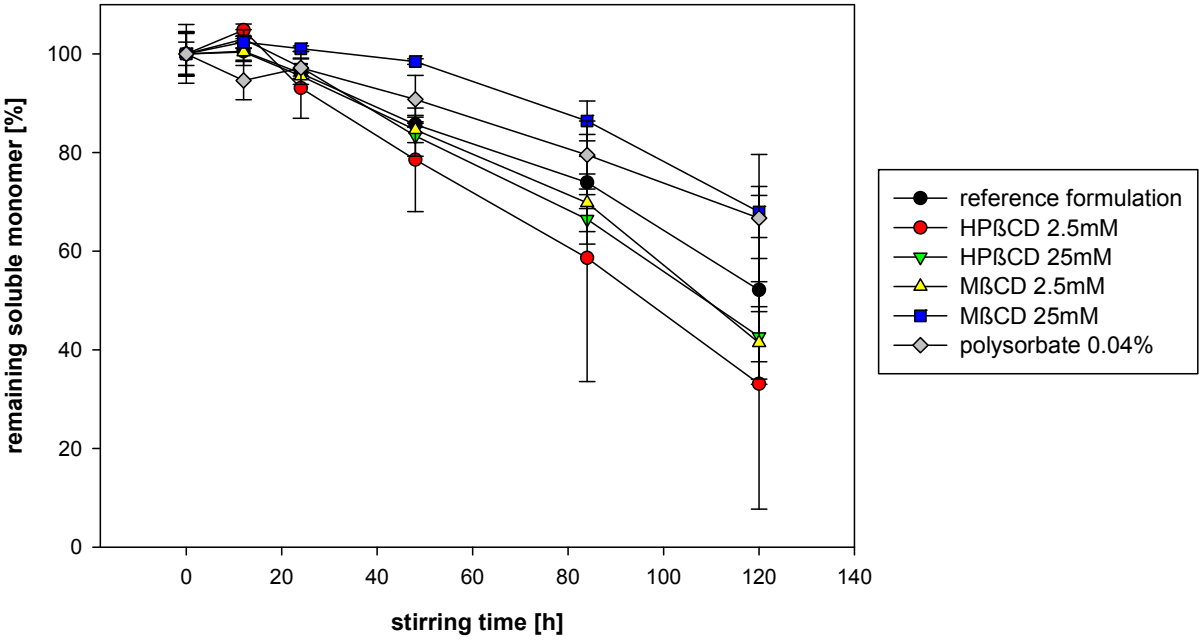

Figure 3.11: Amount of remaining monomer in different mAb-formulations during 120 h of stirring in 2R vials. Each point represents the mean of three samples ± standard deviation.

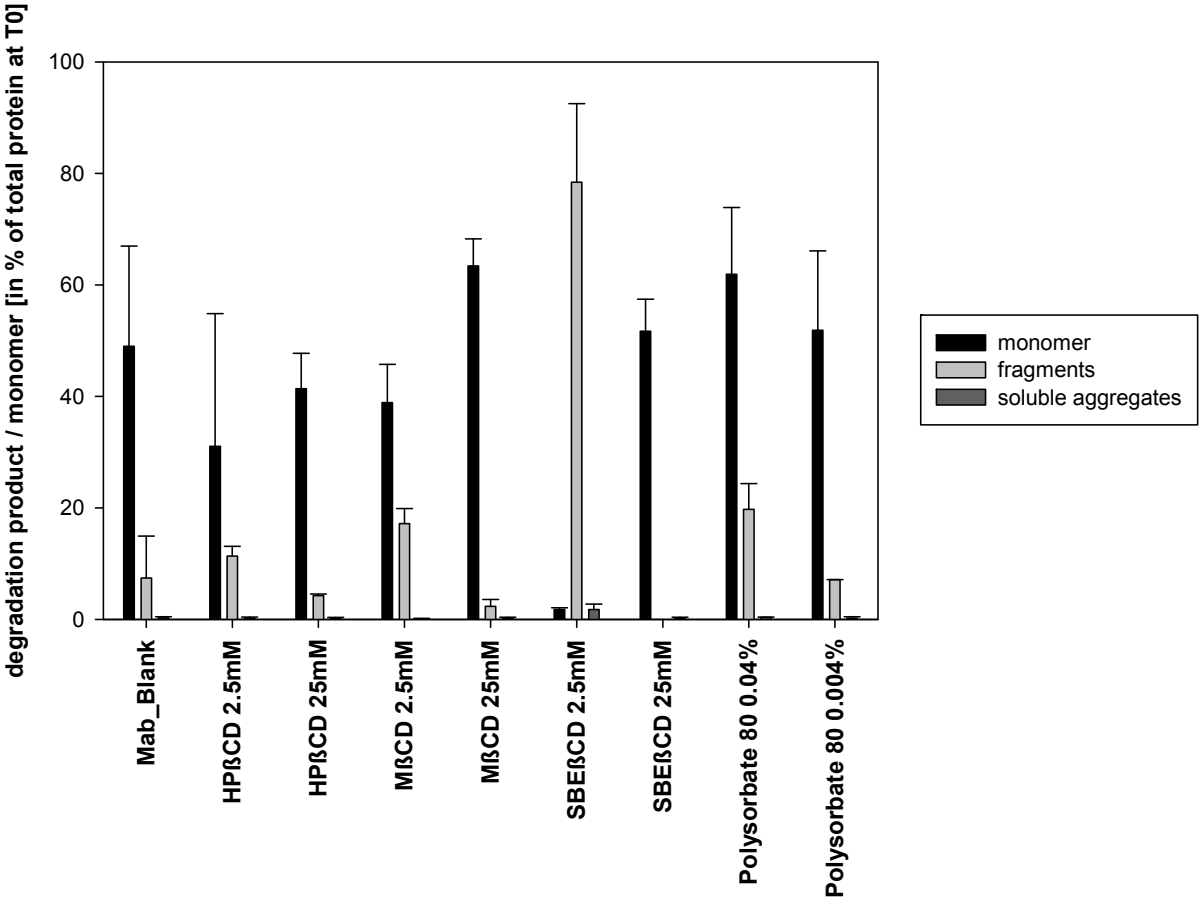

Figure 3.12: Aggregation in different mAb-formulations after 120 h of stirring with Teflon stirrer bars in 2R-vials. Each bar represents the mean of three samples ± standard deviation.

Figure 3.11 also demonstrates that the formulation containing 25 mM MßCD leads to a decreased loss of monomer compared to the reference formulation – in contrast to the other formulations containing CDs (HPßCD 25 mM and 2.5 mM and MßCD 2.5 mM). It can only be speculated at this point why only the high concentration of MßCD leads to a stabilization of the mAb against stirring induced damage – although only to a very limited degree. One explanation could be a higher surface activity of MßCD 25 mM compared to the HPßCD-samples and the lower MßCD-concentration (Chapter 6). Also, an influence of solution viscosity has to be considered since both higher CD-concentrations better stabilize than the respective lower concentration of the CDs. This pattern is also observable for SBEßCD as can be seen in Figure 3.12 where SBEßCD 25 mM results in an amount of remaining monomer that is comparable to the reference formulation whereas SBEßCD 2.5 mM yields almost no remaining monomer. Solution viscosities of the formulations will be discussed in detail at the end of this chapter (section 2.5).

All formulations became visibly turbid after stirring for 12h which can be taken as a hint for the formation of insoluble aggregates. Also the protein recovery-values as determined by SE-HPLC were significantly below 80 % after 120h of stirring which indirectly proves the formation of large amounts of insoluble aggregates (Figure 3.12). As already observed in the agitation experiments at 1.8 mg/mL no soluble aggregates were formed throughout the agitation period and the approximately 8 % soluble aggregates that were present in the starting material vanished, this time presumably by reacting to higher order aggregates. Surprisingly, also significant amounts of fragments were formed in the stirring experiment in contrast to all the agitation studies discussed earlier in this chapter. For instance the reference formulation without further excipients than buffer exhibited 7.4 % of fragments (vs. a negligible amount < 0.1 % before the stirring experiment) after 120 h of stirring (Figure 3.12). This is quite in contrast to earlier reports on stirring induced degradation of an IgG1 for which SE-HPLC-analysis did not reveal increased amounts of fragments[2]. However, it is known that IgG4–antibodies as employed in this study are prone to dissociation into half-antibodies[40] which could have played a role in our experiments. Interestingly, the different excipients have a strong influence on the formation of fragments. This becomes most apparent for the samples containing 2.5 mM SBEßCD because in this formulation the original monomer and soluble aggregates were almost quantitatively cleaved into fragments. Strongly increased amounts of fragments are also observed for the polysorbate 80 formulation (0.04 %) with 19.8 % fragments after the stressing period and the MßCD-formulation (2.5 mM) with 17.2 % fragments. On the other hand some formulations also lead to a reduced formation of fragments when compared to the reference formulation, such as the respective high concentrations (25 mM) of all three CD-derivatives employed which – again – could be related to the higher viscosity of these formulations (section 2.5).

In summary neither cyclodextrins nor polysorbate 80 were capable of completely inhibiting stirring-induced aggregation. The higher concentrations (25 mM) of the CDs lead to a decreased degradation of the mAb both in terms of aggregation and fragmentation. Furthermore it is confirmed that stirring-induced stress results in quite different protein instability patterns than agitation which could be a hint for additional factors such as cavitation or shear to play a role in inducing stirring-induced aggregation. Hence there is a need for further studies investigating causes and mechanisms of stirring-induced aggregation as well as its efficient prevention.

2.7 AGITATION IN THE PRESENCE OF GLASS BEADS

Glass surfaces are among the most widespread surfaces encountered in biopharmaceutical production and most notably also in storage of biopharmaceuticals[41]. Since protein molecules may adsorb to these glass surfaces or to microparticles shed from the glass surfaces[42], partially unfold on these surfaces and then initiate aggregation processes, stable formulations have to be developed that reduce the extent of potential damage on proteins caused by such glass surface exposure. In order to evaluate whether HPßCD also influences adsorption and subsequent aggregation processes induced by glass surfaces an experimental set-up was chosen with the mAb-solution filled into vials without any headspace in the presence of 1.4 g of glass beads (size range 0.25 mm – 0.50 mm) in order to create a large glass-water-interface. To accelerate adsorption and desorption processes to and from this extensive glass surface, vials were agitated on a horizontal shaking device at 198 rpm. In order to make sure that aggregation in the presence of glass beads is not simply due to agitation vials filled with the mAb solution but without glass beads as control samples were agitated as well.

Agitation in the presence of glass beads clearly resulted in an increased level of protein aggregation – this becomes most evident by the formation of insoluble aggregates as can be seen from Figure 3.13: the amount of monomer decreases, dimers and oligomers almost completely vanish and the amount of total soluble mAb-products also decreases. The reference formulation that was agitated in the absence of glass particles shows only very slightly changed amounts of degradation products compared to the sample composition before the agitation period (T0-values). Thus the presence of the glass beads remarkably accelerated the aggregation process.

However, the addition of HPßCD did not completely inhibit aggregation under the conditions chosen. In fact, only the highest HPßCD-concentration (25 mM) led to a higher amount of remaining monomer in solution after the experiment. The two lower concentrations of HPßCD (0.25 mM and 2.5 mM) displayed even lower amounts of remaining monomer than the reference without HPßCD.

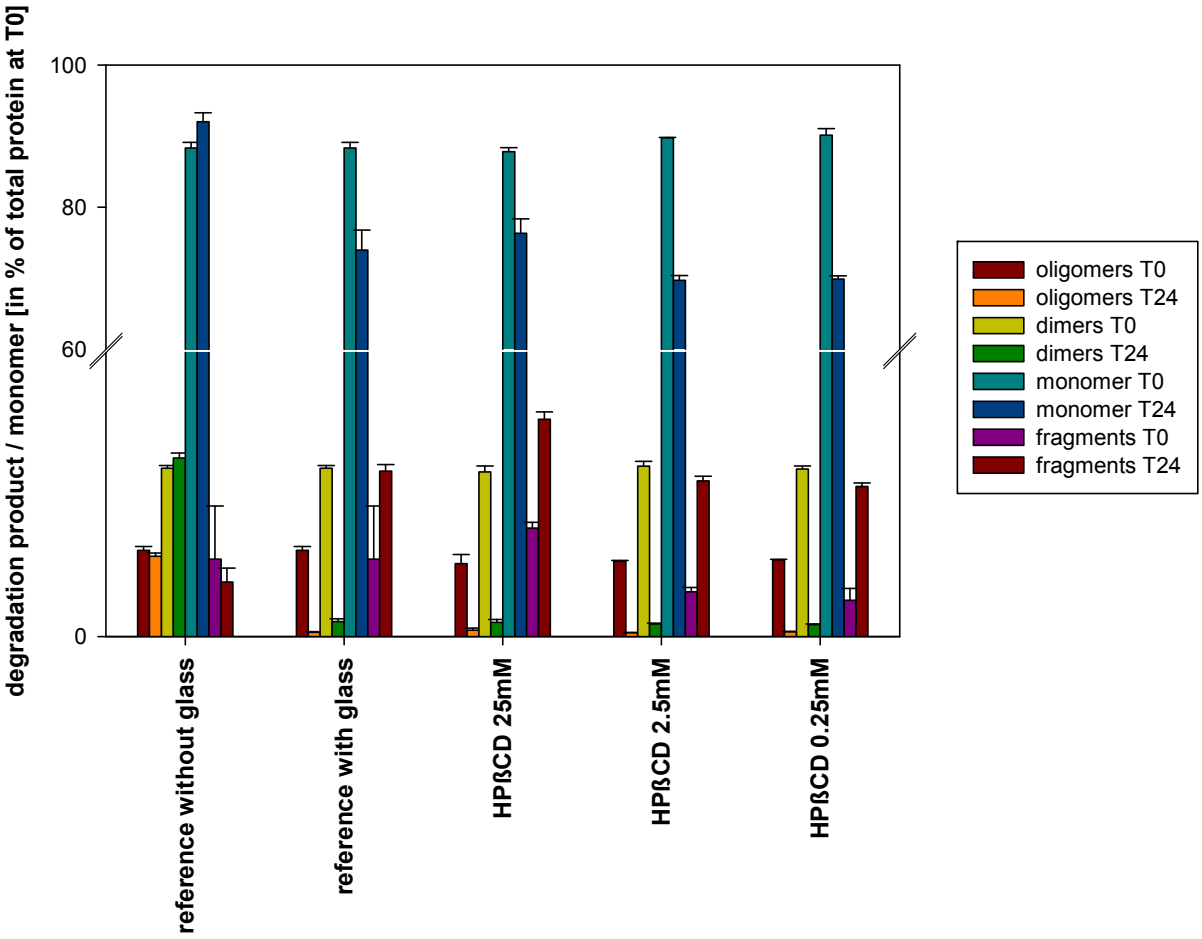

Figure 3.13: Mab-degradation after 24h of agitation in vials in the presence of glass beads to enlarge the water-glass interface. Each bar represents the mean of three samples ± standard deviation.

It has to be noted that in contrast to the agitation experiments in which aggregation was likely induced by the presence of an extensive air-water-interface (chapter 2.1) significant amounts of fragments were formed. The aggregation pattern (no increase of soluble aggregates, large amounts of insoluble aggregates and a remarkable increase of fragments) reminds of the stirring experiment described above (chapter 2.6). For that experiment a synergistic effect of shear in combination with the Teflon^{Tm}-water-interface was postulated as the probable cause of protein instability. It is therefore speculated – due to the very similar aggregation behavior and the occurrence of shear forces also in this experiment caused by the movement of the glass beads in the solution upon agitation – that the protein instability in the presence of glass beads was not only due to adsorption and subsequent unfolding followed by aggregation but also to a synergistic detrimental effect of the large glass-water-interface in combination with shear forces. This assumption is further substantiated by the fact that again the highest HPßCD-concentration shows decreased protein instability in comparison to the other two HPßCD-formulations – a behavior that could be linked to the higher viscosity of the 25 mM HPßCD-formulation and a slower movement of the glass beads associated with lower shear forces (section 2.5).However, the exact cause of protein instability remains unclear. In

conclusion it has to be stated HPßCD under these experimental conditions can decelerate mAb-aggregation only at the rather high concentration of 25 mM.

3 INHIBITION OF FREEZE-THAW-INDUCED AGGREGATION

As described in the general Introduction (Chapter 1) freezing and thawing processes can occur at many different stages throughout the lifecycle of a therapeutic protein product. During storage of protein bulk solutions accidental freezing and subsequent thawing may occur. Assuming increased long-term stability compared to the liquid state, bulk drug substance is routinely stored in the frozen state. On these and numerous further occasions aggregation may result as a consequence of repeated F/T-cycles. Generally different factors contribute to protein aggregation as a result of F/T-cycles: low temperature itself (reduction of hydrophobic interaction in the protein core and exposure of formerly buried protein parts to solvent), formation of an ice-water-interface, high local solute concentration and potential pH-shifts[5].

Comparably few studies on freeze-thawing of monoclonal antibodies – the therapeutically most important group of protein drugs – are available. Kueltzo et al examined the influence of solution factors such as pH, ionic strength and mAb-concentration as well as processing parameters like cooling and warming rates and final temperature after warming on the stability of a model IgG2-antibody[43]. In addition the effects of storage container material and type were also analyzed. It was found out that most of the factors investigated had some effect with aggregation at very low pH (3) being most pronounced probably due to acid-induced denaturation. Furthermore a strong effect of the container material was observed and the commonly used Teflon^{Tm}- and Flexboy^{Tm}– containers turned out to have detrimental effects on protein stability. The study also suggests that for the stabilization at pH-values at which aggregation is mainly driven by adsorption and protein deformation at both the ice-water and the container-interface inhibitors of surface-induced denaturation should be investigated. Another study on mAb-aggregation induced by freeze-thawing aimed at characterizing the physico-chemical-properties of the IgG-aggregates and comparing them to aggregates formed by heating the IgG1-model antibody[44]. It was found that aggregates formed after freeze-thawing were larger in size than those formed after heating and in contrast to the heating-induced aggregates they retained a very native-like structure as determined by applying an array of spectroscopic methods to characterize aggregate-structure. Another study on mAb-aggregation found out that aggregation was minimal at pH 5.5 and greater than 8.0 whereas aggregation was most pronounced at nearly neutral pH[45]. Finally a last study examining the stability of mAbs upon freeze-thawing states that the three monoclonal antibodies tested did not lose any reactivity as determined by an indirect immunofluorescence assay after subjecting the antibodies to 12 freeze / thaw - cycles[46].

However, to date no studies on the prevention of freeze-thawing-induced aggregation of mAbs by the addition of appropriate excipients are available. For other proteins such as IL-1ra[47] or recombinant human Factor XIII[22] the prevention of freeze-thawing-induced

aggregation by the addition of non-ionic surfactants is reported. It can therefore be assumed that also mAbs are stabilized against freeze-thawing-induced aggregation by non-ionic surfactants.

Summing up the published results on freeze-thawing-induced aggregation of pharmaceutical proteins it has to be concluded that freeze-thawing stress poses a major challenge for maintaining protein stability since freeze-thawing stress may occur at many stages during manufacturing and processing of therapeutic proteins and since it may cause significant damage to the protein thereby potentially risking patient safety. Hence there is a need for robust and stable formulations that can resist accidental as well as intentional freeze-thawing-stress without compromising protein stability. Since no studies on mAbs are available examining the prevention of freeze-thawing-induced aggregation by the addition of suitable excipients, CDs that were shown to prevent surface-induced aggregation in the studies discussed earlier were tested for their potential to inhibit freeze-thawing-induced aggregation. Results are compared to formulations containing the non-ionic-surfactant polysorbate 80 that is added to many protein formulations in order to prevent different kinds of surface-induced aggregation.

Figure 3.14 (A) shows the results of a freeze-thawing-study on a monoclonal antibody carried out in the bulk solution formulation buffer (His buffer 20 mM at pH 5.8) in the presence of either HPßCD 2.5 mM, MßCD 2.5 mM or polysorbate 80 0.04 % (equal to a concentration of 0.3 mM). Clearly the mAb did not aggregate to a very large extent since the amount of remaining monomer stayed very close to 100 % throughout the experiment (97.22 % after 15 FT-cycles) and the solutions remained visibly clear after the stressing experiment. Nevertheless a slight stabilization by the addition of MßCD 2.5 mM (100.66 % remaining monomer after the agitation period), HPßCD 2.5mM (99.11 %) and polysorbate 80 (100.87 %) is observable. However, since these differences can hardly be accepted as significantly different, the mAb was dialyzed to a different formulation buffer that was known to afford slightly unfavorable solution conditions[45]: potassium phosphate buffer 20 mM at a pH of 7.4. The results of the freeze-thawing study carried out in this buffer are depicted in Figure 3.14 (B) and (C). As expected aggregation occurs to a much higher extent at pH 7.4 in buffer alone than at pH 5.8 (85.29 % remaining monomer after 15 FT-cycles) in buffer alone. Again, the solutions remained clear and from Figure 3.14 (C) it becomes apparent that the loss of monomer is almost exclusively due to the formation of soluble aggregates (increase to an absolute level of soluble aggregates of more than 16 %) which is different from IgG1-aggregation observed by Hawe et al. that was mainly due to the formation of insoluble aggregates[44]. This difference once again points out that it is hard to define general aggregation patterns and rules for IgGs and that every IgG has to be studied and characterized individually.

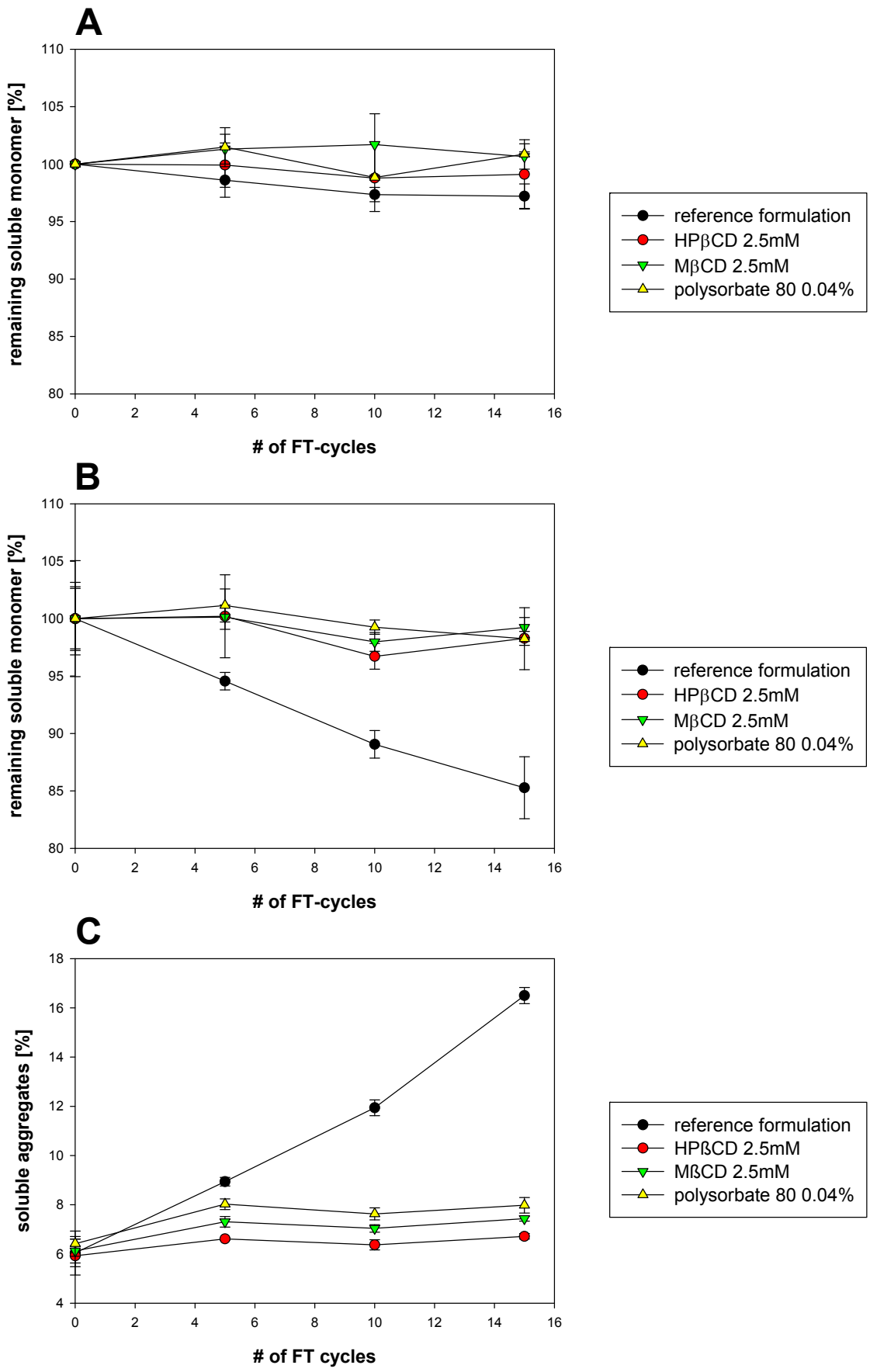

Figure 3.14: Freeze thawing induced aggregation as followed by HP-SEC remaining monomer in different mAb-formulations after up to 15 freeze-thaw-cycles in His buffer pH 5.8 (A) and in potassium phosphate buffer pH 7.4 (B) and soluble aggregates in potassium phosphate buffer pH 7.4 (C). The symbols represent the mean of three samples ± standard deviation

All three formulations containing either a CD or polysorbate 80 suppressed the formation of the soluble mAb-aggregates and completely preserved the mAb-monomer during the freeze-thawing-cycles. Thus to our knowledge for the first time it is demonstrated that low (and therefore nontoxic) concentrations of HPßCD can completely inhibit freeze-thawing-induced mAb-aggregation thereby rendering HPßCD a potentially valuable excipient for the formulation of therapeutic antibodies. Also MßCD was capable of inhibiting surface-induced aggregation. However its use in formulations of therapeutic proteins will be limited due to its parenteral toxicity[48-49]. The influence of both excipients on thermal stability of the mAb will be discussed later in section 4.4. Also the “benchmark”-formulation containing polysorbate 80 at the high concentration of 0.04 % completely preserved the IgG-monomer throughout the stressing-experiment.

In section 2.1 and 2.3 of this chapter all three excipients were already demonstrated to completely suppress aggregation induced by the presence of an extensive air-water-interface. Together with results of an additional freeze-thaw-study at a mAb-concentration of 9.5 mg/mL in which no mAb-aggregation could be observed at all (presumably due to a larger protein to surface-ratio, data not shown) our results point towards a surface-induced aggregation mechanism at an extensive ice-water-interface. This conclusion is in agreement with the assumption by Kueltzo et al. that mAb-aggregation at pH-values that do not favor acid-induced denaturation of the mAb is triggered by the presence of the ice-water-interface[43].

4 INFLUENCE OF CYCLODEXTRINS ON PROTEIN STABILITY AT ELEVATED TEMPERATURES

A potential corollary of the use of CDs as inhibitors of agitation-induced aggregation is that CDs are reported to lead to a decrease of the thermal stability of proteins, presumably due to preferential binding to exposed hydrophobic amino acids in the unfolded state, which is associated with a shift of the folding equilibrium towards the unfolded state. For globular proteins this was demonstrated by Cooper et al[14]. They found that the binding of CDs to exposed side chains destabilizes the native folded form of the protein resulting in a decrease of T_m, as observed in DSC studies. This observation was confirmed, for example, recently by Tavornvipas et al. who reported that the addition of various CDs to lysozyme formulations also led to a decrease in T_m[50]. Thus when binding of CDs to therapeutic proteins occurs, a balance of stabilizing (in terms of aggregation-inhibition by shielding of hydrophobic interaction) and also destabilizing (in terms of a decreased temperature of unfolding and lower thermal resistance of the protein) effects has to be expected. Thus in order to clarify whether the beneficial effect of HPßCD on surface-induced aggregation is compromised by a reduced thermal stability, accelerated stability studies at high temperature (60°C) as well as a long-term study at lower temperatures (4°C, 25°C, 40°C) were carried out.

As already discussed in detail in the introduction non-ionic-surfactants and notably the most commonly applied polysorbates are beneficial for the inhibition of surface-induced aggregation but can lead to accelerated aggregation after long-term storage[7,9-10]. In addition in-situ forming peroxides can be generated and are reported to lead to chemical alterations of the protein which in turn can also increase protein aggregation rates[8]. Therefore alternatives to non-ionic surfactants are required and our data from the surface-induced aggregation experiments suggest that HPßCD may serve as such. However, in order to be an alternative also the disadvantages of non-ionic surfactants during storage have to be overcome by HPßCD as well. Thus a comparison of the long-term-storage effects of HPßCD to the non-ionic-surfactant polysorbate 80 was included into the long-term-storage study as well. To complete the studies on the influence of CDs during thermal stressing of the mAb a storage experiment at 50°C at a higher mAb-concentration (50 mg/mL instead of 1.8 mg/mL) was carried out and thermal stability was also evaluated by performing calorimetric measurements.

4.1 ACCELERATED STABILITY TESTING AT 60°C

In a first study four formulations of different cyclodextrins-derivatives in three concentrations, respectively, were tested for their susceptibility to thermally-induced aggregation (Figure 3.15). After an incubation period of 8 days at 60°C the amount of monomer in the samples without any CDs decreased to about 80 %, which was mainly due to the formation of soluble aggregates (Figure 3.15). In samples containing HPβCD the level of remaining monomer

was slightly higher than in the reference samples. However, samples incubated with MβCD or HPγCD had much greater loss of monomer and high levels of soluble aggregates. SBEβCD-containing samples contained insoluble as well as soluble aggregates.

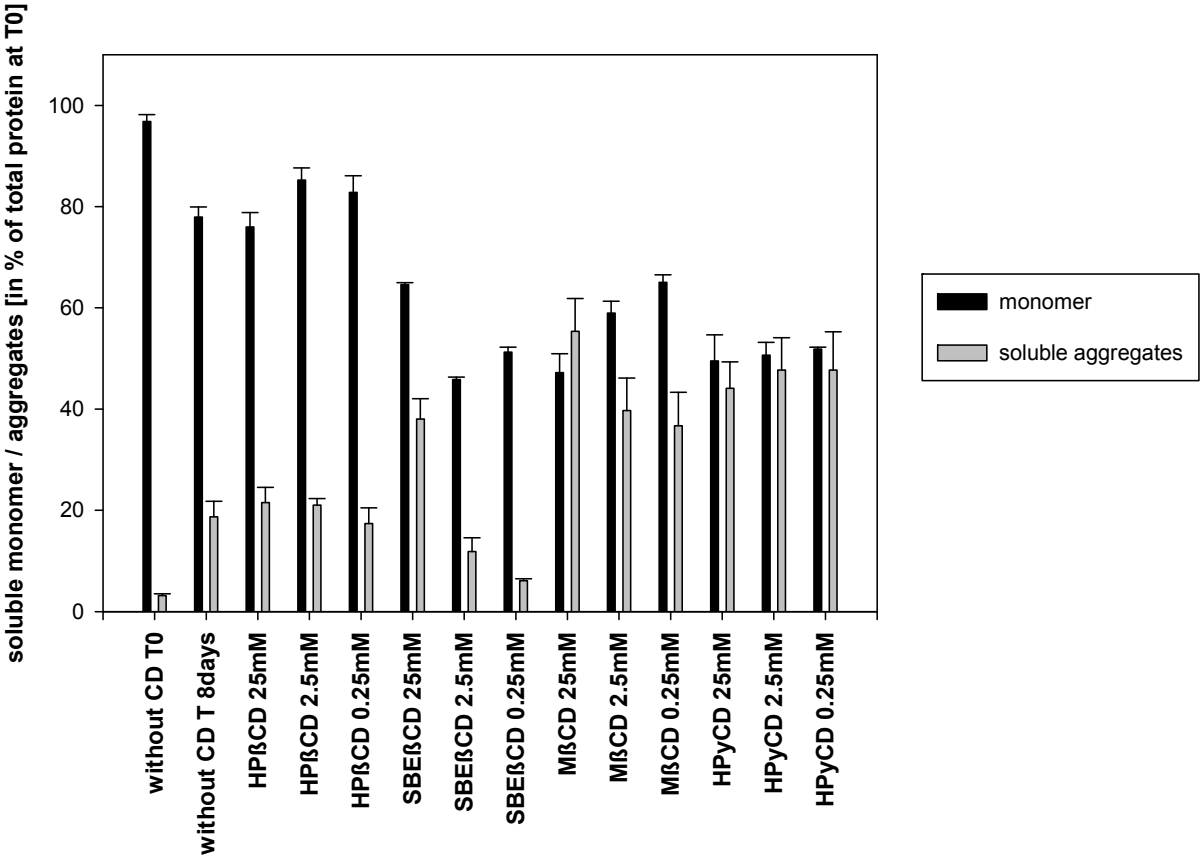

Figure 3.15: Aggregation of IgG after incubation at 60°C for 8 days. The levels of monomer and soluble aggregates as determined by size exclusion chromatography are displayed. Each bar represents the mean of three samples ± standard deviation.

When taken together the results from the agitation (section 2.1) and elevated temperatures experiments, it appears that HPβCD at concentrations of about 2.5 mM in mAb formulations would be effective at inhibiting agitation-induced aggregation while not promoting aggregation at elevated temperature. Because of this very valuable property HPßCD will be the CD-derivative in the focus of all following examinations on the role of CDs as excipients in protein formulation. At this point it remains unclear why some of the CD-derivatives leave mAb-stability at elevated temperature unaltered and others do not. Since SBEβCD was the only ionic CD-derivative tested here the detrimental effects on protein stability could be related to its charge which may lead to shielding of repulsive forces between the protein molecules thereby influencing colloidal protein stability.

4.2 LONG-TERM STABILITY AT 4°C, 25°C AND 40°C

In addition to the accelerated stability study at 60°C for 8 days a long-term storage study for six months was carried out. The goal of this investigation was to evaluate the influence of

HPßCD upon mAb-stability (physical as well as chemical) throughout an extended storage-period reflecting a typical experimental set-up used in protein formulation studies. Storage was carried out at three different temperatures: 4°C, 25°C and 40°C and a broad set of analytical methods was applied to characterize mAb-stability. HP-SEC and SDS-PAGE were used to detect and characterize protein aggregates as well as fragments; IR-spectroscopy was used to monitor conformational changes; turbidity and light obscuration measurements were carried out in order to characterize particle formation and IEF was applied to monitor chemical alterations of the mAb. An HPßCD-formulation at 2.5 mM was compared to an MßCD-formulation which was also shown above to prevent agitation-induced aggregation but also to increase aggregation at 60°C. Furthermore HPßCD-formulations were compared to formulations containing standard excipients for protein-formulations such as sucrose and trehalose (both at 250 mM, which equals 8 % w/v, a typical concentration in protein formulations[6]) and polysorbate 80 in two different concentrations (the same two concentrations as for the earlier agitation studies, both above the cmc). The comparison to polysorbate 80 is of special interest since polysorbates are demonstrated to increase protein aggregation rates upon quiescent storage[8] – in part due to in situ-forming peroxides and subsequent protein aggregation as described earlier. HPßCD was already demonstrated above to possess equal or even superior potential to inhibit surface-induced aggregation compared to polysorbate 80. However, if HPßCD is to be considered as an alternative to polysorbates in protein formulation it has to be made sure that it does not exert negative effects on the long-term stability of proteins.

The results of the SE-HPLC-analysis of the samples after long-term storage are summarized in Figure 3.16. Obviously all formulations exhibit good overall stability as no aggregation occurs at 4°C throughout the incubation period. Also at 25°C no significant loss of monomer in any of the formulations can be observed. Even at the highest storage temperature (40°C) most of the analyzed formulations display no large loss of monomer. The slight loss of monomer in the mAb blank-formulation and in the two CD-formulations can be attributed to the formation of about 3 % fragments (data not shown). The sucrose and the trehalose-formulation completely maintain their original monomer content. However, both formulations containing polysorbate 80 exhibit clearly decreased monomer-contents after the storage period with the formulation containing the higher concentration of polysorbate 80 (0.04 %) showing the most dramatic loss of monomer (roughly 50 % monomer compared to about 92 % in the formulation at the beginning of the storage period). It has to be noted here that some of the formulations, notably the two sugar-containing-formulations and also the polysorbate 80 formulation (0.04 %) appeared remarkably yellow after the storage period. Also their chromatograms (not shown) contained a large extra peak (most pronounced for the sucrose formulation) that was clearly separated from the monomer-fraction but masked

the fragment-peaks. If the extra-peak were also considered a protein-fraction, the recovery of sucrose formulation would be at about 115 % which is obviously an artifact. Therefore the extra peak very likely arises from the buffer of these formulations in which some reaction takes place leading to products that absorb at both 215nm and 280nm. Coloration of His buffer after storage was also observed in an earlier study and linked to the presence of metal and chloride ions in solution[51].

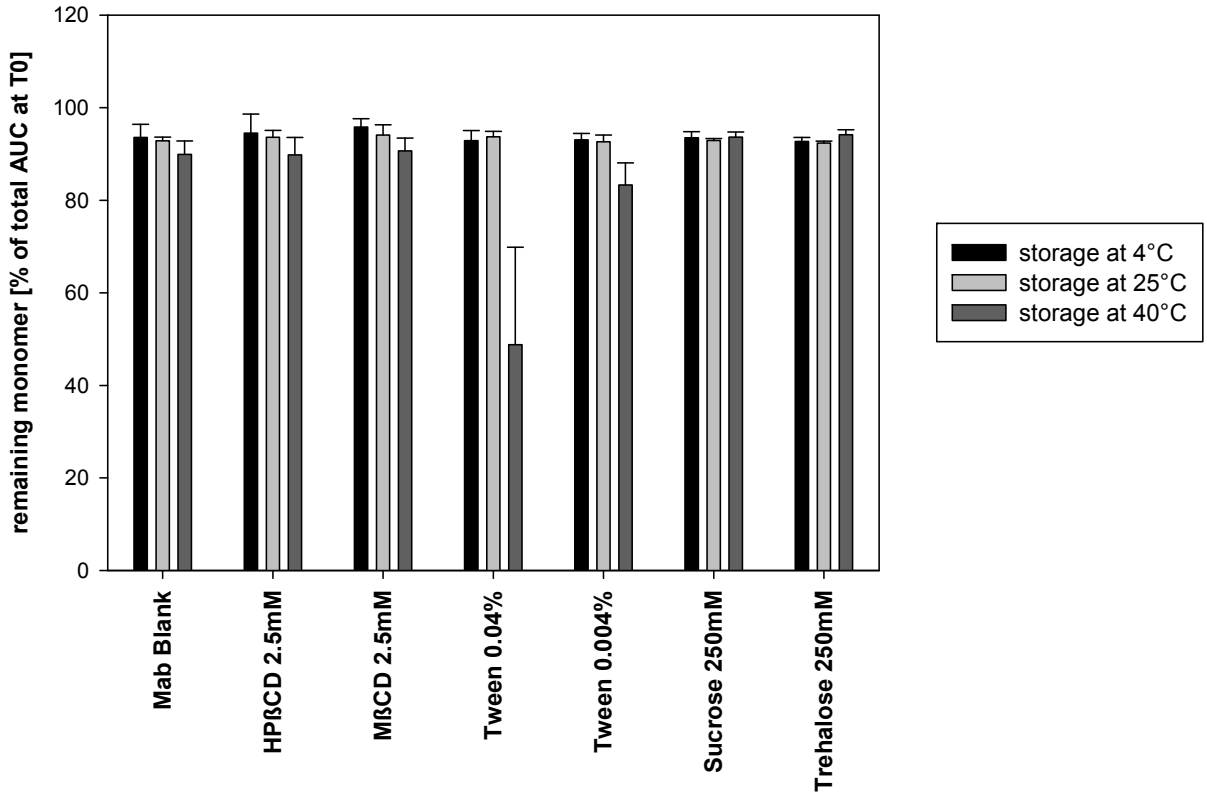

Figure 3.16: Amount of mAb-monomer after long-term storage at 4°C, 25°C and 40°C for six months as determined by HP-SEC. Each bar represents the mean of three samples ± standard deviation.

These results clearly demonstrate that HPßCD at a concentration of 2.5 mM (which was sufficient for complete inhibition of agitation-induced aggregation) is not negatively affecting long-term stability of the mAb since it does not lead to increased levels of aggregates. In this experimental set-up it could not be determined if HPßCD would also be able to stabilize the mAb during long-term storage since aggregation in the reference formulation without any excipients besides buffer is also of negligible extent. Surprisingly also MßCD at a concentration of 2.5 mM leads to no increased aggregation levels which is in contrast to the results of the accelerated stability experiment at 60°C that were discussed above (Figure 3.15) in which the presence of even lower concentrations of MßCD lead to a significant increase in the level of soluble aggregates. A possible interpretation of these results would be that different aggregation pathways play a role at 60°C (which is not far below from the

apparent melting temperature of the mAb of about 75°C, Section 4.4) than at the lower temperatures used for this incubation study[52].

Most importantly these results confirm that polysorbate 80 is problematic for mAb-stability during quiescent storage since – at least at the highest storage temperature of 40°C, which allowed for a clear discrimination – the monomer-content is clearly decreased indicating the extensive formation of aggregates and fragments. As discussed above due to the formation of an extra-peak in the chromatograms of the polysorbate 80 samples that is obstructing potentially formed fragments the exact amount of fragments after the storage period cannot be quantified. The potential reasons for the significant loss of monomer most notably in the mAb-formulation at 0.04 % will be discussed in more detail below together with the results of additional analytical experiments. However already at this point it can be stated that in agreement with earlier findings by other authors the stabilizing effect of polysorbate 80 at interfaces (that was only equal to the effect of HPßCD) in the high concentration of polysorbate is compromised by increased levels of aggregates after long-term storage.

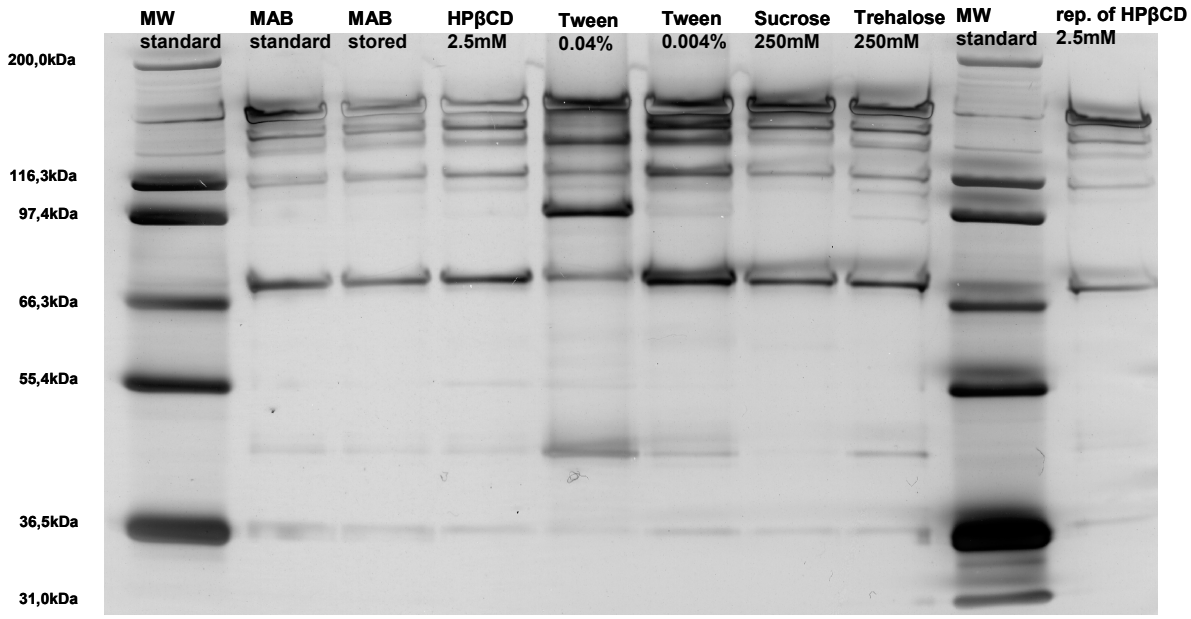

Figure 3.17: SDS-PAGE of mAb after 6 months of storage at 40°C.

In order to further characterize aggregation in the stored samples complementary information was gathered by performing SDS-PAGE. Since no high molecular weight species with a molecular weight greater than 200kDa are visible on the gel (Figure 3.17) it can be concluded that all aggregates present after storage were of noncovalent nature and dissolved into their constituent polypeptide chains during the sample preparation procedure. However, this statement is not precisely true for the polysorbate 80–containing samples since they display band smearing thereby possibly masking the clear detection of high molecular weight bands. The SDS-PAGE results also confirm the results obtained by HP-SEC in that the stored formulations containing HPßCD 2.5 mM, MßCD 2.5mM,

sucrose 250mM and trehalose 250mM are almost indistinguishable from the reference formulation containing no further excipients.

Most interestingly however, a new band at a lower molecular weight than the intact IgG monomer occurs in the stored polysorbate 80-formulations. In order to interpret the bands a look at potential degradation products of IgG4-antibodies is helpful. There are a number of reports on IgG4s exhibiting strongly varying amounts of so-called “half-antibodies”, degradation products consisting of only one heavy and one light chain[40,53-54]. These half-antibodies are only apparent in SDS-PAGE under non-reducing conditions or after other denaturing procedures because they associate via strong hydrophobic interactions. It has been demonstrated that these half-antibodies can be artificially produced by disulfide-bond scrambling during SDS-PAGE-sample preparation[54]. However in the present case, this half-antibody-species is only formed in the presence of polysorbate 80 (the assignment of the band to the half-antibody can be clearly taken when comparing Figure 3.17 to the gels in the cited references) and cannot be found with all the other formulations rendering it unlikely that the band is a pure SDS-PAGE-artifact because sample preparation was the same for all formulations tested. Taylor et al. demonstrate that also the destruction of disulfide bonds and redox-systems can influence the formation of half-antibodies[54]. For instance, in their studies 46 % of the investigated IgG4-antibody was trapped in the intrachain disulfide form after reoxidation. It can therefore be speculated that the presence of polysorbate 80 and the expectable formation of oxidizing species lead to the scrambling of disulfide bonds in this IgG4 resulting in increased levels of half-antibodies.

IgG chemical stability was further characterized by applying IEF, a technique that is sensitive to chemical changes in proteins when the basic and acidic moieties are involved, for instance when deamidation occurs and charge or pI of a protein are altered[55]. Also, different glycoforms of a protein can be separated by IEF. It can be seen in Figure 3.18 that the mAb was chemically altered in all formulations analyzed in this long-term storage study as evident from the different separation patterns of the formulations in comparison to the reference mAb that was not subjected to storage at 40°C (lane 4). It appears that the pI of the IgG was shifted to lower values after the storage period. Since a multitude of possible degradation pathways[56] could lead to such behavior it remains open which reaction caused this pI-shift on a molecular level. In order to solve this matter further techniques such as peptide mapping would be required. In contrast to all other formulations it has to be pointed out that the IgG that was stored in the presence of polysorbate does not only show a slight shift to a lower pI but exhibits massive band broadening (corresponding to a multitude of pI-shifts) in both directions suggesting strong chemical alterations of the mAb in presence of polysorbate. This strong chemical instability of the protein further corroborates the hypothesis that polysorbate-

induced oxidation with subsequent aggregation caused mAb-instability observed in this formulation.

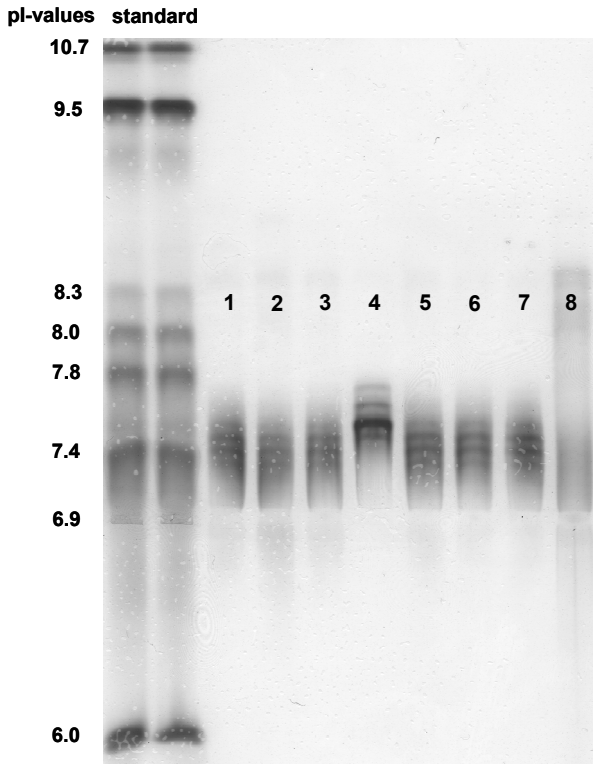

Figure 3.18: IEF-Gel of different mAb-formulations after storage for 6 months in comparison to an unincubated reference: polysorbate 80 0.004 % (1), sucrose 250 mM (2), trehalose 250 mM (3), mAb reference fresh (4), mAb reference after storage (5), HPßCD 2.5 mM (6), MßCD 2.5 mM (7) and polysorbate 80 0.04 % (8).

Formulation stability after storage was also analyzed for the formation of larger aggregates by carrying out turbidity-measurements and particle level measurements. The results of the turbidity measurements are summarized in Figure 3.19. Since the method is not specific for proteins but detects all factors potentially contributing to higher turbidity levels, stored buffer samples were analyzed as well and it is clearly shown that turbidity does not increase due to changes in the buffer systems. It can also be seen that all formulations remain at comparably low absolute turbidity levels (turbidity before storage in all mAb-formulations: 1.5±0.1 FNU, not shown in the chart, highest value after incubation period is 4.8 as can be seen in Figure) indicating that the formation of large aggregates only occurred to a minor extent.

Most importantly, it is further confirmed that HPßCD does not trigger the formation of significant amounts of large particles that would be detectable by turbidimetry as the turbidity after storage of HPßCD-samples does not significantly differ from that of the reference samples without excipients. Increased turbidity values for the polysorbate 0.04 %-formulations indicate the formation of insoluble aggregates.

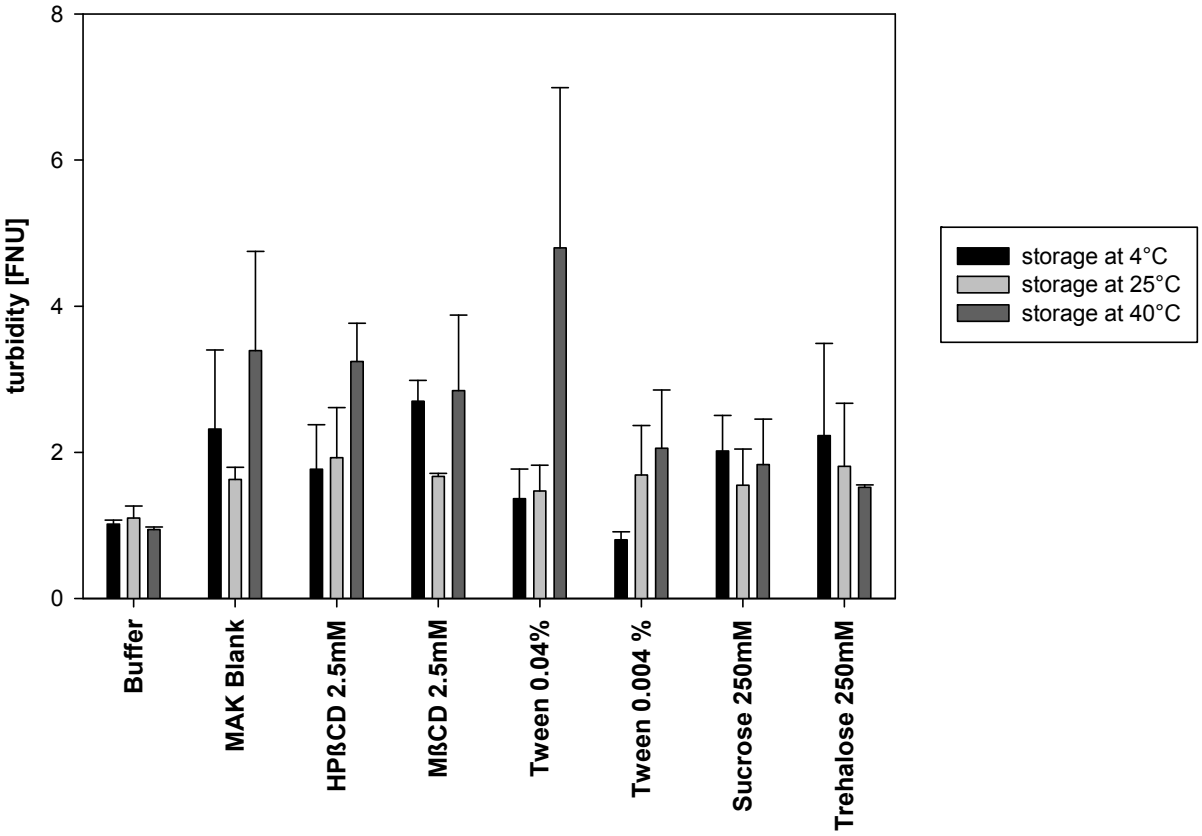

Figure 3.19: Turbidity of seven different mAb-formulations (c=1.8 mg/mL) after 6 months of storage at 4°C, 25°C and 40°C. Each bar represents the mean of three samples ± standard deviation.

The turbidity results are further confirmed by particle measurements as displayed in Figure 3.20, in which the particle levels specified by the pharmacopoeias (particles >25µm and >10µm) and also the amount of particles >1 µm are demonstrated. After storage at all three temperatures and no matter which particle size is selected it is confirmed that HPßCD does not increase aggregation in terms of formation of large aggregates that can be detected as particles. The only exception to this observation seems to be the amount of particles after storage at 25°C in the >25µm-class which is comparably high. However, when taking into account the particle amount results from the other particles size-ranges and storage temperatures this single high value must be attributed to an experimental inadequacy. When analyzing the amount of particles ≥1µm it can be even stated that the amount of particles is reduced by the presence of 2.5 mM HPβCD. Also polysorbate 80 reduced the amount of particles whereas sucrose and trehalose lead to an increase of the amount of particles >1µm. However, the absolute amounts of particles in this study were low (all values after storage are clearly below the specifications for sub visible particles of the European Pharmacopoeia) and the relative fluctuation of the values is large so that a clear trend in either direction cannot be made out. Furthermore it is interesting to note that the formulations with a clearly decreased mAb-stability at 40°C as determined by HP-SEC and SDS-PAGE as

well as IEF, the polysorbate 80-containing formulations, do not exhibit significantly increased particle levels. Hence, the observed mAb-instability in these formulations does not lead to the formation of very large aggregates (≥ 1µm) but smaller, sub visible aggregates that were not directly quantified in the current investigation must have been formed.

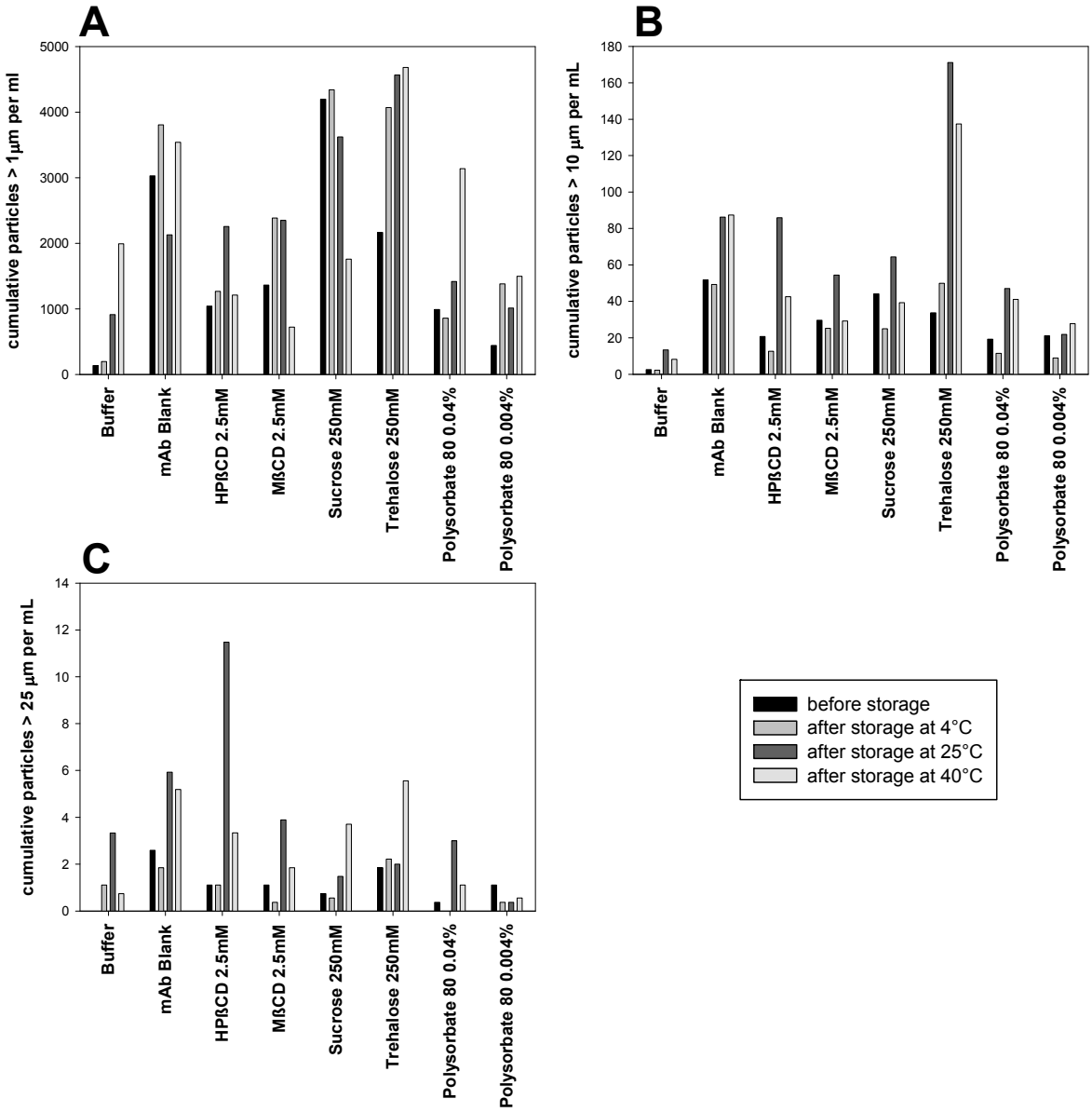

Figure 3.20: Cumulative particles before and after storage of different mAb-formulations with particles > 1 µm (A), particles > 10µm (B) and particles > 25 µm (C).

In order to assess potential changes in IgG secondary structure during long-term storage, IR spectroscopy was applied to compare the secondary structure of the IgG in the different formulations before storage and after storage at 40°C. Figure 3.21 displays the amide I region of the second-derivative transmission IR spectra that has often been described to be sensitive to secondary structural changes[57-58]. In the spectra of the native samples before incubation at 40°C the typical β-sheet-bands with the major band at 1639 cm^{-1} and the weaker band at ca. 1691 cm^{-1} can be found. All spectra recorded after the storage period of 6

months show only slight changes compared to the native spectra with the spectra of the sucrose-formulation being practically identical with the native spectrum and the polysorbate 80-samples showing the relatively strongest alterations. Thus the IgG retained a rather native-like structure in all formulations. The small variations in the β-turn-band region can be attributed to minor concentration differences between the samples. In the spectrum of the polysorbate 80 samples the intensity of the major amide I band at 1639 cm^{-1} is significantly reduced and the intensity of the β-turn-band at 1666 cm^{-1} slightly increases indicating relatively more conformational change in these samples than in the other formulations which is in agreement with the above-discussed results identifying the addition of polysorbate 80 as detrimental to the storage stability of the IgG. However, even in the polysorbate 80-formulations the degree of conformational disturbance is small, observable by the absence of a new band for intermolecular β-sheet and an even stronger reduction of the intensity of the main β-sheet at 1639 cm^{-1} that is usually found in denatured IgG spectra[59].

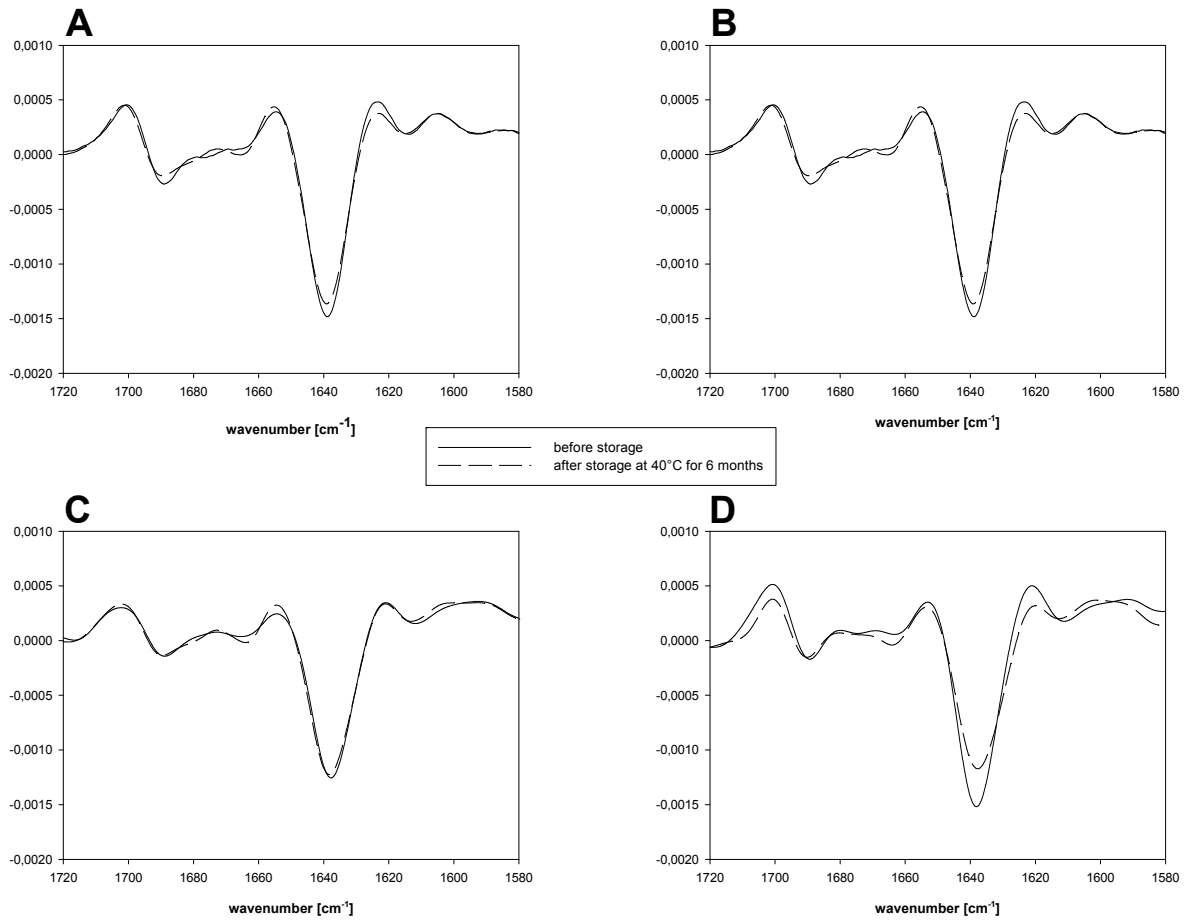

Figure 3.21: Second-derivative IR-spectra of selected mAb-formulations after long-term storage for 6 months at 40°C (n=2) comprising the reference formulation (A) and formulations containing HPßCD 2.5 mM (B), Sucrose 250 mM (C) and polysorbate 0.04 % (D).

In summary the storage study demonstrated that HPßCD is well suited for the use in mAb-formulations because in addition to its beneficial effects in the prevention of surface-induced aggregation it does not exert negative effects on mAb-stability upon quiescent storage: no

formation of soluble aggregates as determined by HP-SEC and SDS-PAGE and no increased extent of particle formation as well as chemical degradation as determined by light blockage and IEF, respectively, were observed in comparison to the reference formulation. Polysorbate 80 formulations in contrast stored under the same conditions showed strongly reduced monomer contents, triggered the formation of a fragmented antibody species and showed stronger secondary conformational changes than the HPßCD formulations. Thus HPßCD offers a promising alternative to polysorbate 80 for stable mAb formulations and should be routinely included in mAb formulation studies including a wide range of structurally different mAbs. Thereby it will be possible to determine whether the observed effects hold generally true for all subtypes of mAbs.

4.3 HIGH PROTEIN CONCENTRATION: ACCELERATED STABILITY AT 50°C

The agitation studies on highly concentrated mAb-formulations (50 mg/mL) discussed above (Section 2.5) were complemented by subjecting the same set of formulations (Table 3.2) to a storage study at 50°C for 16 days.

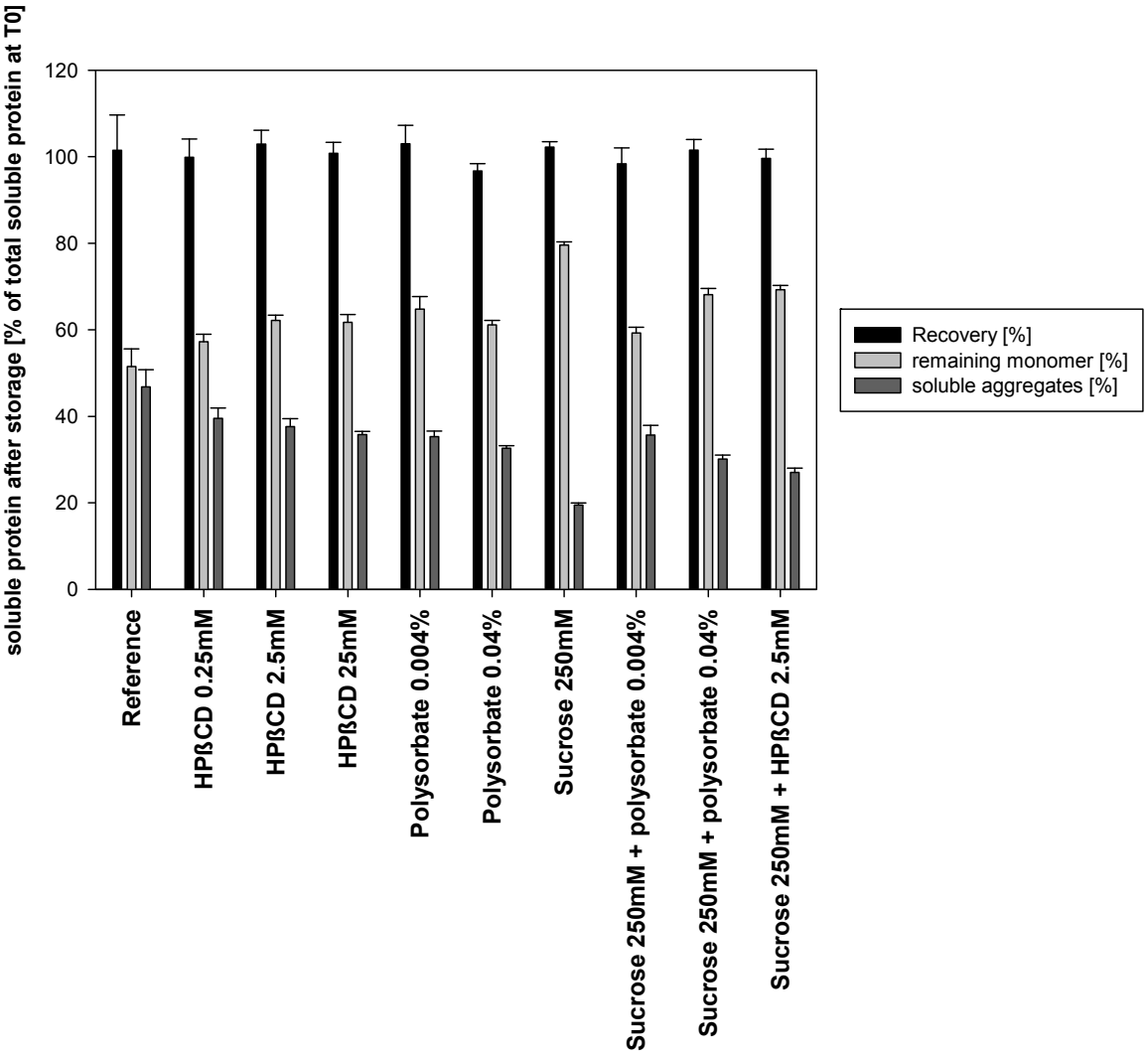

Figure 3.22: Aggregation of IgG (50 mg/mL in His buffer at pH 5.8) after incubation at 50°C for 16 days. The levels of monomer and soluble aggregates and the recovery (total AUC) as determined by HP-SEC are displayed. Each bar represents the mean of three samples ± standard deviation.

In analogy to the studies at lower concentration it was intended to evaluate whether the beneficial effects of HPßCD on the stability of the highly concentrated mAb-formulations against agitation might be compromised by an increased aggregation-rate during storage at elevated temperature. Since the addition of HPßCD to formulations containing sucrose counteracted the aggregation-promoting effects of sucrose during agitation these combined formulations were tested again – this time evaluating if the expectable beneficial effect (due to preferential exclusion) of sucrose on thermal stability of the mAb can be maintained in the presence of HPßCD. As with most previous studies comparisons to polysorbate 80 formulations were also carried out since polysorbates are currently included in most

commercial mAb-formulations – at high as well as at lower concentration[6]. In order to gain further inside into the mechanism of aggregation potential conformational changes of the mAb in all formulations were monitored by FTIR-spectroscopy as well as by second-derivative UV spectroscopy before and after the incubation period.

During the incubation period none of the samples became visibly turbid and also the total protein recovery after 16 days of incubation at 50°C stayed at approximately 100 % in all formulations (Figure 3.22). Thus no significant amounts of insoluble aggregates were formed. A slight loss of soluble protein could only be observed in the formulation containing polysorbate 80 at the rather high concentration of 0.04 % which could be due to the formation of a small amount of insoluble aggregates.

However, in all formulations significant amounts of soluble aggregates were formed (Figure 3.22 and Figure 3.23). Aggregates were in a size range from dimer to higher order oligomers with the larger soluble aggregate fraction increasing over time. As with agitation stress at 50 mg/mL and as with incubation at 60°C (at the lower concentration of 1.8 mg/mL) no formulation completely suppressed the formation of soluble aggregates. Nevertheless all formulations containing an excipient besides buffer showed decreased levels of soluble aggregates after 384 h of incubation – even the formulations containing polysorbate 80. This result was somehow unexpected since up to this point HPßCD was only shown to not negatively affect mAb-stability during storage at elevated temperature but not to stabilize it against thermal stress. Also it appears unexpected that polysorbate 80 with no further excipients increases thermal stability of the mAb which is quite in contrast to the results of the long-term incubation at 40°C described above and other reports on increased aggregation rates during quiescent storage in the presence of polysorbate[10]. However this analysis is only correct when selecting the values after 384h of storage. At earlier time points (Figure 3.23) of the experiment some formulations like the polysorbate 0.04 %-formulation show slightly higher amounts of soluble aggregates than the reference formulation. Hence not every excipient stabilizes the mAb at every time point of the experiment.

Clearly, the formulation containing sucrose and no further excipient leads to the thermally most stable formulation since the lowest amount of aggregates was determined for this formulation – at all time points of the experiment. This behavior can likely be attributed to the property of sucrose to stabilize proteins in solution via the preferential exclusion mechanism[60]. The addition of polysorbate 80 to the sucrose-formulation leads to increased levels of soluble aggregates in comparison to the formulation containing sucrose alone which is in agreement with the observation that polysorbate can increase the levels of aggregation upon quiescent storage[7]. In contrast, the combined use of HPßCD and sucrose inhibits aggregation during thermal stressing only to a slightly lesser degree than the use of sucrose alone and this combination of excipients turns out to be the overall second-most effective

one in the entire storage study. Thus HPßCD compromises the stabilizing effect of sucrose to a lesser degree than polysorbate 80 does.

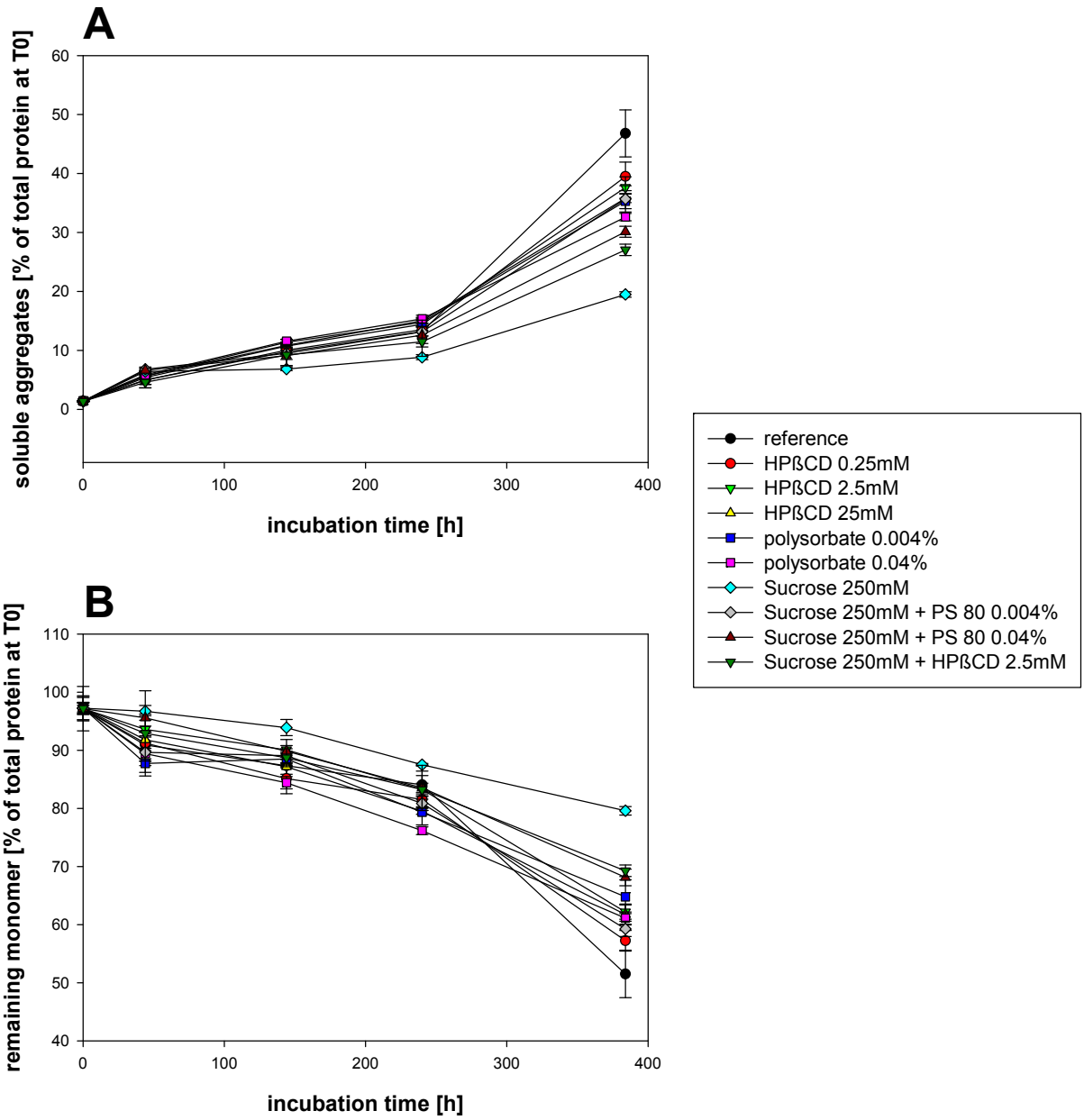

Figure 3.23: Soluble aggregates (A) and remaining monomer (B) in 50 mg/mL IgG-formulations during storage at 50°C for 16 days. Symbols represent the mean of three samples ± standard deviation.

In summary when combining the results of this short-term study with the results of the agitation study at 50 mg/mL it has to be concluded that the most stable protein formulation appears to be a combination of HPßCD and sucrose combining both advantages of the two excipients: the stabilization against aggregation at the air-water-interface by HPßCD and the increase of thermal stability in solution *via* preferential exclusion in the presence of sucrose.

To gain insight into the potential route of aggregation during storage, and hence also mechanism for inhibition, secondary structural changes in the protein molecules were monitored by using IR-spectroscopy. Figure 3.24 shows the second-derivative IR-

transmission spectra of the IgG in solution before and after subjecting it to the 50°C-incubation for 384h (16 days). It can be seen that no significant shifts within the characteristic IR bands are apparent when comparing the spectra. Therefore it has to be assumed that the mAb-molecules maintain a very native-like secondary structure throughout the experiment and that they presumably did not expose large hydrophobic regions by unfolding before aggregating. These findings are in accordance with the results obtained earlier on the secondary structure of the mAb-precipitates created by agitation from the lower-concentrated mAb-formulations which were very native-like, too. In contrast to the IR-spectra obtained from the supernatant of the highly-concentrated agitated formulations the mAb-solution obtained after storage at 50°C contained very large amounts of soluble aggregates (>40 % for the reference formulation in Figure 3.24 (A)) but nevertheless the presence of these aggregates only leads to minor spectral changes. For comparison Figure 3.24 (B) shows the IR-spectra of the mAb-formulation in presence of sucrose (the formulation that was best stabilized and showed the lowest amount of aggregates after the incubation period) and it can be seen that the intensity of the Amide I-band at 1639 cm^{-1} decreases to a smaller extent than in the reference formulation without any excipients which could be taken as a hint that less structural alterations occurred in the presence of sucrose.

In summary the mAb maintains a very native-like secondary structure during storage at 50°C and therefore structural changes on a secondary structural level do not explain the aggregation mechanism and hence also not the stabilization mechanism by sucrose and other excipients.

Figure 3.24: Area-normalized 2nd derivative IR transmission spectra of 50 mg/mL IgG-formulations before (solid line) and after storage at 50°C for 384 h (dashed line) with no excipients except for buffer (A) and in the presence of 250 mM sucrose (B). n=2

Since the aggregation mechanism of the mAb under investigation could not be elucidated by IR-spectroscopy further investigations focusing on possible tertiary structural changes in the

mAb were carried out. A general picture of the tertiary-structural situation in a protein can be obtained by using second-derivate UV absorbance spectroscopy since the positions of the peaks in a 2DUV-spectrum are sensitive to changes in the polarity of microenvironment of the hydrophobic amino acids in a protein[43,61-62].

Figure 3.25 (A) shows representative spectra of the mAb that were obtained before and after the incubation period. The spectra strongly resemble those obtained in an earlier study on an IgG2 antibody exhibiting five characteristic peaks at the almost identical wavelenghts[43]: 252-Phe, 259-Phe, 276-Tyr, 284-Tyr/Trp and 292-Trp. It can be seen that for the reference formulation the spectrum is slightly changed after the incubation period. Changes in peak-intensity are most pronounced in the 284-Tyr/Trp and 292-Trp-region. Since these peaks were also shown to be very sensitive indicators of changes in the microenvironment of the amino acids their shifts were analyzed in more detail (Table 3.3 and Table 3.4). Obviously the peak shifts were not very large with a maximum of 0.09 nm for the reference-formulation which is small compared to complete unfolding upon thermal denaturation resulting in a peak shift of 0.8nm for an IgG2[43]. Nevertheless due to the very good reproducibility of the experiment the differences must be assigned to significant deviations from the native tertiary structure of the mAb.

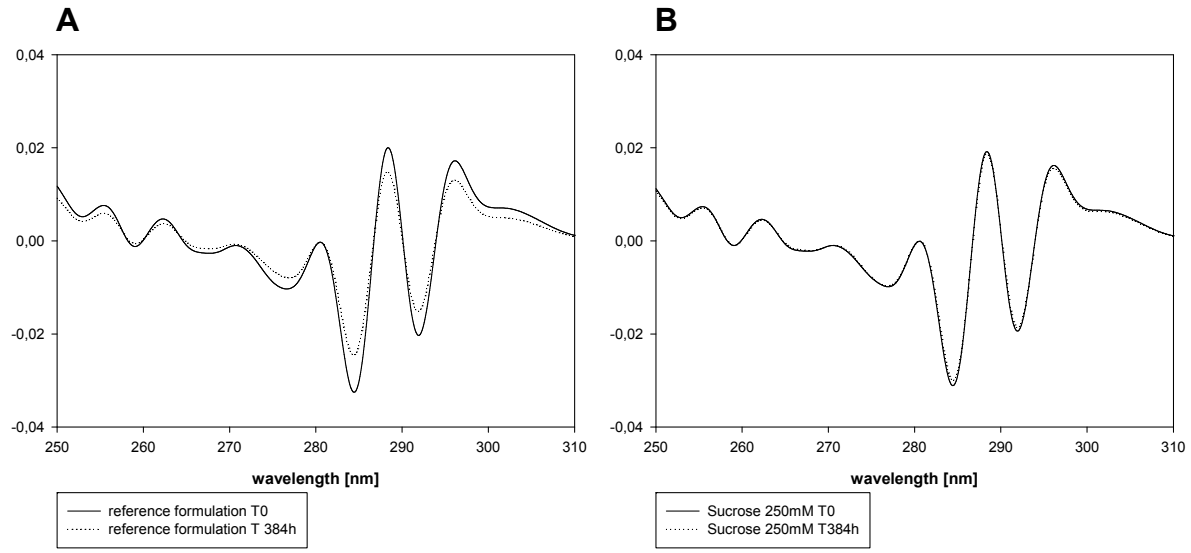

Figure 3.25: Representative 2DUV-spectra: second derivative spectra of mAb (50 mg/mL) samples before and after incubation at 50°C for 384 h of the reference-formulation without excipients besides buffer (A) and the formulation containing 250 mM sucrose (B).

All peak shifts occurred to shorter wavelengths which means that the polarity of the microenvironment increases and the protein changes to a less compact structure leading to subsequent aggregation[43,61]. This conclusion is further substantiated when comparing the mAb-spectrum of the sucrose-formulation before and after incubation at 50°C. Here almost no alterations in the spectrum after incubation are recognizable leading to the conclusion that the mAb maintains its native tertiary structure in the presence sucrose. This observation is in

agreement with the negligible wavelength-shifts of 0.03nm as listed in Table 3.3 and Table 3.4. As described above the sucrose formulation also showed the lowest degree of aggregation throughout the incubation period which further proves that partial unfolding on a tertiary level of the mAb goes along with mAb-aggregation. Species with native secondary structure and slightly perturbed tertiary structures, which is typical of partially unfolded molecules, have also been found to lead to aggregation of other proteins, such as rh-GCSF[63]. It also has to be noted here that HPßCD does not negatively influence mAb-conformation during storage at 50°C. The peak-shifts observed for the HPßCD-formulations are somewhere in between the values obtained for the reference formulation and the sucrose-formulations indicating even a slight stabilization of the mAb.

In summary it can be stated that also at a higher mAb-concentration (50 mg/mL as compared to 1.8 mg/mL for the earlier studies) HPßCD does not decrease the thermal stability of the mAb – neither in terms of a higher propensity to aggregation nor does it lead to a decreased conformational stability of the mAb. Hence the suppression of agitation-induced aggregation of HPßCD is not compromised by a reduced thermal stability. It could also be shown that mAb-aggregation during storage at 50°C likely proceeds *via* a partially unfolded species of the mAb.

Table 3.3: Tertiary structural characterization of the IgG before and after storage at 50°C for 384 h by second-derivative UV-spectroscopy. Peak positions of the 292 nm-Trp-peak and the differences before and after storage are listed (n=2).

Formulation	***Peak position T0 [nm]***	***Peak position T 384h [nm]***	**Difference [nm]**
Reference	291.92±0.01	291.84±0.00	0.09
HPßCD 0.25 mM	291.93±0.00	291.83±0.01	0.10
HPßCD 2.5 mM	291.93±0.01	291.87±0.00	0.06
HPßCD 25 mM	291.93±0.01	291.85±0.01	0.09
Polysorbate 0.004 %	291.93±0.01	291.85±0.00	0.08
Polysorbate 0.04 %	291.93±0.00	291.86±0.01	0.07
Sucrose 250 mM	291.93±0.01	291.90±0.01	0.03
Sucrose+PS low	291.93±0.01	291.86±0.01	0.07
Sucrose+PS high	291.93±0.01	291.88±0.00	0.06
Sucrose+HPßCD 2.5 mM	291.93±0.01	291.87±0.00	0.06

Table 3.4: Tertiary structural characterization of the IgG before and after storage at 50°C for 384h by second-derivative UV-spectroscopy. Peak positions of the 284 nm-Tyr/Trp-peak and the differences before and after storage are listed (n=2).

Formulation	*Peak position T0 [nm]*	*Peak position T 384h [nm]*	Difference [nm]
Reference	284.44±0.00	284.37±0.00	0.07
HPßCD 0.25 mM	284.43±0.01	284.36±0.01	0.07
HPßCD 2.5 mM	284.43±0.01	284.39±0.00	0.05
HPßCD 25 mM	284.43±0.01	284.38±0.01	0.06
Polysorbate 0.004 %	284.45±0.01	284.37±0.01	0.07
Polysorbate 0.04 %	284.44±0.01	284.39±0.00	0.06
Sucrose 250 mM	284.44±0.00	284.41±0.01	0.03
Sucrose+PS low	284.44±0.01	284.38±0.01	0.06
Sucrose+PS high	284.44±0.00	284.40±0.00	0.04
Sucrose+HPßCD 2.5 mM	284.44±0.00	284.38±0.01	0.06

When combining the results from the studies carried out at a mAb concentration of 50 mg/mL (agitation and storage at 50°C) it can be concluded that HPßCD leads to decreased aggregation rates during agitation presumably due to aggregation prevention at the interface while sucrose promoted aggregation during agitation. During storage at 50°C HPßCD leads to a slight stabilization of the mAb whereas sucrose significantly decreased the extent of aggregation. Interestingly when combining the two excipients the advantages of both could be preserved at a time leading to a formulation that is stable in the bulk and against interfacial stresses. It is therefore suggested to more extensively investigate this combination of excipients for a larger number of different antibodies and to also apply even broader analytical techniques notably concerning the characterization of particle formation.

4.4 Effects of CDs on the apparent melting temperature of mAb

It is reported that the reduction of agitation-induced aggregation by non-ionic surfactants might be due to an increase in the thermodynamic stability of a protein[64]. In order to investigate whether this behavior might also play a role for the stabilization of the IgG by CDs, the apparent melting temperature of the mAb was measured in presence and absence of CDs.

First, fluorescence spectroscopy was employed to record melting curves. The resulting curves show a good overlay between two formulations containing 25 mM of either HPβCD or MβCD and the formulation containing IgG in buffer alone (Figure 3.26). Thus the apparent melting temperature as determined by this method (monitoring of tertiary structural transitions) remains unaffected even in the presence of high concentrations of HPßCD.

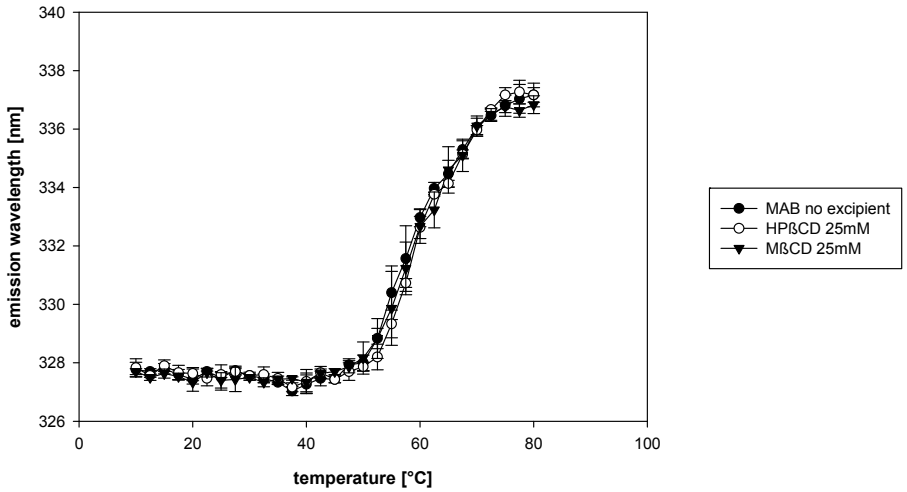

Figure 3.26: Shift of the IgG maximum emission wavelength during temperature ramping. Comparison between formulations containing HPβCD, MβCD and the IgG in presence of buffer only. Symbols represent the mean of three samples ± standard deviation.

Microcalorimetric measurements further confirmed results from the fluorescence spectroscopy experiments (Table 3.5). The apparent melting temperature by microcalorimetry for the IgG in buffer alone is nearly identical to those for the protein in samples with CDs. Thus, it appears that CDs have no measurable effect on the thermodynamic stability of the IgG. Together with the findings shown in Figure 3.26 it can be concluded that a change in thermodynamic stability is not an explanation for the aggregation inhibition of the mAb in the presence of CDs. These results can be interpreted as an indication that no binding between CDs and the IgG occurs which is in contrast to some previous publications in which T_m is reported to decrease due to a preferential binding of CDs to the partially unfolded state of the protein and a subsequent shift of the folding equilibrium[14].

Table 3.5: Melting temperatures and enthalpies of unfolding of formulations containing CDs, polysorbate 80, sugars and a reference formulation as determined by microcalorimetry. The mean ± standard deviation is listed (n=2).

Formulation	Apparent T_m [°C]	$\Delta H_{unfolding}$ [kcal/mol]
His 20 mM no excipient	75.64 ± 0.12	834.16
HPβCD 2.5 mM	75.73 ± 0.07	856.33
MβCD 2.5 mM	75.69 ± 0.15	861.99
Tween80 0.004 %	75.62 ± 0.16	880.54
Tween80 0.04 %	75.64 ± 0.08	885.05
Sucrose 250 mM	76.54 ± 0.10	916.51
Trehalose 250 mM	76.86 ± 0.15	866.83

Interestingly, the apparent T_m is increased by about 1°C in the presence of the sugars and sugar alcohols that were used for the agitation studies discussed in the previous sections. However, this increase in thermodynamic stability does not correspond to increased stability against aggregation at the air-water interface. In contrast, agitation-induced aggregation is even more pronounced in the presence of these sugars and sugar alcohols (Figure 3.6). Thus thermodynamic stability of the IgG in the bulk solution probably plays a minor role for the explanation of the aggregation behavior at interfaces.

5 INFLUENCE OF CYCLODEXTRINS ON THE VISCOSITY OF MAB FORMULATIONS

The determination of the solution viscosity of the 1.8 mg/mL formulations was carried out as an attempt to get insight into potential reasons of mAb-stabilization by CDs. There are literature reports that relate decreased rates of aggregation to an increase of solution viscosity restraining the motion of the protein backbone[5]. This phenomenon was observed with protein solutions after the addition of polymers. The results of our investigations are displayed in Table 3.6. It becomes obvious that at an HPβCD-concentration of 2.5 mM (sufficient for complete inhibition of IgG-aggregation in the agitation-experiments) viscosity is not significantly increased and thus it is unlikely that viscosity is a determining factor for IgG-stabilization. In addition the LMW-sugar formulations (250 mM) as well as SBEβCD (25 mM) show a clear increase in viscosity by more than 20 %, but however the propensity of the IgG to aggregate at the air-water-interface is not reduced. Thus it is concluded that the observed stabilization of the IgG-formulation against agitation-induced aggregation is not related in any way to changes in solution viscosity.

However, a correlation of the viscosity values to the results of the stirring studies (section 2.6) and the agitation study in the presence of glass beads (section 2.7) can be assumed: in both experiments CDs at low concentrations of 2.5 mM were shown to have no inhibitory effect on aggregation, whereas at a higher concentration of 25 mM (for which an increased viscosity was observed) a slight reduction of aggregation was detected. This correlation can be taken as a hint that the increase in solution viscosity rather than a specific stabilization mechanism is responsible for decreased mAb-aggregation rates in those experimental set-ups.

Table 3.6: Viscosity of selected mAb-formulations (1.8 mg/mL) in the presence of different concentrations of HPßCD, SBEßCD and Sucrose. Each value represents the mean of three samples±standard deviation.

Excipient	Excipient concentration [mM]	Viscosity [mPas]
IgG in His 20 mM no excipient	0	1.010±0.0003
HPβCD	0.25	1.0091±0.0013
HPβCD	2.5	1.0226±0.0038
HPβCD	25	1.1211±0.0013
SBEβCD	0.25	1.0138±0.0081
SBEβCD	2.5	1.0228±0.0063
SBEβCD	25	1.2386±0.0054
Sucrose	250	1.2920±0.0111

Additional viscosity measurements were carried out on CD-mAb formulations at the high mAb-concentration of 50 mg/mL, since viscosity is known to be a critical factor for the

development of highly concentrated mAb-formulations[15]. Figure 3.27 clearly demonstrates that HPßCD at its aggregation-inhibiting concentration of 2.5 mM does not increase the inherently elevated viscosity of the mAb-formulation. In analogy to the results obtained for the measurements at the lower mAb-concentration of 1.8 mg/mL viscosity starts to be measurably increased beginning at a concentration of 25 mM cyclodextrin. Also sucrose at 250 mM adds to the viscosity of the highly concentrated mAb-formulation. Surprisingly, the absolute amount of the viscosity increase by the addition of 250 mM sucrose is higher when added to the 50 mg/mL mAb-formulation (0.46 mPa*s) than when added to the 1.8 mg/mL mAb-formulation (0.28 mPa*s) which further confirms the difficulty of adding sucrose to mAb-formulations with an already inherently high viscosity. Most importantly, it has to be noted that the most stable formulation, the combination of HPßCD 2.5 mM and sucrose 250 mM, does not exhibit an increased viscosity compared to the mAb-formulation containing sucrose only. The absence of an increase in viscosity further confirms the potential of HPßCD 2.5 mM and its combination with sucrose as promising formulation for highly-concentrated mAb-formulations.

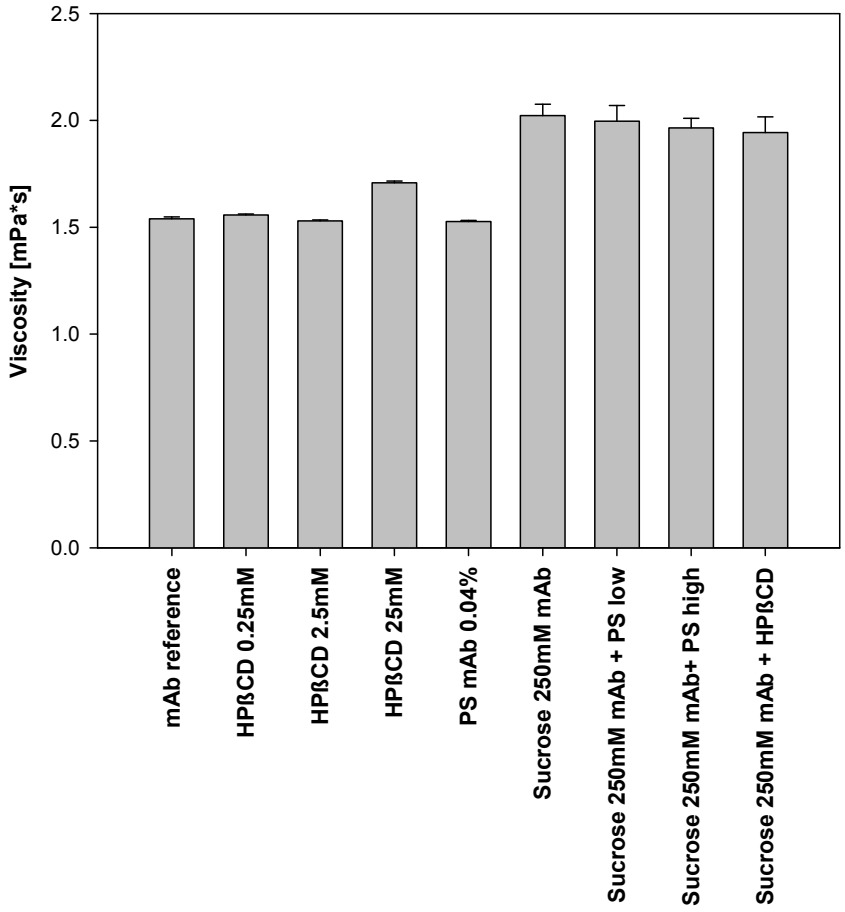

Figure 3.27: Viscosity of mAb-formulations (50 mg/mL) in presence of different excipients as determined by . Each bar represents the mean value of 20 repeated measurements ± standard deviation.

6 SUMMARY AND CONCLUSIONS

In this chapter the results of studies investigating the use of CD-derivatives in the formulation of monoclonal antibodies were discussed. First a variety of different cyclodextrin-derivatives was evaluated regarding their potential to stabilize an IgG-antibody against different stressing conditions. The most promising CD-derivative of these investigations - HPßCD - was evaluated in more detail as a formulation alternative to polysorbate 80.

To our knowledge for the first time, it was demonstrated that HPβCD is well suited to inhibit aggregation of an IgG monoclonal antibody at the air-water interface. In contrast to other CD-derivatives investigated in our studies, the addition of HPβCD did not negatively affect IgG-stability during storage at elevated temperature (60°C) in the absence of agitation, which renders HPßCD the most promising of the CD-derivatives tested in our studies. Remarkably low (≥ 2.5 mM corresponding to 0.35 % (m/m)) and thus presumably nontoxic concentrations of HPβCD were satisfactory for complete inhibition of IgG-aggregation at the air-water-interface.

Agitation studies also demonstrated that low molecular weight sugars and sugar alcohols that are known to be preferentially excluded from the protein surface (sucrose, trehalose, mannitol and sorbitol) even promoted aggregation and thus show a completely different behavior than the oligosaccharide HPβCD. Therefore it can be concluded that HPβCD exerts its stabilizing mechanisms against aggregation at the air-water interface by different means than low molecular weight sugars that are commonly used in protein formulation.

In a rather high concentration of 0.04 % (clearly above the CMC) polysorbate 80 provided the same, complete degree of stabilization against agitation-induced aggregation as HPßCD at 2.5 mM (i.e. 0.35 %). However, when employed in a lower concentration of 0.004 % polysorbate 80 (that is still in a typical concentration range employed in mAb-formulations) the formation of soluble aggregates was observed.

HPβCD at the comparably low concentration of 2.5 mM also stabilizes the IgG against aggregation induced by freeze-thawing stress. It is therefore demonstrated that stabilization against aggregation is not limited to the air-water-interface, but that it is also achievable at other surfaces such as the ice-water-interface.

It was also found out that sulfobutylether-CD-derivatives are generally not suitable for mAb-formulation - independent of their ring size or degree of substitution. Since all other non-charged β- and γ-CD-derivatives turned out to at least partially stabilize the mAb against surface-induced aggregation, it is assumed that the negative charge of the SBE-CDs plays an important role for the explanation of the inability of these ionic derivatives to preserve mAb-stability.

A long term storage study for six months conducted at 4°C, 25°C and 40°C confirmed that HPßCD is well suited for the use in mAb-formulations because in addition to its beneficial

effects for the prevention of surface-induced aggregation it does not exert negative effects on mAb-stability upon quiescent long-term storage. Polysorbate 80 formulations that were stored under the same conditions as the HPβCD-containing formulation showed strongly reduced monomer contents, triggered the formation of a fragmented antibody species and showed stronger secondary conformational changes than the HPßCD formulations. Thus, since HPβCD is not associated with the known disadvantages of polysorbates (formation of micelles, generation of peroxides during storage and subsequent protein aggregation as confirmed by these studies) they pose a valuable alternative to polysorbates in mAb-formulation.

At high protein-concentration (50 mg/mL) HPβCD was also capable of reducing mAb-aggregation at the air-water interface. However, a complete suppression of mAb-aggregation, as observed at the lower concentration (1.8 mg/ml), was not achievable at high mAb-concentration. However, the stress conditions chosen for agitation at high mAb-concentration were harsher than those at low mAb concentration. A combination of HPβCD and sucrose turned out to better stabilize the mAb against agitation-induced aggregation than a combination of the mAb and polysorbate 80. In combination with an additional incubation study of the highly-concentrated mAb-formulations at 50°C it could be shown that sucrose was able to preserve its stabilizing effect during storage at elevated temperature also in the presence of HPβCD whereas HPßCD was able to completely counteract the aggregation-promoting effect of sucrose during agitation.

Spectroscopic investigations (IR-spectroscopy) after agitation-induced aggregation revealed only very minor structural changes in the aggregated mAb-samples, which suggests that aggregation does not proceed *via* substantially unfolded mAb-species. In turn this presumably also means that the mechanism of aggregation-inhibition does not proceed *via* direct binding of the CD to the partially unfolded mAb and subsequent blocking of hydrophobic interaction between the mAb-molecules. Since also the thermal stability of the mAb as determined by microcalorimetric measurements and fluorescence spectroscopy ramps was unaltered in the presence of HPβCD, no evidence for direct binding between HPβCD and the mAb in the bulk solution could be gathered by the results presented in this chapter.

In conclusion it is suggested that CDs should be routinely included in formulation studies during the development of liquid IgG-formulations in order to evaluate whether the observed tendencies apply to wider number of different antibody-subtypes. In addition the influence on potential chemical degradation of the mAb-molecules should be characterized in more analytical detail.

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CHAPTER 4

CYCLODEXTRINS AS EXCIPIENTS FOR THE STABILIZATION OF RECOMBINANT HUMAN GRANULOCYTE COLONY STIMULATING FACTOR (RH-GCSF) AND RECOMBINANT HUMAN GROWTH HORMONE (RH-GH)

1 INTRODUCTION

In Chapter 3 it was demonstrated that CDs, notably HPβCD, have numerous beneficial effects on IgG-stability that render them potentially useful excipients for protein formulation. In brief, HPβCD was shown to stabilize a mAb against surface-induced aggregation, e.g. during agitation or freeze-thaw stress. Also, in contrast to the commonly employed polysorbates, HPβCD does not decrease thermal stability of the IgG nor does it lead to accelerated aggregation rates during quiescent storage of the protein at elevated temperature.

In this chapter the role of CDs, notably of HPβCD, for the prevention of protein aggregation is investigated using two further model proteins that are structurally very different from the mAb that was used for the previous investigations. The first model protein is recombinant granulocyte colony stimulation factor (rh-GCSF), a cytokine with a four-helix bundle structure and a molecular weight of 18.8 kDa that is prone to aggregation, notably at pH-values significantly above pH 4[1]. Since rh-GCSF contains five aromatic amino acids (two Trp and three Tyr) and partial unfolding of the protein was shown to occur prior to aggregation[2] it seems reasonable to test CDs as stabilizing agents for rh-GCSF under the assumption that CDs are capable of incorporating exposed aromatic amino acid residues on the partially unfolded protein into the hydrophobic cavity thereby preventing rh-GCSF aggregation.

Recombinant human growth hormone (rh-GH) was included into the studies as a further model protein since its interaction with different CD-derivatives had already been proven. Also, its property to be stabilized against aggregation by CDs had already been indicated[3-5]: the studies on Rh-GH can therefore be regarded as a set of “positive control” experiments. By comparing the experimental results obtained with rh-GH to the results of the studies with the two other model proteins it was expected to achieve general conclusions on the mechanism of protein stabilization by CDs. A further aspect of the studies on rh-GH is to confirm literature observations for pharmaceutically relevant stress conditions, as the majority of the aggregation experiments on rh-GH described in literature so far were carried out under pharmaceutically not relevant stress conditions, e.g. at pH 2.5 in presence of high amounts of salt[4].

In order to keep the results clearly laid-out and comparable, the two additional model-proteins discussed in this chapter were subjected to the same set of stressing conditions as the mAb. Agitation experiments in centrifugal tubes were conducted to evaluate the effect of the CDs on aggregation induced by the presence of an air-water-interface. Freeze-thaw studies were carried out to evaluate the stabilization potential at the air-water interface and against further stress factors occurring during repeated cycles of freezing and thawing (for details the reader is referred to the introduction). Finally the formulations were stored at elevated temperature (50°C) in order to check whether potential inhibitory effects on surface-induced aggregation by CDs are counterbalanced by accelerated aggregation during quiescent storage at elevated temperature and reduced thermal stability or whether, in contrast, stabilization against aggregation at elevated temperature can be observed.

2 RECOMBINANT GRANULOCYTE-COLONY-STIMULATING FACTOR (RH-GCSF)

Few studies have been performed so far investigating the behavior of rh-GCSF when subjected to agitation and an extensive air-water interface. In studies on PEG-GCSF it was found that there is an inverse relationship between concentration of the protein and susceptibility to agitation-induced aggregation[6]. In addition, it was determined that the addition of polysorbate 20 ameliorated the agitation-induced degradation of the protein but abrogated the aggregation that occurred during quiescent storage at 29°C pointing into a similar direction as the observations obtained with the IgG in Chapter 3. Since a later work has found that the aggregation mechanism of PEG-GCSF is very similar to that of rh-GCSF it can be assumed that the findings for PEG-GCSF apply to rh-GCSF in a similar manner[7], thereby again pointing out the limitations of polysorbates and the need for an alternative to this group of stabilizers against surface-induced aggregation. To the best of our knowledge no studies are described in literature regarding the effect of FT-cycles on rh-GCSF aggregation.

2.1 AGITATION-INDUCED AGGREGATION

Proteins are exposed to the air-water interface in a multitude of situations during their lifecycle, for instance during mixing or shipping. As explained in detail in the general introduction, the adsorption of proteins to the air-water interface and the subsequent unfolding open up new reaction pathways, as compared to the bulk solution, that accelerate aggregation processes. Therefore it is important to understand and control aggregation at the air-water interface. Experimentally aggregation of a therapeutic protein at the air-water interface can be mimicked by agitation studies.

In the following agitation studies rh-GCSF was investigated at a concentration of 1.5 mg/ml – a concentration that had previously been used many times in other studies mainly evaluating the aggregation behavior of rh-GCSF under physiological conditions[2,8]. Three different CD-

derivatives were included into the studies: HPβCD and SBEβCD as the two derivatives that are currently used in approved parenteral products[9] and MβCD as a derivative that, besides HPβCD, proved to be efficient for the inhibition of surface-induced aggregation of the IgG as discussed in Chapter 3. The formulations were compared to two formulations containing polysorbate 80 (in a higher and a lower concentration, both above the CMC of polysorbate 80[10-11]) as a typical non-ionic surfactant that is frequently employed in protein formulations for the inhibition of agitation-induced aggregation. From Figure 4.1 it can be seen that agitation at 1100 rpm in vertically oriented centrifugal tubes on a Thermomixer™ decreases the amount of monomer in the reference formulation without any excipients besides buffer to about 86 %. As apparent from the results of a second agitation study on rh-GCSF (Figure 4.2) this loss of monomer is mainly due to the formation of high molecular weight aggregates, since soluble aggregates as determined by HP-SEC make up only 3.4 % of the original amount of protein before the agitation period.

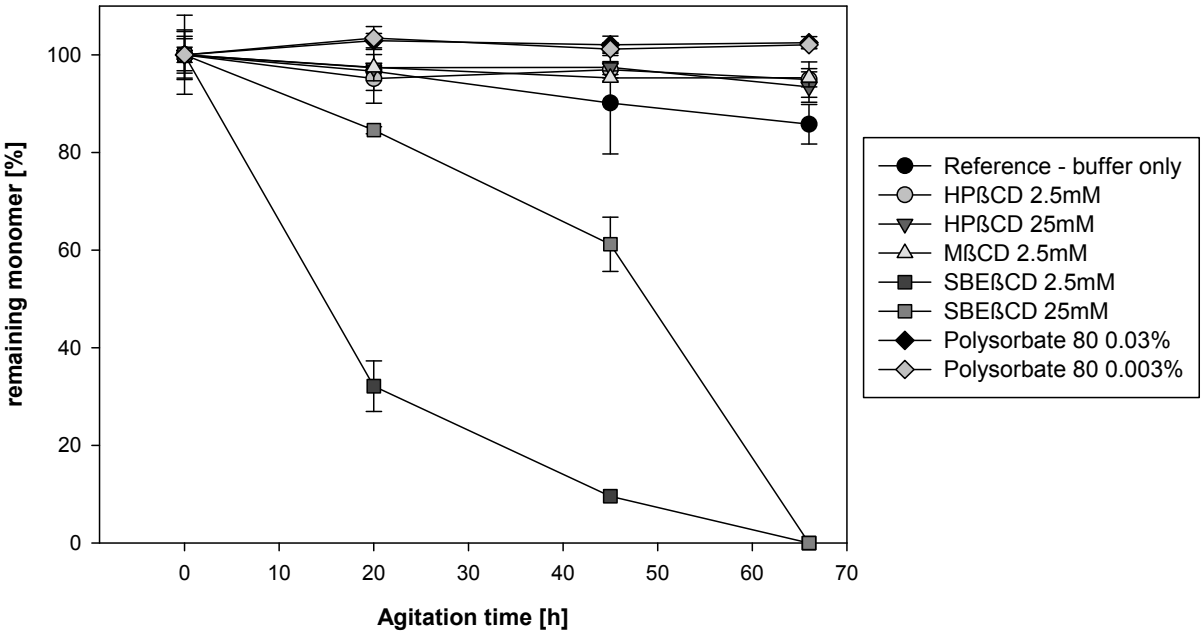

Figure 4.1: Aggregation in different rh-GCSF-formulations throughout an agitation-period of 66 h at 1100 rpm as determined by HP-SEC. All formulations had a pH of 4 and rh-GCSF was present at 1.5 mg/ml. Each value represents the average of three samples ± standard deviation.

Clearly the presence of polysorbate 80 completely suppressed the formation of aggregates throughout the agitation period. Even the low concentration of polysorbate 80 employed in this study (0.003 %, still above the CMC of polysorbate 80 at 0.0012 %[11]) was sufficient for a complete protection against agitation-induced aggregation. HPβCD at both concentrations as well as MβCD at a concentration of 2.5 mM also stabilized rh-GCSF against aggregation at the air-water-interface, however to a slightly smaller degree than polysorbate 80 with approximately 95 % of remaining monomer at the end of the agitation period. As with the studies on the mAb, this is the first published demonstration that HPβCD and MβCD stabilize

rh-GCSF against aggregation. Again, it is remarkable, that comparably low concentrations of the CDs (2.5 mM which equals about 0.4% w/w) are sufficient to reach the maximally stabilizing potential. In contrast, the ionic CD-derivative included in the study, SBEβCD, failed to stabilize rh-GCSF against aggregation at the air-water interface and even deteriorated the situation compared to the reference without excipients: after 66 h of agitating in the presence of 2.5 mM as well as 25 mM SBEβCD all rh-GCSF monomer is converted into aggregates. This increased susceptibility to agitation-induced aggregation in the presence of SBEβCD was also observed for the mAb.

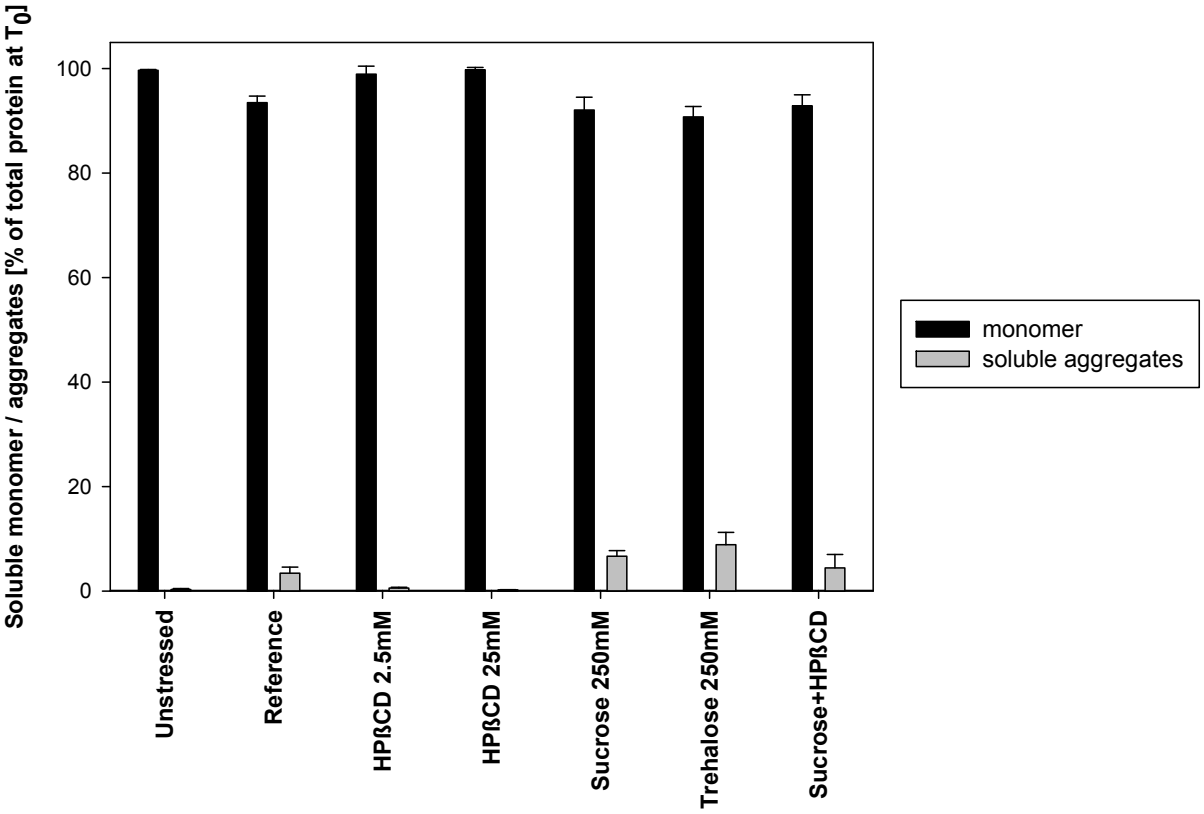

Figure 4.2: Comparison of the level of rh-GCSF-aggregation after 72 h of agitation at 1100 rpm in formulations containing HPβCD to formulations containing sucrose, trehalose and a combination of sucrose 250 mM and HPβCD 2.5 mM. Rh-GCSF concentration was 1.5 mg/ml and the formulations were buffered in acetate at pH 4. Each bar represents the mean of three samples ± standard deviation.

In a second agitation study, the stabilizing potential of CDs was compared to the effects of low molecular weight sugars representing state of the art excipients for protein formulation[12]. As shown in Figure 4.2, in contrast to HPβCD and MβCD for nearly complete stabilization against agitation-induced aggregation was observed, the presence of 250 mM sucrose or trehalose has no beneficial effect on rh-GCSF stability. The presence of these low-molecular weight sugars even accelerates aggregation at the air-water interface compared to the reference formulation containing no excipients besides buffer mainly by the formation of soluble aggregates. This result is quite in contrast to earlier works observing a clear

thermodynamic stabilization and consequently a reduction of aggregation of rh-GCSF in the presence of sucrose[2,8]. In these studies the role of sucrose was analyzed and it was found out that sucrose reduces the population of structurally expanded rh-GCSF molecules by preferential exclusion thereby slowing the aggregation reaction. Obviously the postulated mechanism of stabilization that governs rh-GCSF stability in solution, does not apply to the protein's susceptibility towards surface-induced aggregation. Possibly the presence of an extensive air-water-interface gives way to further aggregation pathways[13]. It can be argued on a theoretical basis that the presence of preferentially excluded excipients, such as sucrose decreases the free energy of unfolding of a protein adsorbed to the air-water-interface. This might explain the negative effects on protein stability observed for sucrose and trehalose.[13]. This assumption is substantiated by the fact that also the mAb – although in terms of size and structure very different form rh-GCSF – showed increased aggregation rates during agitation in the presence of preferentially excluded excipients. From the comparison of the effect of CDs and LMW-sugars it can be concluded that CDs although chemically also classified as sugars act completely different from the disaccharides employed for this study. In contrast to the IgG, the acceleration of aggregation in the presence of sucrose could only be partially counteracted by the addition of HPβCD to the sucrose formulation, as demonstrated in Figure 4.2.

2.2 FREEZE-THAW STUDIES

Freeze-thawing stress poses a major challenge to maintaining protein stability since freeze-thawing stress may occur at multiple stages, intentionally and also unintentionally, during manufacturing, processing and storing of therapeutic proteins and since it may cause significant damage to the protein thereby potentially risking patient safety. So far no studies are available examining the prevention of freeze-thawing-induced aggregation of rh-GCSF by the addition of suitable excipients. From studies on other proteins it is known that polysorbates are useful excipients for minimizing the level of protein aggregation during acute freeze-thaw studies, such as published for recombinant hemoglobin[14]. However, the very same study also denotes that during quiescent long-term storage of recombinant hemoglobin the addition of polysorbate 80 induced aggregation. Thus, once again, the need for excipients stabilizing against surface-induced aggregation, e.g. during freeze-thaw-studies, while not hampering long-term storage stability, is apparent.

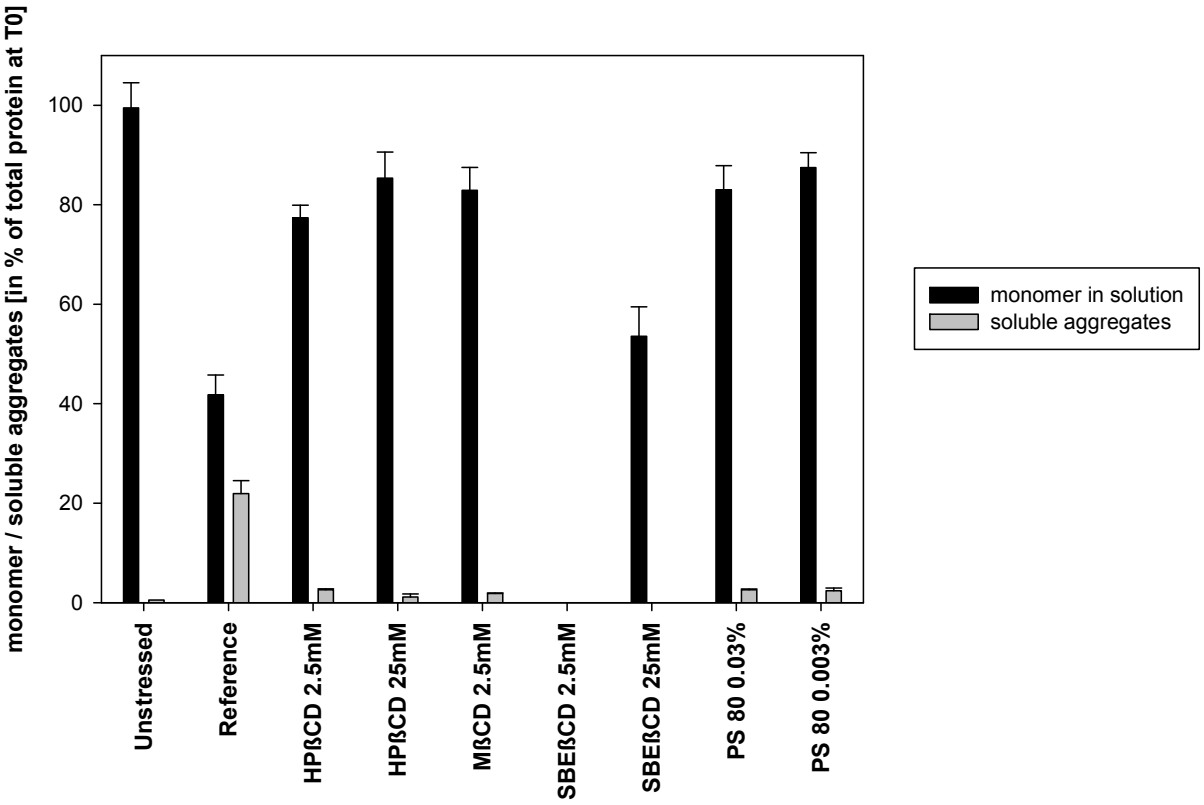

Figure 4.3: Remaining monomer and soluble aggregates in different rh-GCSF formulations after 15 F/T-cycles as determined by HP-SEC. Each bar represents the mean of three samples ± standard deviation.

The potential of HPβCD, MβCD and SBEβCD for the inhibition of freeze-thaw induced aggregation of rh–GCSF is demonstrated in Figure 4.3. After the 15 F/T-cycles the amount of remaining rh-GCSF monomer in the reference formulation without stabilizing excipients decreased to approximately 40 % of the original content and a significant amount of soluble aggregates was formed (22 %). In contrast the presence of HPβCD and MβCD F/T-induced aggregation was strongly reduced: the lower concentration of HPβCD (2.5 mM) was able to preserve 77 % of the original amount of monomer whereas the higher HPβCD-concentration led to a preservation of 85 % of the original amount of protein. In contrast to observations on the mAb, in this experiment an HPβCD concentration of 2.5 mM was not yet sufficient for achieving the maximally stabilizing potential of HPβCD since the higher concentration better protected rh-GCSF from aggregation. MβCD prevented rh-GCSF from surface-induced aggregation already at a lower concentration than HPβCD. In contrast SBEβCD (Captisol^{TM}) at its lower concentration even accelerated rh-GCSF-aggregation throughout the experiment very much in line with the results obtained for the mAb. After 15 F/T-cycles no remaining monomer, not even soluble aggregates, could be determined any more by HP-SEC. However, the higher concentration of SBEβCD (25 mM) led to a slight stabilization of the protein, as compared to the reference formulation, with 54 % remaining monomer after the F/T-cycles. As expected also polysorbate 80 suppressed rh-GCSF aggregation. The extent of the stabilization was comparable to that achieved by HPβCD and MβCD. It has to be

noted that all stabilizing excipients almost completely inhibited the formation of soluble aggregates. In addition, as evident from Figure 4.4 the addition of HPβCD, MβCD or polysorbate 80 led to a complete aggregation-suppression for up to 10 F/T-cycles and only after more than 10 cycles aggregation could be observed in these formulations. Since 15 F/T-cycles represent rather harsh stressing conditions that are unlikely to occur to that extent during the processing and storage of biopharmaceuticals, it can be assumed that by the addition of CDs complete stabilization against F/T-induced aggregation is afforded. Except for the results on the formulation containing 25 mM SBEβCD all these results are qualitatively in agreement with results obtained for F/T-studies on the mAb at pH 7.4, confirming the general effectiveness of CDs for the stabilization against surface-induced aggregation of structurally different proteins.

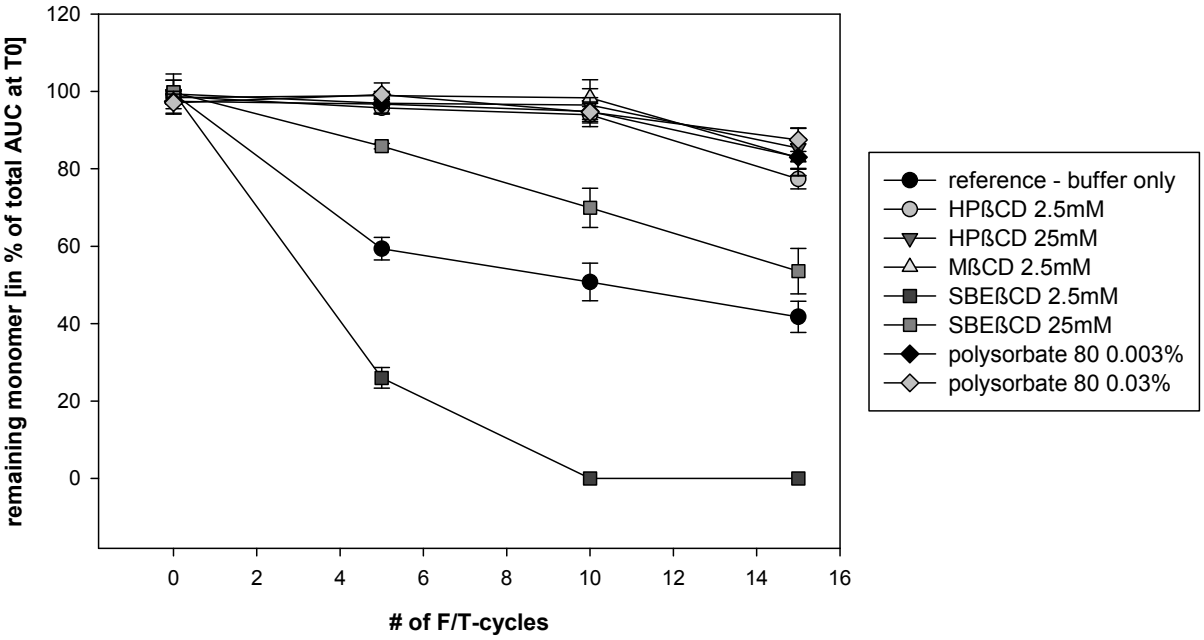

Figure 4.4: Loss of rh-GCSF monomer in different formulations throughout 15 F/T-cycles as determined by HP-SEC. Each value represents the mean of three samples ± standard deviation.

In summary it is demonstrated here that HPβCD and MβCD are very useful excipients for the stabilization of rh-GCSF since they are capable of inhibiting agitation- as well as F/T-induced aggregation. As with the earlier studies on the mAb an incubation at elevated temperature study was conducted in order to check whether the beneficial effects against surface-induced aggregation are counterbalanced by a decreased storage-stability that is widely described in literature for polysorbates[14-15] and which could also be expected when assuming that CDs indeed preferentially bind to the partially unfolded state of rh-GCSF, thereby reducing the thermal stability of the protein in solution.

2.3 INCUBATION AT 50°C

Generally, the factors controlling rh-GCSF stability in solution are well understood and extensively published[1-2,16]. In brief, rh-GCSF aggregates rapidly at neutral pH in the liquid state, but can be stored without any signs of aggregation for up to two years at 2-8°C at acidic pH (e.g. pH 3.5). Interestingly the protein's free energy of unfolding and its conformation do not significantly differ at the two solution conditions. The reason for the different susceptibility to aggregation can be found in the colloidal stability of rh-GCSF: at low pH repulsive forces dominate protein interaction whereas at neutral pH intermolecular attraction forces of rh-GCSF molecules foster aggregation. Since the first step of aggregation of rh-GCSF involves a structurally expanded species, sucrose as a preferentially excluded thermodynamic stabilizer is capable of inhibiting rh-GCSF aggregation[1]. Little data has been published so far on the aggregation behavior of rh-GCSF at elevated temperature. The above cited studies were carried out under "physiological conditions" (37°C in the presence of salt). For PEG-GCSF studies at 45°C are available in which a direct concentration-dependence of aggregation was determined[6]. The following study was carried out at pH 4 corresponding to the pH of the formulation of most commercial rh-GCSF formulations. Incubation was performed at 50°C. At pH 4.0 colloidal stability is high and the rate-limiting step in the aggregation process is initial dimer formation prior to the formation of higher order aggregates.[1]

2.3.1 AGGREGATION

In Figure 4.5 A and B the levels of monomeric rh-GCSF remaining in solution throughout an incubation study at 50°C for 230h are shown. In part A of the figure the reference formulation containing rh-GCSF in acetate buffer is compared to formulations containing three different CD-derivatives in two concentrations, respectively. Rh-GCSF stored without stabilizing excipients showed a continuous decrease of remaining monomer content, reaching 60 % after 230 h of incubation. It becomes apparent that SBEβCD at both concentrations led to a very rapid loss of monomeric protein: within one day of incubation all monomeric protein was lost. No soluble aggregates could be determined in the SBEβCD-formulations (data not shown) implying that only insoluble aggregates were performed. Also MβCD at the 25 mM concentration accelerated aggregation compared to the reference. However, rh-GCSF aggregation was not as drastic as in the SBEβCD formulations. The monomer content decreased almost linearly over time reaching about 15 % after 230 h of incubation. The two HPβCD-formulations and the lower concentrated MβCD-formulation showed almost no difference to the reference-formulation. However, at the end of the incubation period a slight stabilization could be observed, but the observed difference was small and should therefore not be over interpreted. Thus in conclusion HPβCD had no effect on the aggregation behavior of rh-GCSF at elevated temperature whereas MβCD (in its high concentration)

accelerated aggregation, confirming conclusion from the investigations on the IgG. Taking into consideration the results of the agitation study and the F/T-study, HPβCD is again identified as the most promising CD-derivative tested, since it inhibits interfacial aggregation while not accelerating aggregation during storage at elevated temperature.

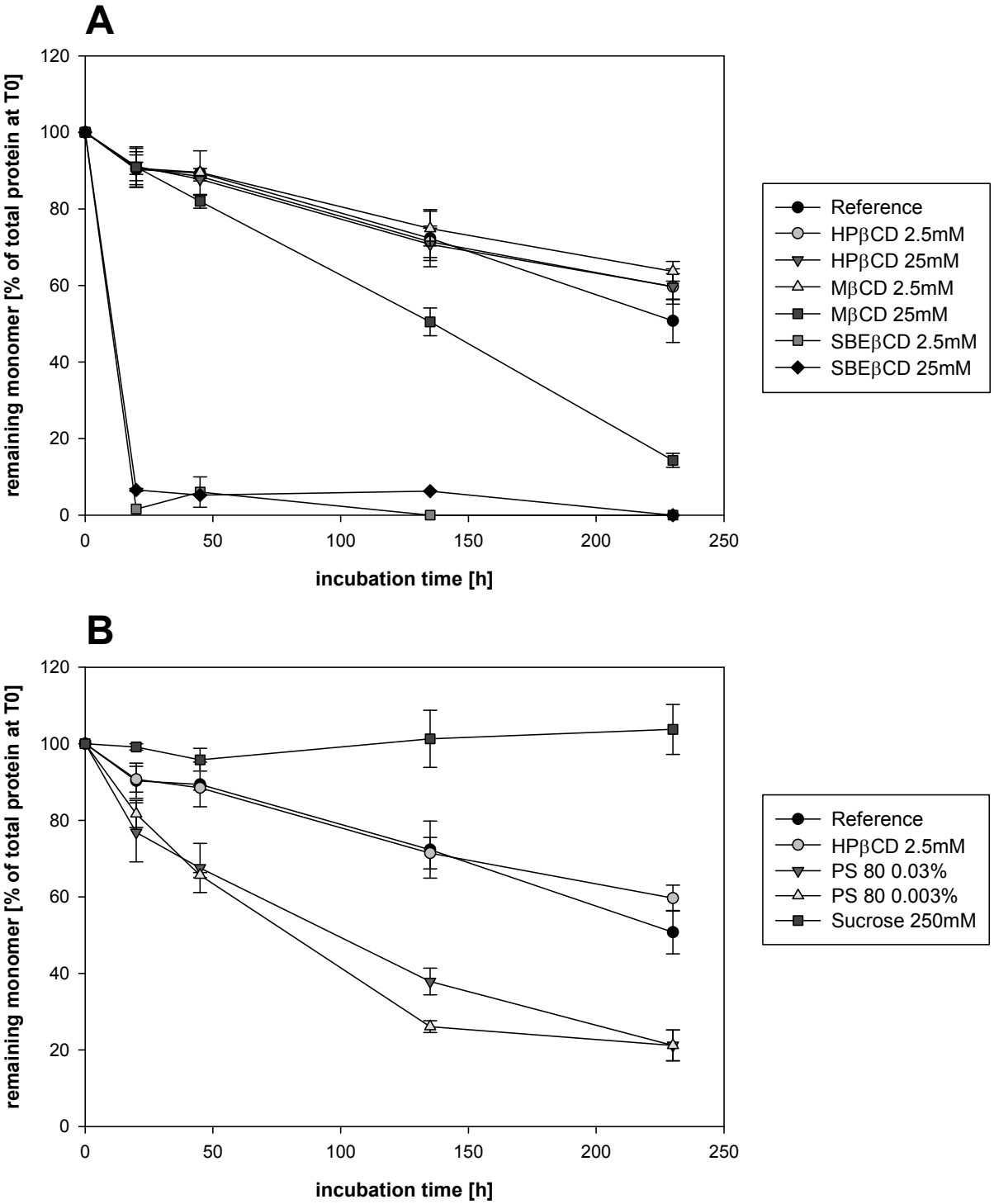

Figure 4.5: Loss of rh-GCSF-monomer throughout an incubation period of 230 h at 50°C as determined by HP-SEC. Rh-GCSF was formulated at 1.5 mg/ml at a pH of 4.0. Each value represents the mean of three values ± standard deviation. In part (A) the comparison of the reference formulation to CD-formulations is depicted, and in part (B) the comparison of the reference formulation to polysorbate 80 and sucrose is shown.

Figure 4.5 B depicts a comparison of the reference formulation without stabilizers to rh-GCSF formulations containing either polysorbate 80 at two concentrations, sucrose or HPβCD. The sucrose formulation did not exhibit any aggregation throughout the storage period whereas the two formulations containing polysorbate 80 demonstrated significantly increased aggregation rates with only slightly more than 20 % remaining monomer after storage. Interestingly, polysorbate 80 concentration had almost no influence on the extent of aggregation. Again, the observations and conclusions from the investigation of the IgG were qualitatively confirmed. The positive effects of polysorbate 80 against surface-induced aggregation are counterbalanced by increased aggregation rates during quiescent storage whereas sucrose as a preferentially excluded excipient increases rh-GCSF stability during storage but leads to increased amounts of aggregates during agitation. HPβCD has no effect on aggregation after storage at 50°C and therefore its stabilizing effect against surface-induced aggregation is not counterbalanced by reduced storage stability.

2.3.2 CONFORMATIONAL STABILITY AND MICROCALORIMETRIC DATA

Since all tested formulations were of low pH and low ionic strength (besides those containing ionic SBEβCD) colloidal stability is expected to be high. Thus, differences in conformational stability should be governing the different aggregation propensity of rh-GCSF in the various formulations observed during storage at 50°C. In order to verify this hypothesis the conformational stability of rh-GCSF in solution after the 230 h incubation period was assessed using FTIR. In addition, the thermodynamic stability of selected formulations was determined by microcalorimetry prior to storage, in order to elucidate whether CDs, as suggested by literature, lead to a shift of the folding equilibrium towards more unfolded forms due to the CDs' preferential binding to unfolded states of the protein under investigation.

In Figure 4.6 the FTIR second derivative spectra of rh-GCSF prior to and after 230 h storage at 50°C of four selected formulations are compared. Since the IR-transmission cell used for the recording of these spectra requires particle-free solutions all formulations were centrifuged after storage and only the remaining supernatant was analyzed. Most of the stored solutions showed only minor amounts of soluble aggregates after storage (< 3 %) except for the polysorbate 80 0.03 %-samples (16.3 %) and the MβCD 25 mM-samples (11.4 %) which means that the IR-spectra only partially reflect perturbed secondary structure in aggregates but for the most part actually represent the more or less structurally perturbed rh-GCSF monomer after storage.

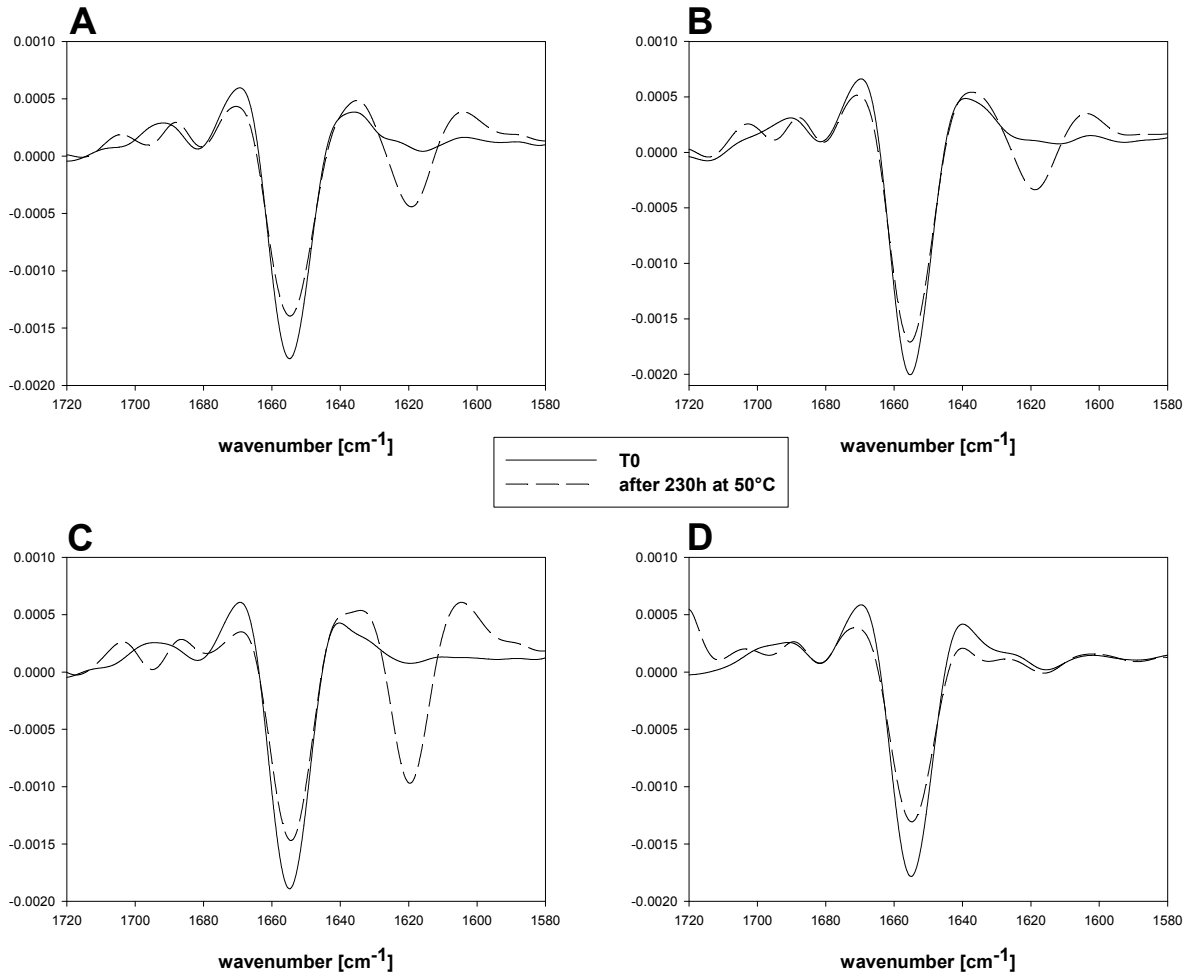

Figure 4.6: Area normalized second-derivative IR-transmission spectra of selected rh-GCSF formulations after storage for 230 h at 50°C (n=2) comprising the reference formulation (A) and formulations containing HPßCD 2.5 mM (B), polysorbate 80 0.03 % (C) and sucrose 250 mM (D).

In Figure 4.6 A, B and C it can be seen that after storage the formation of a new band at 1620 cm^{-1} occurred which is attributed to intermolecular β-sheet-formation[2,8,17]. Concomitantly, the α-helical band at 1656 cm^{-1} decreased in its intensity indicating the loss of native secondary structure. The reference formulation (A) showed a very comparable degree of secondary structural disruption as the HPβCD 2.5 mM formulation (B) coinciding with the observation of comparable aggregate levels in these formulations. In contrast, the polysorbate 80-formulation exhibited a distinctly stronger β-sheet. The formation of the β-sheet band was not observed for the sucrose formulation as evidenced by Figure 4.6 D. Figure 4.6 is complemented by Table 4.1 in which the change in the ratio of the second derivative peak intensities at 1620 cm^{-1} and at 1656 cm^{-1} is listed for all the formulations tested in this investigation. The ratios confirmed the visually observed trends from the spectra: the strongest changes in secondary structure occurred in the polysorbate 80-formulation and in the MβCD 25 mM formulation (spectra not shown) whereas HPβCD showed a loss of secondary structure that is even slightly smaller than that of the reference.

The disaccharides sucrose and trehalose completely preserved the native secondary structure.

Table 4.1: Change in ratio of IR second derivative peak intensities at 1620 cm^{-1}/1656 cm^{-1} before and after storage at 50°C in various rh-GCSF formulations. All values are obtained from the average spectra of two independent samples.

Formulation	1620 cm^{-1}/1656 cm^{-1}-ratio before incubation	1620 cm^{-1}/1656 cm^{-1}-ratio after incubation
Reference	-0.06	0.30
HPβCD 2.5 mM	-0.06	0.17
HPβCD 25 mM	-0.05	0.29
MβCD 2.5mM	-0.07	0.30
MβCD 25 mM	-0.04	1.03
SBEβCD 2.5 mM	-0.10	n/a
SBEβCD 25 mM	-0.04	n/a
Polysorbate 80 0.003 %	-0.04	0.39
Polysorbate 80 0.03 %	-0.05	0.65
Sucrose 0.5 M	-0.05	-0.03
Trehalose 0.5 M	-0.09	0.00

In conclusion it can be stated that the addition of HPβCD had no significant effect on the secondary structure of rh-GCSF which is again in agreement with the results obtained for the mAb. As also widely described in literature, the addition of preferentially excluded excipients to solutions of rh-GCSF, such as sucrose and trehalose, favored a more compact conformation of the protein which led to a very minor loss of secondary structure and no aggregation as determined by HP-SEC. On the other hand, surface-active polysorbate 80 caused a major loss of secondary structure which is in agreement with accelerated aggregation as determined by HP-SEC. In earlier works on rh-GCSF aggregation under physiological conditions it was speculated that the transition to β-sheet is a result of aggregate formation and not due to the monomeric protein assuming a β-sheet "template" conformation prior to aggregation[2]. Our results on rh-GCSF aggregation at 50°C point into a different direction when keeping in mind that only the supernatant with very little amounts of soluble aggregates (e.g. in the reference formulation) also showed a prominent β-sheet band at 1620 cm^{-1}. Thus it seems likely that a secondary structural transition of the monomeric protein occurs prior to aggregation.

In addition to IR-experiments for the detection of secondary structural changes, second-derivative UV absorbance spectroscopy was applied for the detection of tertiary

conformational changes. The method exploits changes in the polarity of microenvironments of the aromatic amino acids Tyr and Trp of which rh-GCSF contains three and two per molecule, respectively[2]. Therefore both, conformational changes exposing the amino acids to the solvent to varying degrees and the polarity of the solvent itself potentially influence the protein spectra[2,18]. Since for example the addition of 0.9 % benzyl alcohol resulted in a change in peak positions of the model compounds N-acetyl-tyrosinamide and N-acetyl-tryptophanamide, it has to be assumed that the addition of much higher quantities of sugars and cyclodextrins in our studies must also result in changes of the solvent polarity[8]. Therefore a direct comparison of the different formulations and an evaluation of the effect of the added excipients on the tertiary structure of rh-GCSF only, was not possible, because the signal would always be also influenced by the excipients' influence on solvent polarity. However, a comparison of the tertiary structure of rh-GCSF before and after incubation at 50°C and hence monitoring the effect of storage on each formulation was possible after subtraction of the appropriate background spectra.

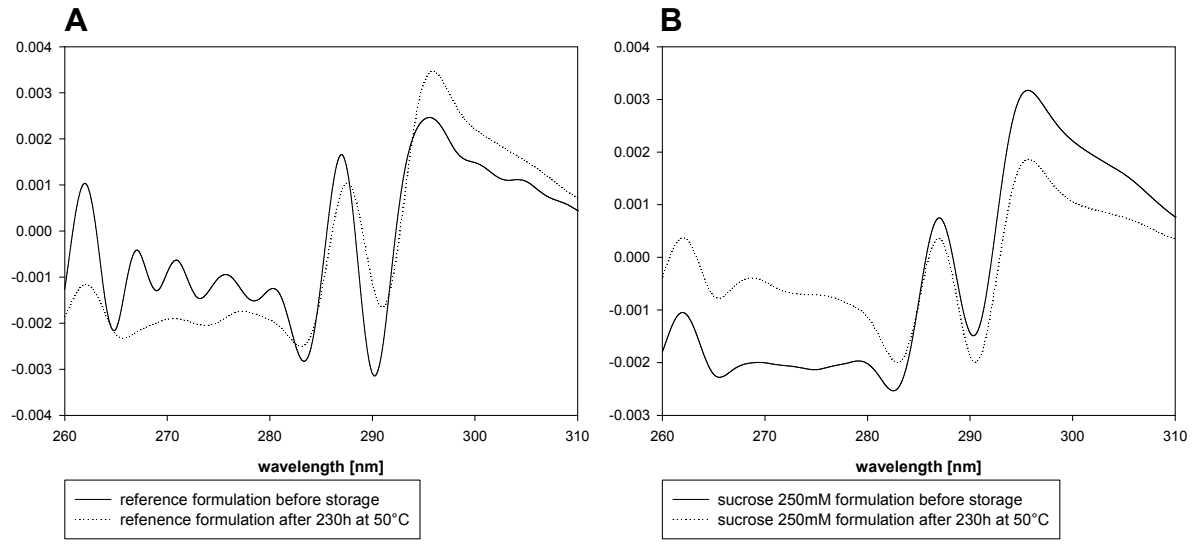

Figure 4.7: Representative UV-spectra: second derivative spectra of rh-GCSF samples before and after incubation at 50°C for 230 h of the reference-formulation without excipients besides buffer (A) and the formulation containing 250 mM sucrose (B).

The second-derivative UV-spectra of two representative formulations are shown in Figure 4.7. A red shift of the Trp-band relative to the spectrum before storage could be observed in both formulations indicating that tryptophan in the stored protein solution was in a more hydrophobic environment than before storage[19]. This result was somehow surprising, since one would anticipate higher solvent exposure due to partial unfolding upon thermal stress. A look at Table 4.2 and into literature offers an explanation for this behavior. Krishnan et al. recorded the spectra of isolated dimer-fractions (containing 0.12 mg/ml dimer) of rh-GCSF after separation by HP-SEC and also found a clear red-shift of the Trp-band and the Tyr-band[2]. This means that in the dimers Trp and Tyr are less solvent-exposed than in the rh-GCSF monomer. In the present study a similar trend was observed: Trp was most strongly

red-shifted in those formulations containing the highest amount of dimer (MβCD 25 mM, polysorbate 80 % and SBEβCD 2.5 mM) which could interpreted in a way that the red shift recorded after storage reflected dimer formation and rather not unfolding on a tertiary structural level prior to the formation of insoluble aggregates.

Most importantly it has to be concluded that the presence of HPβCD in both concentrations does not affect tertiary structure more strongly than the reference formulation. Therefore, no shift towards a more unfolded conformation of rh-GCSF could be observed in the presence of HPβCD which can also be taken as a further hint that no interaction between rh-GCSF and HPβCD takes place in solution.

Table 4.2: Tertiary structural characterization of rh-GCSF before and after storage at 50°C for 230 h by second-derivative UV-spectroscopy. Peak positions of the 290 nm-Trp-peak and the differences before and after storage are listed (n=2).

Formulation	Trp-position before incubation [nm]	Trp-position after incubation [nm]	Difference [nm]
Reference	290.21	290.94	0.73
HPβCD 2.5 mM	290.44	291.10	0.66
HPβCD 25 mM	290.44	290.90	0.46
MβCD 2.5 mM	290.42	290.94	0.52
MβCD 25 mM	290.30	291.56	1.26
SBEβCD 2.5 mM	291.56	293.00	1.44
PS 80 0.003 %	290.47	291.46	0.99
PS 80 0.03 %	290.14	291.15	1.01
Sucrose 0.5 M	290.32	290.50	0.18
Trehalose 0.5 M	290.34	291.05	0.71

Since it is reported that the reduction of agitation-induced aggregation by non-ionic surfactants can be due to an increase in the thermodynamic stability of a protein[20] and to find out whether this behavior might contribute to the stabilization of rh-GCSF by CDs or whether thermodynamic stability is rather unaffected or even decreased by the addition of CDs (potential shift of the folding equilibrium towards a more unfolded form due to preferential binding to the unfolded state[21]), the apparent melting temperature of selected rh-GCSF formulations was measured in presence and absence of CDs (Table 4.3).

The apparent melting temperature of rh-GCSF as determined by microcalorimetry in buffer alone was nearly identical to that for the protein in the presence of HPβCD. Thus HPβCD has no measurable effect on the thermodynamic stability of rh-GCSF. This finding leads to two consequences: it can be concluded that a change in thermodynamic stability is not an

explanation for the inhibition of surface-induced aggregation of rh-GCSF in the presence of HPβCD. Secondly, this result can also be interpreted as an indication that no binding between CDs and rh-GCSF occurs because a preferential binding of CDs to the partially unfolded state of the protein would have resulted in a subsequent shift of the folding equilibrium associated with a decrease in T_m[21].

Interestingly, the apparent T_m was decreased by about 2°C in the presence of MβCD which also stabilized against aggregation at the air-water interface but which at higher concentrations negatively affected storage stability at 50°C. This behavior could be due to two reasons: either direct binding in solution occurs between MβCD and rh-GCSF thereby shifting the folding equilibrium of rh-GCSF, or this CD-derivate is more surface-active than HPβCD leading to the inherent thermodynamically destabilizing effects of surface-active excipients on proteins in the bulk solution in the same way as discussed in the introduction for polysorbates[13,22].

From Table 4.3 it can also be seen that SBEβCD led to a very pronounced decrease of apparent T_m by more than 10°C. This finding was very much in agreement with the results of the stress studies in which SBEβCD proved to be detrimental to rh-GCSF stability during both, agitation and quiescent storage. Apparently the strong decrease of conformational stability of rh-GCSF explains the undesired effects of SBEβCD. In addition, an effect on colloidal stability, due to the shielding of the repulsive forces between the rh-GCSF-molecules at pH 4 by the ionic excipient might play a role.

It has to be noted here that thermal unfolding of all samples was irreversible due to the formation of aggregates during heating – therefore the term "apparent T_m" was used.

Table 4.3: Apparent melting temperatures of different rh-GCSF-CD formulations as determined by microcalorimetry. Rh-GCSF was formulated at 0.5 mg/ml and pH 4 and experiments were carried out once. A high degree of reproducibility of the results (SD < 0.1°C) was demonstrated in a separate experiment (data not shown).

Formulation	Apparent T_m [°C]
Reference	64.89
HPβCD 10 mM	64.83
MβCD 10 mM	62.68
SBEβCD 10 mM	53.85

In summary, when using polysorbate 80 and sugars a compromise has to be struck in the formulation of rh-GCSF: stabilization at the interface by polysorbate 80 is very well possible as evidenced by complete stabilization against agitation-induced aggregation and good stabilization against F/T-induced stress. However, the use of the strongly surface-active

polysorbate is accompanied by increased aggregation of rh-GCSF during quiescent storage in solution. This is very likely an inherent problem of surface-active excipients as discussed in the introduction and also in Chapter 6. The opposite phenomenon can be observed when using sugars for rh-GCSF formulation: increased aggregation after agitation and complete stabilization in the bulk as evidenced by the data presented in this chapter.

Our results demonstrate that a possible solution to this predicament could be provided by the usage of HPβCD. The excipient leads to a clear stabilization against surface-induced aggregation (comparable to that of polysorbate 80) while leaving storage stability in solution and thermodynamic stability of rh-GCSF unaltered.

These results also allow some speculations on the mechanism of rh-GCSF stabilization by HPβCD. The fact that HPβCD did not lead to a destabilization in the bulk and that also T_m was left unaltered by the addition of HPβCD render direct binding in the bulk solution unlikely. Furthermore it can be speculated that HPβCD is not strongly surface-active, since high concentrations of the surface active MβCD and also of the surface-active polysorbate 80 do lead to a destabilization in the bulk. It appears that additional mechanisms of stabilization of rh-GCSF by HPβCD against surface-induced aggregation could play a role. These mechanistic questions will be addressed in detail in the following chapters.

2.4 Effect of HPβCD on rh-GCSF aggregation under physiological conditions

To complement the typical stress conditions chosen for accelerated stability testing, harsh agitation conditions, extensive freeze-thaw cycles and storage at elevated temperature, a study investigating the influence of HPβCD on rh-GCSF-aggregation under physiological conditions was conducted. The study was based on a number of reports in literature that rh-GCSF readily aggregates under physiological conditions (pH 7, 10 mM phosphate buffer and 150 mM NaCl, 37°C) within a couple of days[1-2].

It was also reported that under these conditions rh-GCSF exists in equilibrium with a partially unfolded conformation with increased solvent-exposure of Trp 58 that has an increased propensity to aggregate[23]. We therefore assumed that by the addition of HPβCD, the incorporation of the solvent-exposed Trp into the CD-cavity at pH 7 could be expected. Thereby shielding of hydrophobic protein interaction and a reduction of the degree of aggregation compared to the reference formulation without HPβCD was anticipated. In order to allow for a clear evaluation of the potential benefit of HPβCD-addition, a sucrose-containing formulation was included as a “benchmark formulation” into the studies.

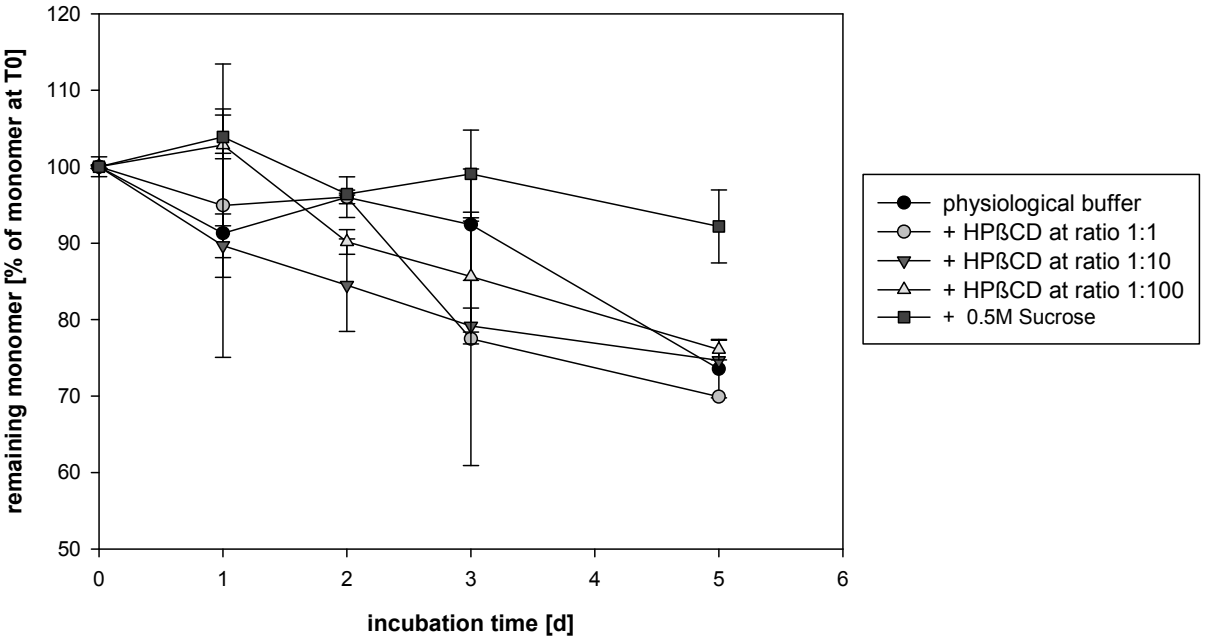

Figure 4.8: Rh-GCSF aggregation under physiological conditions (10 mM sodium phosphate and 150 mM sodium chloride at pH 7.0 and 37°C) in formulations containing different ratios of rh-GCSF : HPβCD as determined by HP-SEC. A reference formulation consisting of rh-GCSF in physiological buffer only and a formulation containing 0.5 M sucrose are also included. Each value is the mean of three samples ± standard deviation.

In Figure 4.8 the loss of monomer over an incubation period of 5 days is shown. It can be seen that in the reference formulation about 25 % of monomer were lost within the incubation period. From reduced recoveries in SE-HPLC the conclusion can be drawn that most of the aggregates formed are of insoluble nature (data not shown). Obviously the addition of HPβCD to the rh-GCSF formulations did not significantly alter the extent of aggregation. In contrast, the formulation containing sucrose was clearly stabilized in good agreement with literature[2]. Hence the anticipated stabilization of rh-GCSF by the addition of HPβCD due to the shielding of hydrophobic protein interaction did not occur. Different explanations could explain this behavior. The degree of rh-GCSF partial unfolding could have been too subtle for the Trp58-residue to be sterically accessible by HPβCD and thus no incorporation into the CD-cavity was possible. Another explanation would be that other mechanisms besides partial exposure of this amino acid govern rh-GCSF aggregation under physiological conditions and therefore CDs are generally not suitable to prevent rh-GCSF-aggregation under physiological conditions.

3 EXPERIMENTS WITH RECOMBINANT HUMAN GROWTH HORMONE (RH-GH)

Having demonstrated in the previous sections that CDs, notably HPβCD, can serve as valuable excipients for the stabilization of rh-GCSF and a mAb, rh-GH was investigated as a third model protein. Although some studies on this matter have already been published[4,24] the reasons to investigate the aggregation-behavior of rh-GH in the presence of CDs within this project were several-fold.

First of all the conditions under which the effect of CDs on rh-GH-aggregation was investigated so far are unlikely to occur during situations typically encountered during manufacturing, processing and storage of the protein. For example Otzen et al. chose acidic conditions (pH 2.5) in the presence of 1M NaCl in order to provide a "convenient assay" for aggregation, exploiting the fact rh-GH populates a partially folded A-state at low pH with native-like secondary structure but loss of tertiary structure leading to a high propensity of rh-GH to aggregate in the presence of NaCl[4]. In another study on the suppression of rh-GH aggregation a molten globule intermediate of rh-GH was artificially created by the presence of 4.5M GdmHCl[5]. Upon dilution and subsequent refolding significant aggregation occurred and the effect of various CD-derivatives on rh-GH aggregation was evaluated[24]. In addition the study also investigated the influence of CDs on vortexing-induced aggregation of rh-GH: by vigorously vortexing rh-GH for only 60s insoluble aggregates were created. Finally, also the effect of CDs on chemical degradation of rh-GH was investigated by subjecting the protein to a 3 % hydrogen peroxide treatment and incubating it at 37°C. All the cited conditions are somehow stressful to the protein but do not necessarily reflect conditions that occur during industrial processing. We therefore conducted agitation studies for the duration of two days mimicking exposure to the air-water-interface as experienced during mixing and shipping processes and also performed freeze-thaw studies simulating either intentional freezing of bulk drug substance or accidental freezing of the drug, e.g. occurring during refrigerated storage.

A second reason to study the aggregation behavior of rh-GH in the presence of CDs was to provide a basis for later follow-up studies that will evaluate whether CDs might be suitable to suppress aggregation-related immunogenicity of rh-GH. Since in an earlier study on rh-GH it was determined that aggregates in commercial formulations of rh-GH were immunogenic in naïve adult and neonatally primed mice, as were aggregates provoked in these formulations by exerting freeze-thawing and agitation stress, it is of particular interest to evaluate if CDs are capable of reducing the amount of aggregates under similar conditions[25]. Large insoluble aggregates as well as smaller soluble oligomers, together very likely acting as adjuvants as well as antigens caused the strongest immune response in the cited study. Therefore a special focus of our studies was laid on these types of aggregates by including asymmetrical field flow fractionation (AF4) as a analytical method into the investigations that is capable of

detecting all these types of aggregates in a single run[25-26]. In the study performed by Fradkin et al. high hydrostatic pressure was used as a means to reduce the aggregate burden in the stressed rh-GH samples which also lead to a reduction of immunogenicity[25]. However, the reduction of aggregation was accompanied by a remarkable increase in the amount of deamidated rh-GH. The addition of CDs in contrast could provide a way to inhibit the formation of aggregates (and therefore immune responses) *a priori* and at the same time not compromising rh-GH chemical stability.

A third reason to study rh-GH was the fact that in contrast to the mAb and to rh-GCSF a number of studies were already available describing the interaction of rh-GH with various CD-derivatives[4,24]. It is known that even in the native state rh-GH exposes a large number of aromatic amino acids thereby offering access to β-CD derivatives for the inclusion of the aromatic residues into the hydrophobic CD-cavity[4]. NMR data have confirmed aromatic amino acids as predominant binding sites for β-CD[4]. Therefore, by comparing the aggregation behavior of rh-GH in the presence of CDs, as a protein that is known to bind to CDs in solution, to the aggregation behavior of the two other model-proteins, it was expected to achieve conclusions on the mechanism of stabilization also of the other two model proteins and to allow for some general conclusions on the mechanism of stabilization.

Finally – since that is one of the overall goals of this thesis – the effects of HPβCD and SBEβCD are directly compared to those of polysorbate 80 which is an excipient that was several times already proven to be effective to prevent surface-induced aggregation of rh-GH[11,14-15,27-28]. However, to date no direct comparisons between the effect of polysorbate 80 and CDs are available in literature[29-30].

3.1 AGITATION STUDY

Initially an agitation study in centrifugal tubes vertically placed on a Thermomixer™ was carried out for 48 h. The reference formulation consisted of a Histidin buffer and Mannitol. The reason for choosing this formulation was that this very formulation was also used for the immunogenicity studies published by Fradkin et al.[25] and since it was intended to study the influence of HPβCD on the formation of potentially immunogenic aggregates as many parameters as possible were kept identical to the published investigations. It can be seen from Figure 4.9 that after an agitation period of 48 h the monomer content of the control sample (without excipients that stabilize against surface-induced aggregation) decreased to about 50 % and distinct amounts of aggregated species were formed. From Figure 4.10 it is conceivable that these aggregates were of insoluble nature as they are not detected as peaks quantifiable by HP-SEC.

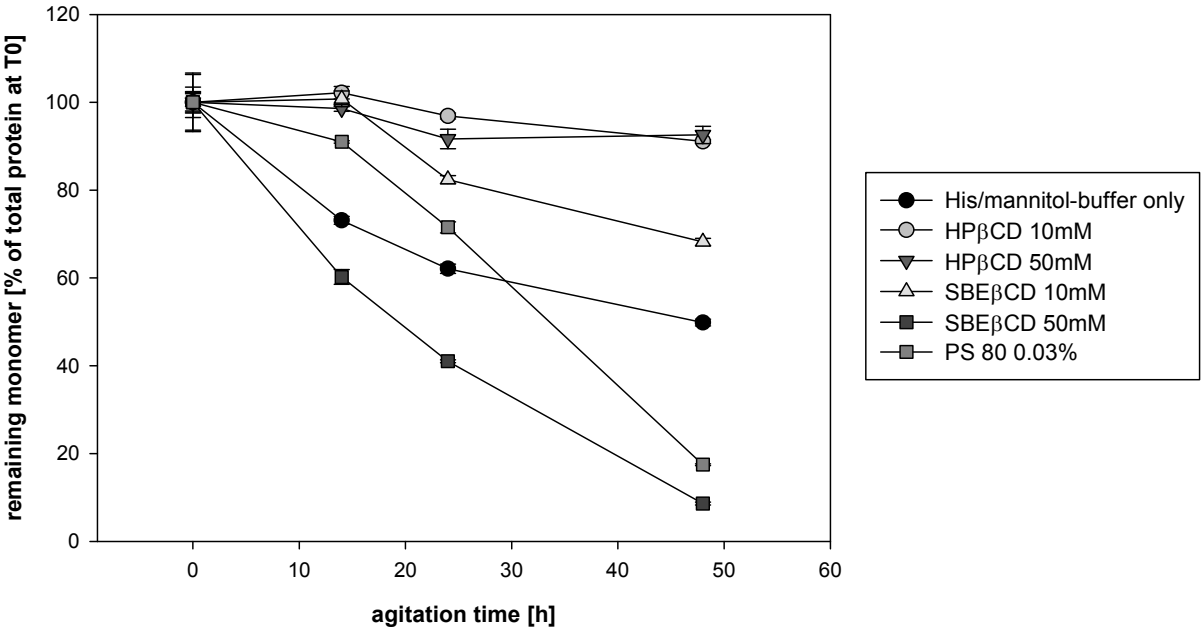

Figure 4.9: Remaining monomer of rh-GH during agitation in centrifugal tubes for a period of 48 h as followed by HP-SEC. All values are the mean of three samples ± standard deviation.

These findings were confirmed by a comparison of fractograms obtained by AF4-analysis (Figure 4.11) in which it can be seen that the reference sample obtained after 24h of agitation showed a clearly reduced monomer content and significant amounts of aggregated species were formed eluting after the monomer peak. In addition the void peak showed a clearly increased intensity indicating increased amounts of aggregate species larger than ~1µm[26,31] or larger than 600-700 nm (according to personal communication with Wyatt Technology Europe GmbH) eluting in the so-called steric hyperlayer mode. These aggregate species were reported to potentially trigger immune reactions by Fradkin et al.[25]. Clearly HPβCD stabilizes rh-GH against aggregation at the air-water-interface since after the agitation-period more than 90 % of the original monomer content can be detected in solution (Figure 4.9). No significant difference with regard to the stabilizing effect can be observed between the 10 mM and the 50 mM concentration of HPβCD. Also these HP-SEC results were confirmed by an AF4-analysis (Figure 4.11) after 24h of agitation. Clearly, the formulation containing HPβCD in its 50 mM-concentration very much resembled in its composition the native sample before the start of the experiment. Also the amount of insoluble aggregates as detected by the steric hyperlayer mode (in the size range that is relevant for immunological considerations[32]) was not increased underlining the potential of HPβCD to prevent immunogenicity of rh-GH formulations *per se*. Interestingly, in its 10 mM concentration also SBEβCD stabilized rh-GH against agitation-induced aggregation in contrast to observations made from studies with the mAb and rh-GCSF. The extent of aggregation-suppression was smaller than that observed by the addition of HPβCD but it was still significant leaving about 68 % of monomer in solution after the 24 h incubation period. In contrast, the high concentration of SBEβCD (50 mM) accelerated aggregation leaving only

about 9 % remaining monomer in solution after 48h. Therefore SBEβCD both stabilizes and destabilizes rh-GH in a concentration-dependent manner. Polysorbate 80 showed an ambiguous behavior. Within the first 24h of agitation it exhibited a clear reduction of aggregation when compared to the reference sample. However, after 48h the extent of aggregation dramatically increased and the formulation turned then out to be less stable against aggregation at the air-water interface than the reference formulation. Thus depending on the length of the experiment, polysorbate 80 accelerates or decelerates aggregation of rh-GH. From Figure 4.10 it can be seen that in contrast to all other formulations the presence of polysorbate 80 led to massively increasing amounts of soluble aggregates over time.

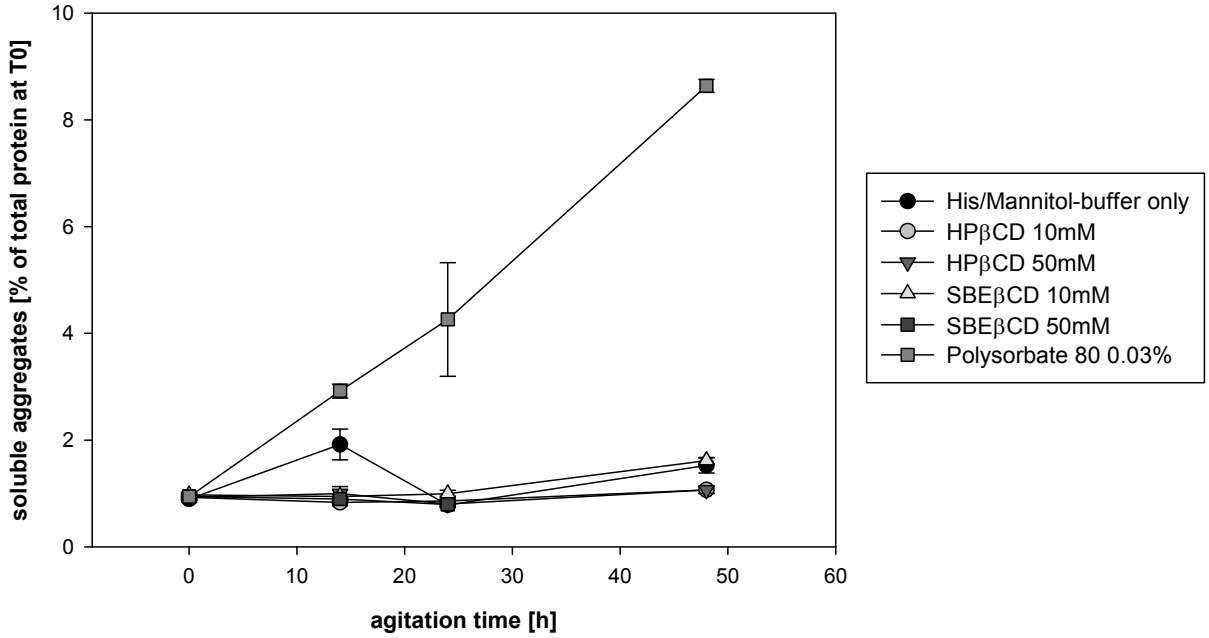

Figure 4.10: Formation of soluble aggregates of rh-GH throughout an agitation period of 48 h as followed by HP-SEC. All values are the mean of three samples ± standard deviation.

The observation that HPβCD better stabilized rh-GH against agitation-induced aggregation than other CD-derivatives is in accordance with an earlier study that investigated the effect of different CD-derivatives on rh-GH aggregation after vigorous vortexing for 60s[24]. Also the amount of HPβCD necessary to achieve the maximally stabilizing effect (10 mM) agrees with our results. The authors of the earlier work on the suppression of rh-GH-aggregation speculate that the effectiveness of HPβCD to prevent interfacial aggregation is due to its surface-activity and that HPβCD acts in a manner similar to that of surfactants[24]. In support of their theory, a linear correlation of the surface-tension of differently substituted HPβCD-species and the amount of aggregates is shown. However, the surface tension values presented for HPβCD (about 52 mN/m for the highest degree of substitution) are much higher than those published for polysorbate 80 (about 40 mN/m)[10]. Therefore polysorbate 80 has to be considered a lot more surface-active than HPβCD and nevertheless in the present study less effective rh-GH stabilization against aggregation at the air-water interface than by

HPβCD was observed. Based on our results we therefore rather suggest that the pure presence of HPβCD at the interface along with its capability to form inclusion complexes with exposed hydrophobic residues on the rh-GH-molecule[4] explain the stabilization of rh-GH – and not competitive displacement from the interface. A certain surface-activity of HPβCD presumably helps to "scavenge" partially unfolded rh-GH molecules directly at the surface and therefore renders HPβCD more efficient than other CD-derivatives in the prevention of agitation-induced aggregation. However, surface-activity alone and a resulting decrease of the concentration of rh-GH at the interface due to competition at the interface can not explain the observed stabilization.

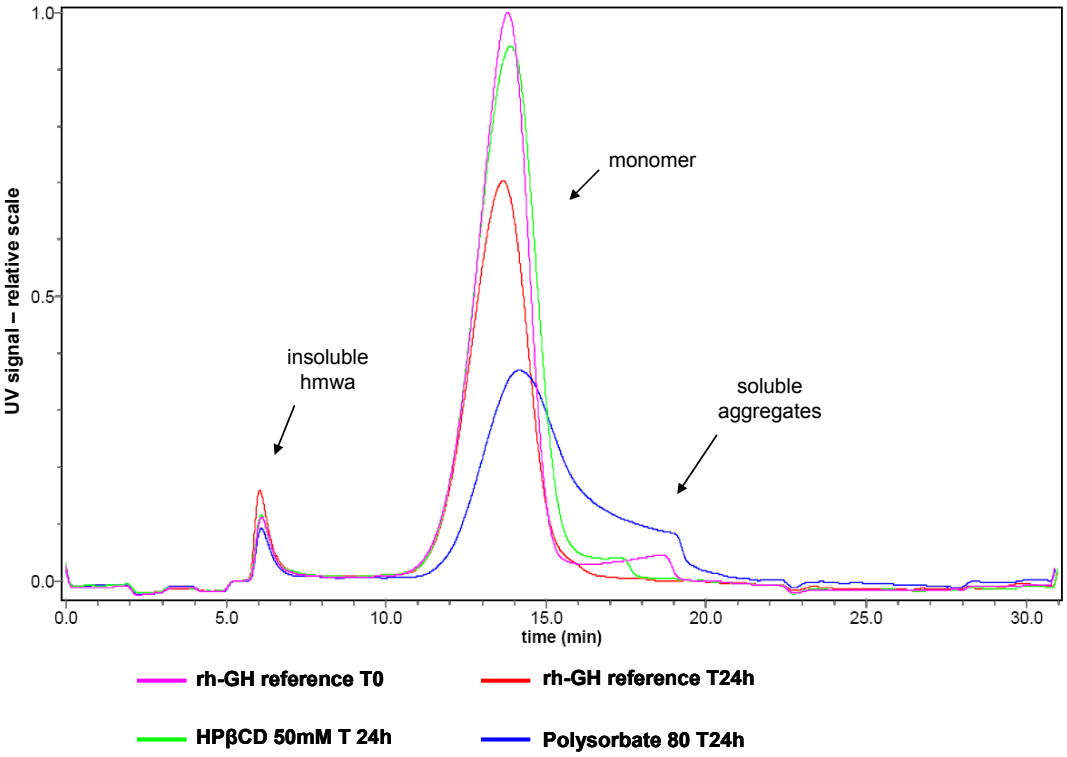

Figure 4.11: Representative AF4-fractograms of different rh-GH formulations after 24h of agitation by asymmetric field-flow fractionation.

This assumption is supported by the fact that also for SBEβCD stabilization against agitation-induced aggregation was observed– at least at its lower concentration (10 mM). This finding is quite in contrast to the studies on the mAb and rh-GCSF in which SBEβCD always led to increased aggregation rates (except for a single formulation during F/T-stress of rh-GCSF with 25 mM SBEβCD). Since SBEβCD (Captisol™) possesses practically no surface-activity (see Table 3.1 of Chapter 3) rh-GH is most probably stabilized by direct interaction with SBEβCD resulting in shielding of exposed hydrophobic amino acids on the protein rather than competition at the air-water interface. At high SBEβCD-concentration apparently other effects govern aggregation besides the incorporation of hydrophobic amino acids in the hydrophobic cavity, leading to increased aggregation rates.

The inhibition of agitation-induced aggregation of rh-GH by polysorbate 80 was discussed many times in literature[30,33]. Looking at only the two first time points of the experiment (after 12 h and 24 h agitation, respectively) rh-GH aggregation was reduced by the addition of polysorbate 80 being in good accordance with literature findings. However, after 48h an increase in the rate of aggregation was observed, suggesting that rh-GH aggregation at the air-water interface in the presence of polysorbate 80 could be characterized by a "lag-phase" with little aggregation at first followed by massive aggregation afterwards. The polysorbate 80 concentration chosen for the experiment reflects a typical polysorbate 80 concentration in protein formulations (i.e. clearly above the CMC, nearly identical to the high concentration chosen in the mAb and rh-GCSF-experiments at which polysorbate 80 proved to be efficient for aggregation inhibition). However, Katakam et al. report that complete suppression of rh-GH-aggregation by polysorbate 80 was achieved at a concentration of 0.1 %[33]. Therefore concentration seems to be a critical factor in the stabilization of rh-GH by polysorbate 80 and the unexpected results in our experiment could be due to a sub-optimal concentration for the stabilization against agitation-induced aggregation. Nevertheless, in conclusion, since HPβCD showed a more efficient reduction of aggregation in both concentrations investigated here the comparison between the two excipients, polysorbate 80 and HPβCD, points towards superiority of HPβCD for the stabilization of rh-GH under stress conditions that rh-GH might very well be subjected to during manufacturing and shipping of the formulations.

3.2 FREEZE-THAW STUDY

In a further attempt to evaluate whether CD-derivatives are suitable to stabilize rh-GH under pharmaceutically relevant stress conditions, freeze-thaw studies were carried out. After 15 cycles of freezing the samples to -80°C by immersing them into liquid nitrogen and thawing them at room temperature the amount of monomer in solution as determined by HP-SEC is decreased to 63 % of the content prior to the freeze thaw stress in the reference formulation (Figure 4.12). From Figure 4.13 it can be seen that aggregation is almost exclusively due to the formation of insoluble aggregates that cannot be detected by HP-SEC as no soluble aggregates could be detected. In contrast to the agitation-experiments none of the samples showed high levels of soluble aggregates (below 2 % in all samples at all time points). A complete stabilization against freeze-thaw induced rh-GH aggregation could be observed in the formulations containing HPβCD (Figure 4.12). Interestingly, almost complete aggregation suppression was also achieved by the addition of SBEβCD to the rh-GH formulations with 93 % and 97 % remaining monomer for the 10 mM and the 50 mM-concentration of SBEβCD, respectively. Polysorbate 80 also stabilized rh-GH against F/T-induced aggregation. However, the degree of aggregation suppression was significantly smaller than that achieved by the two CD-derivatives.

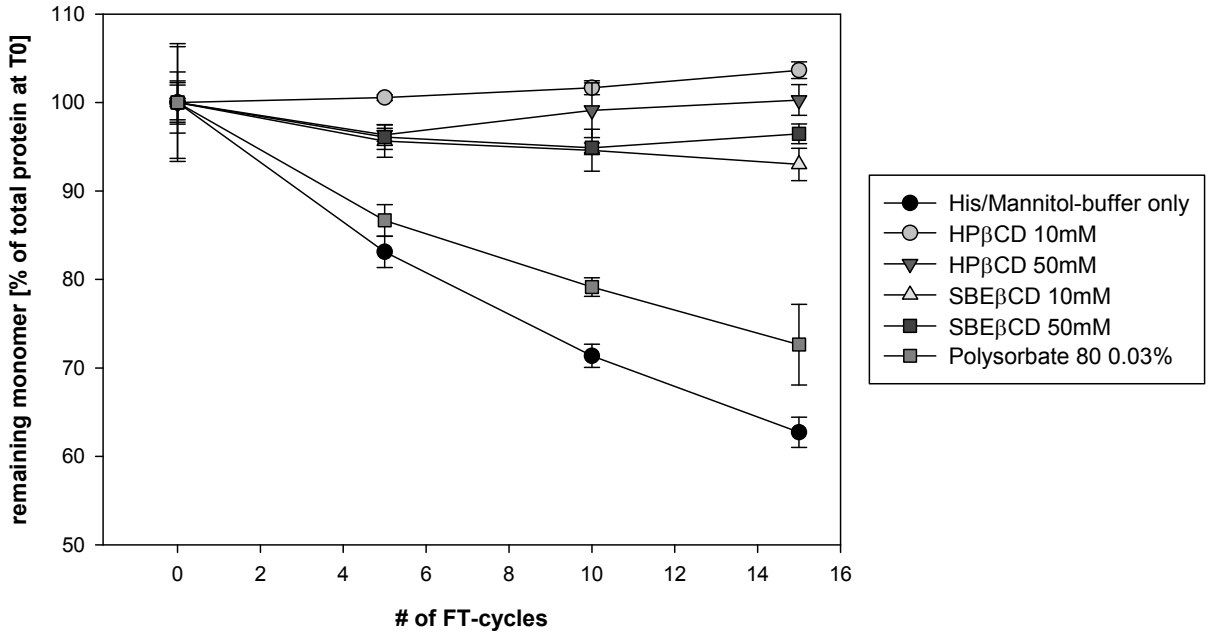

Figure 4.12: Loss of rh-GH-monomer during 15 F/T-cycles as followed by HP-SEC. All values are the mean of three samples ± standard deviation.

For the first time it is demonstrated here that CD-derivatives are capable of stabilizing rh-GH against aggregation induced by repeated freeze-thawing cycles. Since F/T-induced aggregation is caused by the presence of an interface (ice-water interface) this is a further demonstration that HPβCD and SBEβCD are capable of inhibiting surface-induced rh-GH aggregation. However, the result of this study is very much in contrast to the results of the F/T-studies carried out with a mAb and rh-GCSF. In those studies SBEβCD accelerated protein aggregation whereas in this case both concentrations of SBEβCD investigated lead to a clear almost complete stabilization of rh-GH against F/T-induced aggregation. Very likely this difference can be explained by the fact, that SBEβCD (which is not surface-active, as mentioned above and which therefore cannot stabilize *via* competition at the interface) is capable of binding to rh-GH[4], thereby masking potential hydrophobic interaction between the rh-GH molecules. The more surface-active HPβCD is capable of reaching the surface in higher concentrations thereby more efficiently interacting with the surface-unfolded rh-GH at the location of their origin. This property translates into an even more effective suppression of rh-GH aggregation by HPβCD.

Another important difference to the earlier experiments on rh-GCSF and the IgG lies in the observation that the CD-derivatives tested here more effectively stabilized the protein under investigation against surface-induced aggregation than polysorbate 80 at a reasonably high concentration (more than 10-fold above the CMC, efficient at this concentration to inhibit F/T-induced aggregation of rh-GCSF and the mAb).

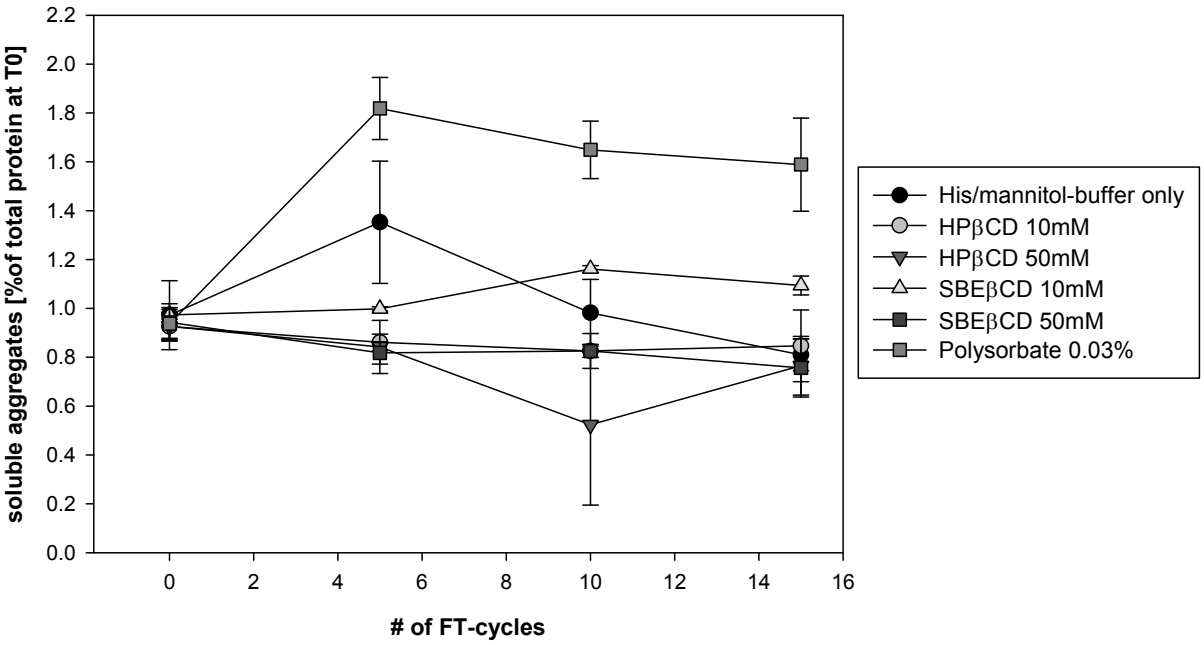

Figure 4.13: Formation of soluble aggregates of rh-GH during 15 F/T-cycles as followed by HP-SEC. All values are the mean of three samples ± standard deviation.

Since rh-GH-aggregates formed by F/T-stress were reported to trigger immune reactions in mice[25] it has to be concluded again, that the addition of HPβCD to the formulations is a promising way to prevent unwanted immunogenicity of rh-GH formulations. In contrast to reducing the aggregate-burden after the stress has occurred, as done by the application of high hydrostatic pressure[25], HPβCD provides a low-aggregate formulation at all stages of the processing and storage of rh-GH solutions. We therefore suggest the effect of HPβCD on rh-GH immunogenicity to be tested in appropriate animal models.

3.3 INCUBATION AT 50°C

In order to provide a complete set of data on the influence of CDs on rh-GH under stress conditions also a short-term incubation study at 50°C was carried out. The same formulations as for the agitation and F/T-studies were investigated for aggregation throughout a period of one month. As already observed with some of the mAb-formulations described in the previous chapter, the samples appeared yellow after the storage period. In addition their chromatograms (not shown) contained a large extra peak (most pronounced for the polysorbate-formulation) that - in contrast to mAb-experiments – did overlay with the monomer-peak and masked the fragment-peaks. If the extra-peak were also considered a protein-fraction the recovery of polysorbate formulation would be at about 150 %, which is clearly an artifact result. Therefore it is concluded that the extra peak arises from the buffer of these formulations, in which a reaction takes place that leads to products adsorbing at both 215nm at 280nm. Coloration of His buffer after storage was also observed in an earlier study and linked to the presence of metal and chloride ions in solution[34]. Since it was impossible to

analyze samples in which this phenomenon occurred, the evaluation of the experiment ends after 72h. The results are demonstrated in Figure 4.14.

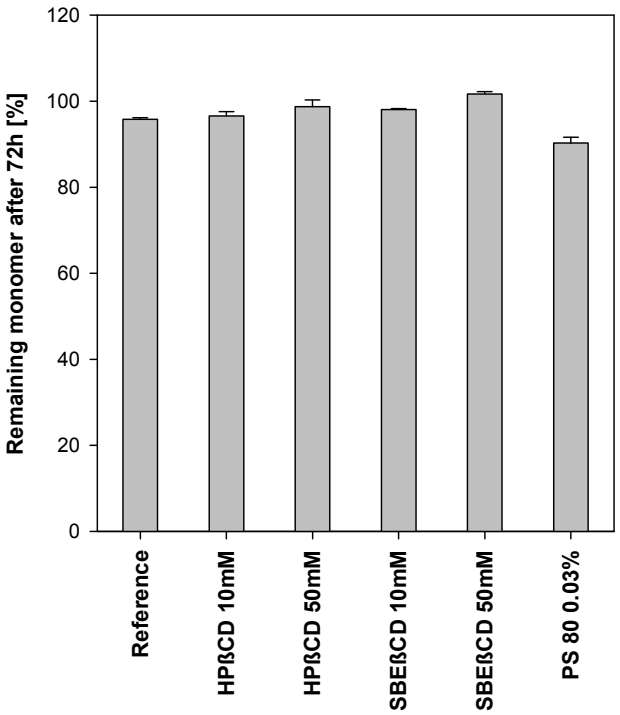

Figure 4.14: Remaining monomer after storage of rh-GH at 50°C for 72h as determined by HP-SEC. Each bar represents the mean value of three samples ± standard deviation.

After this short period of time only a small fraction of the original rh-GH was aggregated in the reference formulation since the monomer-content as determined by HP-SEC is still about 95.7 %. All formulations containing CD-derivatives (either HPβCD or SBEβCD in a 10 mM or a 50 mM-concentration, respectively) exhibited a slightly smaller or even no loss of monomer with the higher concentration inhibiting aggregation more effectively than the lower concentration and SBEβCD protecting rh-GH to a higher degree than HPβCD, respectively. The only formulation showing a higher loss of monomer is the polysorbate 80 formulation with only about 80 % remaining monomer after 72 h.

Again, it has to be concluded, that in contrast to the studies with the mAb and rh-GCSF, for rh-GH stabilization is observed by SBEβCD. Since this stabilization of rh-GH by SBEβCD is observed for all three different stress conditions it can be assumed that there is a common underlying principle governing stabilization. Due to the very different stressing conditions and the fact that interaction has already been reported in literature[4-5], it is likely that the interaction between exposed hydrophobic amino acid residues on the rh-GH molecule and the hydrophobic CD-cavity is that general stabilizing principle.

The results obtained with regard to the comparison between HPβCD and polysorbate 80 are in agreement with the results obtained for the mAb and rh-GCSF: during quiescent storage polysorbate 80 leads to increased levels of aggregates – a problem that is never observed with HPβCD, rendering it a promising excipient in protein formulation. Even more, in this

case not only was there no negative effect of HPβCD on rh-GH stability during quiescent storage, as observed with the IgG and rh-GCSF, but even a stabilizing effect is observed.

4 SUMMARY AND CONCLUSIONS

The results of accelerated stability studies with two further, structurally different, model proteins, recombinant granulocyte colony stimulating factor and recombinant human growth hormone, are discussed in this chapter. A set of three different stressing conditions (agitation, repeated freeze-thawing and quiescent storage at 50°C) was exerted on the model proteins and the influence of different CD-derivatives as well as further excipients that are commonly used in protein formulation, i.e. disaccharides and polysorbate 80, on protein aggregation was analyzed.

The results obtained with rh-GCSF qualitatively very well agreed with the results obtained for the IgG, the first model protein discussed in the previous Chapter 3. The CD-derivate showing the highest degree of protein stabilization was again HPβCD, which at relatively low (2.5 mM) and therefore non-toxic concentrations well inhibited interfacial aggregation that was provoked by either agitation (exposure to the air-water interface) or F/T stress exposing the protein to the ice-water interface. At the same time HPβCD did not compromise the quiescent storage stability of rh-GCSF at elevated temperature, thereby rendering it superior to MβCD which also showed good stabilization during agitation but led to increased aggregation rates during storage at elevated temperature. The only ionic CD-derivative tested, SBEβCD, again turned out to be detrimental to rh-GCSF stability during both, agitation and quiescent storage at 50°C further confirming trends observed from the studies on the IgG. Rh-GCSF formulations containing polysorbate 80 were also well protected against interfacial aggregation, but also showed dramatically increased aggregation rates during quiescent storage in accordance with the results obtained for the IgG. Therefore, although structurally a very different protein, rh-GCSF qualitatively confirms the trends observed for the mAb and further underlines that HPβCD is a very promising excipient for protein formulation that is capable to overcome shortcomings that polysorbate 80 suffers from.

The third model protein investigated in this thesis, rh-GH, was known to expose hydrophobic amino acid residues even in its native state and binding between cyclodextrins and rh-GH had been described in literature[4-5]. This structural particularity of rh-GH also translated into a different aggregation profile when compared to the two other model proteins. With few exceptions rh-GH was stabilized against aggregation by all CD-derivatives against all stressing conditions. This behavior renders binding between the CDs and rh-GH as common stabilizing principle under the very different stressing condition very likely. Conversely these findings also render binding between the two other model proteins and the stabilizing CD-

derivatives unlikely, since the stabilizing effect strongly depended on the stress conditions being applied.

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CHAPTER 5

INTERACTION BETWEEN CYCLODEXTRINS AND PROTEINS IN BULK SOLUTION

1 INTRODUCTION

In their ability to inhibit protein aggregation at interfaces, as discussed in detail in Chapters 3 and 4 of this thesis, cyclodextrins resemble non-ionic surfactants. Because CDs, like non-ionic surfactants, are known to interact with hydrophobic parts of proteins and because some CD-derivatives are also known to be surface-active like non-ionic surfactants, the hypothesis that CDs function in manners analogous to non-ionic surfactants in protein formulations served as starting point for the mechanistic investigations in this and the following chapter. The experimental approach discussed in Chapters 5 and 6 is intended to identify or exclude the potential mechanisms by which CDs stabilize the investigated therapeutic proteins against aggregation at the air-water-interface. In the present chapter the role of cyclodextrin-protein interaction for aggregation inhibition will be studied, while the next Chapter 6 will be dedicated to the investigation of the behavior of cyclodextrins at the air-water interface.

In most research articles the suppression of protein aggregation by cyclodextrins is attributed to the CDs' capability to incorporate suitably sized, exposed, hydrophobic amino acid residues of proteins into the hydrophobic CD-cavity, thereby blocking the potential hydrophobic protein-protein interface[1-5]. However, the unambiguous detection of binding between cyclodextrins and proteins is analytically challenging, since it is a rather weak interaction, which is characterized by binding affinities only in the mM-range[6-9]. So far CD-protein binding has been directly proven for only very few therapeutic proteins, i.e. insulin[6,10] and rh-GH[4,11], which is also a third model protein investigated in this thesis. In these studies, a clear and plausible relation between binding of cyclodextrins to proteins and aggregation inhibition of therapeutic proteins by the same cyclodextrin derivatives could be demonstrated.

In addition to the few interaction studies with therapeutic proteins, interaction of cyclodextrins with model peptides, which are easier to study due to their reduced complexity, thereby serving as model for larger proteins, was demonstrated[7,12,13]. Studies on model peptides included the di-peptide Trp-Gly[12], Aspartame[12], Melittin[13] and [D-Trp[6], Des-Gly[10]] LHRH[7].

A limited number of studies was dedicated to the investigation of the structural basis of the interaction between proteins and cyclodextrins. A comprehensive overview of the studies is given in Chapter 1 (Table 1.3) of this thesis. It seems that notably aromatic amino acids residues, such as those found in Phe, Tyr, His and Trp allow a fit into the hydrophobic moiety of β-CD and its derivatives[11]. Generally, as suggested by Aachmann et al.[14], the accessibility of solvent exposed hydrophobic amino acids by cyclodextrins influences the propensity for complexation with CDs and thus potential shielding of hydrophobic protein-protein

interaction. However, interaction is not limited to aromatic amino acids residues. Also linear chains, as for example in Ile, were found to interact with the tighter α-cyclodextrin cavity, however with a weaker affinity.

The range of analytical techniques capable of monitoring of protein-cyclodextrin interactions comprises direct fluorescence titration[11] (intrinsic steady-state fluorescence) as well as fluorescence lifetime measurements[11,13], NMR-measurements[10,14,15], isothermal titration calorimetry[7], dilution microcalorimetry[8], electrospray-ionization mass spectrometry[10] and competitive spectrophotometry[12]. However, most of the applied analytical techniques suffer from certain short-comings. For example, fluorescence spectroscopy can only be carried out in very dilute solutions in order to avoid inner filtering effects[16]. However, by dilution the situation as present in protein formulations (with regard to surface charges, conformational and colloidal stability etc.) is altered. Regarding NMR-data, interpretation can be ambivalent at times and the technique is not suitable for testing a large number of combinations of cyclodextrin-derivatives and proteins[14]. Isothermal titration calorimetry (ITC) has the disadvantage that in order to detect weak interactions, such as those between cyclodextrins and proteins, very high concentrations of the protein are necessary, again altering the system as compared to the typical protein formulation[17]. Exploratory first ITC-experiments carried out with rh-GCSF showed that even at a protein concentration of 4.04 mg/ml (concentration of the bulk solution which is by far higher than that of the formulated drug product) no interaction with SBEβCD or HPβCD could be detected (data not shown).

In search of a sensitive technique, that requires only moderate amounts of protein and that allows the investigation of a significant number of binding partner combinations in a reasonable amount of time, it was decided to evaluate surface plasmon resonance spectroscopy (SPR) as a tool for monitoring the interaction between the three therapeutic model proteins investigated in this thesis and various cyclodextrin-derivatives. Detection by surface-plasmon resonance spectroscopy is based on changes in optical properties of a surface layer upon binding of macromolecules[18-20]. One of the binding partners is immobilized at the surface and the respective binding partner is flowing over the surface. The amounts of substance needed are small, for both the immobilized component as well as for the complementary binding partner. The SPR signal allows to follow binding reaction in real-time. Although SPR is primarily employed for the monitoring of highly specific biological binding processes, such as antibody-antigen-interactions[21], it has also been previously used for the description of rather weak and transient carbohydrate-protein binding reactions with affinities in the milimolar range[19,22,23]. Even the binding of cyclodextrins to a protein has already been followed by SPR[24]. However, the bacterial transporter protein under investigation was a protein that specifically binds to cyclodextrins thereby allowing *bacillus subtilis* to utilize cyclodextrins as a carbon source, which represents quite a different situation

than the potential, rather unspecific interaction between therapeutic proteins and cyclodextrin-derivatives as formulation excipients.

Basically, two different experimental set-ups can be chosen for the investigation of cyclodextrin-protein interaction: the immobilization of the cyclodextrin or the immobilization of the protein. For two reasons it was decided to immobilize the protein. First, the immobilization of a protein is a more straightforward approach than the immobilization of the carbohydrate since the immobilization of a sugar to a dextran surface usually requires the derivatization of the carbohydrate molecule[20,25] whereas the immobilization of a protein via its lysine residues does not require derivatization[26]. Second, since three model proteins (IgG, rh-GCSF and rh-GH) were to be tested as potential protein binding partners, but up to ten different CD-derivatives were available for testing, it is obviously more convenient and associated with fewer expenses to immobilize the three proteins. The proteins were immobilized on a carboxymethylated (CM5®) chip to form a flexible hydrogel providing an environment suitable to study the binding process in conditions very close to those found unbound in solution[18].

The aim of the investigations was to first evaluate SPR as an analytical tool to monitor the weak and transient cyclodextrin-protein interactions. This evaluation was carried out using rh-GCSF as a model protein. A number of control experiments were performed: comparisons to other methods that are known to be capable to monitor weak interaction such as fluorescence spectroscopy or surface acoustic wave sensors were carried out. Also, the comparison of cyclodextrin binding to that of linear sugars without cavity in order to evaluate the necessity of the hydrophobic CD-cavity for binding was included into the experiments. Moreover, it was intended to determine the affinity of potential interactions, the concentration range in which cyclodextrins interact with the model protein and also to evaluate whether solution conditions, such as pH, influence the binding properties. In a second step, the SPR-experiments were extended to the other two model proteins, IgG and rh-GCSF, in order to correlate the binding pattern with the stability profiles that were obtained by accelerated stability testing, as discussed in Chapters 3 and 4.

Mass spectrometry was also included into the studies as a further analytical tool, notably due to its potential to provide useful information about the stoichiometry of the cyclodextrin-protein binding reaction[6,27-29]. In general, mass spectrometry is a valuable tool for the detection of noncovalent bio molecular complexes[30,31]. Notably electrospray ionization mass-spectrometry (ESI-MS) with its unique capability to also preserve weaker non covalent bonds upon transferring the complexes from the solution phase into the gas phase was shown to be useful for the characterization of non covalent protein complexes[31]. Therefore ESI-MS was included into the investigations of CD-protein interaction as a further analytical tool in order to identify combinations in which cyclodextrins and therapeutic proteins may form complexes.

However, care should be taken when trying to correlate the MS gas-phase results to the binding-situation in solution. There are reports that show that correlation is not necessarily given[30]. Therefore the investigations by ESI-MS will focus on the potential of ESI-MS as method to detect the non-covalent cyclodextrin-protein complexes. The results of the mass spectrometric investigations are discussed in the last section of this chapter.

2 SURFACE PLASMON RESONANCE SPECTROSCOPY FOR MONITORING CD-PROTEIN INTERACTION – EVALUATION OF METHODOLOGY USING RH-GCSF AS MODEL PROTEIN

For the monitoring of binding between cyclodextrins and proteins surface plasmon resonance spectroscopy (SPR) was used as a new technique for this purpose. The initial investigations were carried out using rh-GCSF as a model protein. The aims of the following investigations were two-fold: first the general evaluation of SPR as a new analytical technique that is suitable to monitor weak carbohydrate-protein interactions and secondly to describe the binding between various cyclodextrin-derivatives and all three model proteins in order to be able to correlate these results to the accelerated stability studies.

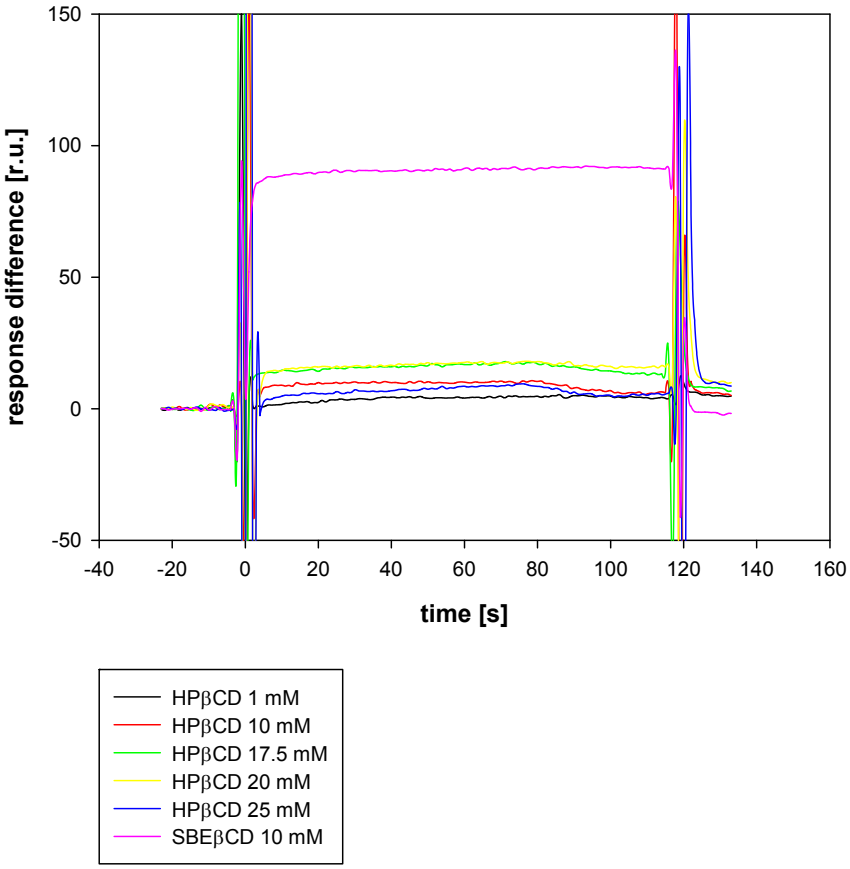

Figure 5.1: Representative SPR-sensorgram depicting the interaction between increasing amounts of HPβCD (1 mM to 25 mM) to immobilized rh-GCSF. HPβCD was injected for 120 s at a flow rate of 30 µL/min. The interaction between 10 mM SBEβCD to immobilized rh-GCSF under the same experimental conditions is included as comparison. The experiment was conducted at pH 4 in a 10 mM sodium acetate buffer.

An SPR setup was chosen that allowed for convenient monitoring of the binding of a significant number of CD-derivatives to rh-GCSF in real-time. Rh-GCSF was immobilized to the derivatized dextran surface layer of a CM 5® chip via amine linkage of the lysine residues of the protein. Solutions of cyclodextrin derivatives were subsequently flushed over the immobilized protein. In order to create identical conditions in the reference cell and in the measuring cell (expect for the presence of the immobilized protein), the reference cell was also activated using EDC/NHS (as described in detail in Chapter 2) and then deactivated using Ethanolamine (instead of protein as in the measuring cell). After immobilization of the protein in the measuring cell and deactivation of the reference cell approximately 2000 response units (RU) remained on the chip and were the "baseline" for the following experiments. Therefore, taking into account the molecular weights of rh-GCSF and HPβCD, a theoretical maximal response of 149 RU can be calculated for the potential binding of HPβCD to the immobilized rh-GCSF, when assuming a 1:1 binding stoichiometry between cyclodextrin and protein.

The first obvious observation of the measurements was that cyclodextrin solutions lead to a rather large bulk signal (not shown). This signal change occurred in both cells and it is due to changes in the refractive index of the solution flowing over the surface of the chip. It does reflect any form of binding[32]. The bulk signals were in an order of magnitude of 2000 RU for a 10 mM HPβCD solution. This means that a potentially specific binding response would always be the difference of two large signals in the measuring and the reference cell (with the slightly higher response recorded in the measuring cell and the difference between the signal in the measuring cell and the signal in the reference cell being the actual binding signal) thereby necessarily leading to some imprecision of the results.

For some cyclodextrins, such as methyl-β-cyclodextrin, γ-cyclodextrin and hydroxypropyl-γ-cyclodextrin, no response after referencing could be observed (sensorgrams not shown) indicating that no binding of these CD-derivatives to rh-GCSF took place. For other cyclodextrin-derivatives, such as SBEβCD, α-CD and also HPβCD (Figure 5.1), a reproducible response of strongly varying extent could be observed. The by far strongest binding signal was observed for the only ionic derivative included into the examinations, SBEβCD. Figure 5.1 compares the binding signals that were obtained by 10 mM SBEβCD and increasing concentrations of HPβCD (1 mM to 25 mM). The binding signal of HPβCD increases with higher concentrations of HPβCD and reaches a maximum at 17.5 mM. Interestingly, at 25 mM a decreased binding signal is observed. It was tried to calculate a steady-state binding affinity from the signals obtained for HPβCD (not shown). However, due to the relatively small concentration range of the interaction and the unclear steady-state response signal (the signal never reaches a clear steady state at higher concentrations, see

Figure 5.1) the quality of the fits remained poor and the description of binding for such weak interactions had to remain on a qualitative level.

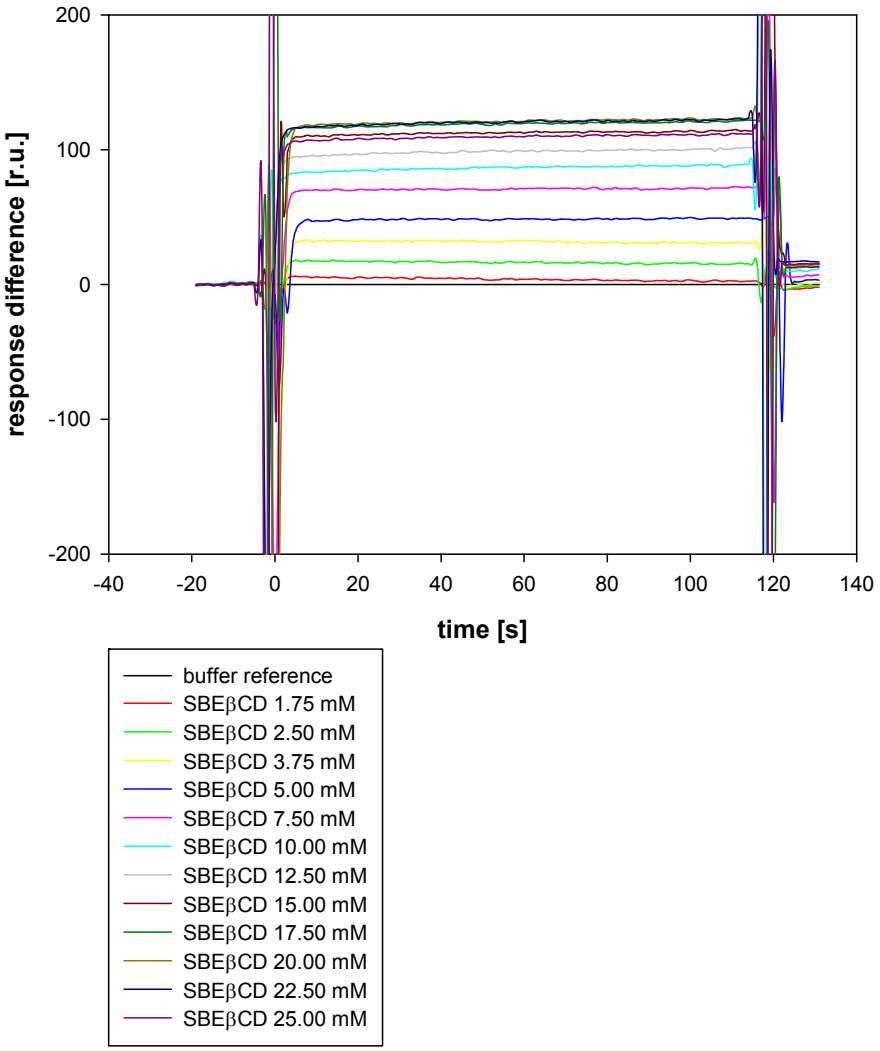

Figure 5.2: Overlaid sensorgrams depicting the interaction between increasing amounts of SBEβCD (1.75 mM to 25 mM) and immobilized rh-GCSF. SBEβCD was injected for 120 s at a flow rate of 30 µL/min. The experiment was conducted at pH 4 in 10 mM sodium acetate buffer.

For SBEβCD a significantly stronger binding signal than for HPβCD was observed: a response of about 80 RU at a concentration of 10 mM was observed for SBEβCD compared to a response of about 10 RU for HPβCD at the same concentration. In part, the stronger signal can be explained by the higher molecular weight of SBEβCD as compared to HPβCD (2163 g/mol compared to 1400 g/mol accounting for a signal increase by a factor of 1.54), but the stronger binding signal also clearly expresses a higher degree of interaction between SBEβCD and rh-GCSF than for HPβCD and rh-GCSF. In Figure 5.2 it can be seen that a signal with clearly observable steady-state response values is obtained over a much wider concentration range than for HPβCD (1.75 mM to 25 mM without a decay of the signal at very high concentrations as observed for HPβCD). Furthermore the reproducibility of the signal was better and a clear steady state-response could be determined at all investigated concentrations.

From the overlaid sensorgrams in Figure 5.2 the average steady state plateau response levels (R_{eq}) were read off as a measure of the concentration of the complex at equilibrium state, which is a necessary value for the equilibrium analysis of binding and the subsequent calculation of the binding affinity. Further parameters for the determination of equilibrium binding constants are the concentrations of the free interactants at equilibrium[19]. In this case the concentration of the free analyte (CD-derivative) is approximately equal to the concentration of the CD-solution being injected onto the immobilized protein. The concentration of the free ligand (rh-GCSF) can be calculated from the concentration of the complex and the total surface binding capacity. Equilibrium analysis was hence carried out in an analogous manner to standard Scatchard plots[33]. R_{eq} values were plotted against the concentrations of the SBEβCD-solutions (Figure 5.3). The resulting R_{eq} vs. concentration curve was fitted assuming a simple 1:1-Langmuir-binding model. The curve with the relatively lowest χ^2 has been applied for the calculation of the steady-state binding affinity (Figure 5.3). A value of K_d = 9.28*10^{-3} mol/L was obtained in good agreement with other literature values calculated for the binding between cyclodextrins and proteins (refer to Table 1.3 in Chapter 1).

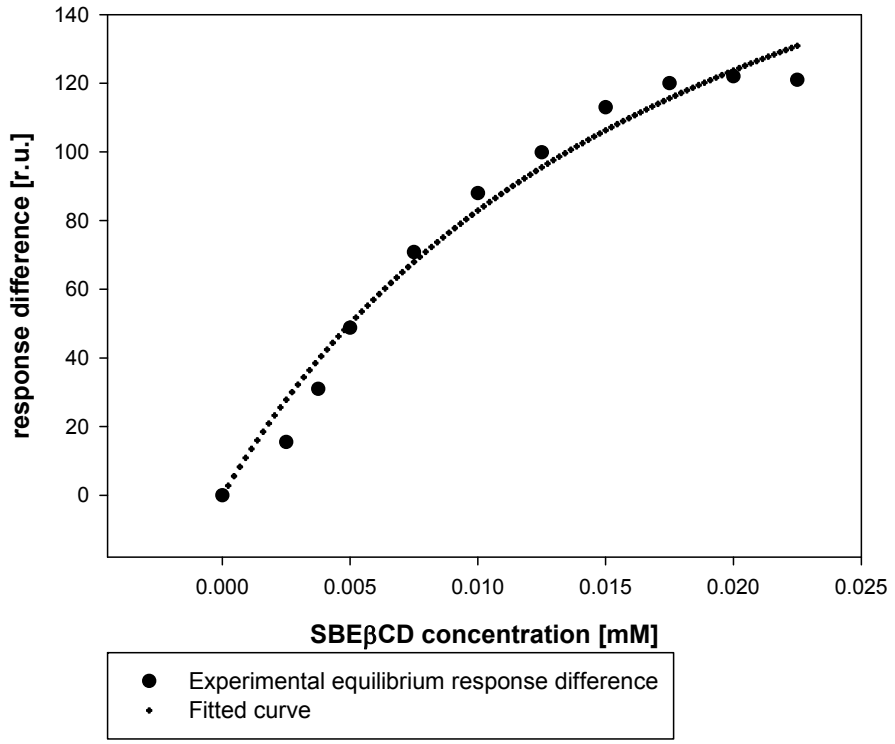

Figure 5.3: Analysis of the response signals of the SPR-sensorgrams presented in Figure 5.2 and determination of the binding affinity of SBEβCD to rh-GCSF assuming a simple one site saturation binding model. A binding constant of K_d = 9.28*10^{-3} mol/L was obtained.

In order to ensure comparability of the different sensorgrams and in order to exclude that changes on the chip lead to aberrations in the results SBEβCD at a concentration of 10 mM was included into all further experiments with rh-GSCF as a positive control and as a standard.

2.1 EXCLUSION OF UNSPECIFIC INTERACTION

The binding of cyclodextrin derivatives to rh-GCSF was compared to the binding of maltoheptaose to rh-GCSF. Maltoheptaose is a linear sugar consisting of seven units of 1a, 4e-linked oligomers of α-D-glucopyranoside, i.e. it contains the same number and linkage of glucose-molecules as the cyclic β-CD-molecule, except that Maltoheptaose does not possess a hydrophobic cavity like cyclodextrins[34]. Therefore the investigation of maltoheptaose binding was supposed to serve as a way to determine the relevance of the CD-cavity for interaction with rh-GCSF and to differentiate between specific interaction and unspecific interaction the CD-derivatives and rh-GCSF that is not due to the incorporation of suitably sized residues on the protein into the hydrophobic protein cavity. In Figure 5.4 it can be seen that the addition of 2.5 mM maltoheptaose to the immobilized rh-GCSF did not yield a measurable signal after referencing. Therefore no binding of maltoheptaose to rh-GCSF occurred. It is hence likely that the observed binding of SBEβCD, HPβCD and α-CD to rh-GCSF is indeed due to the presence of the CD-cavity and not the results of unspecific carbohydrate-protein interaction.

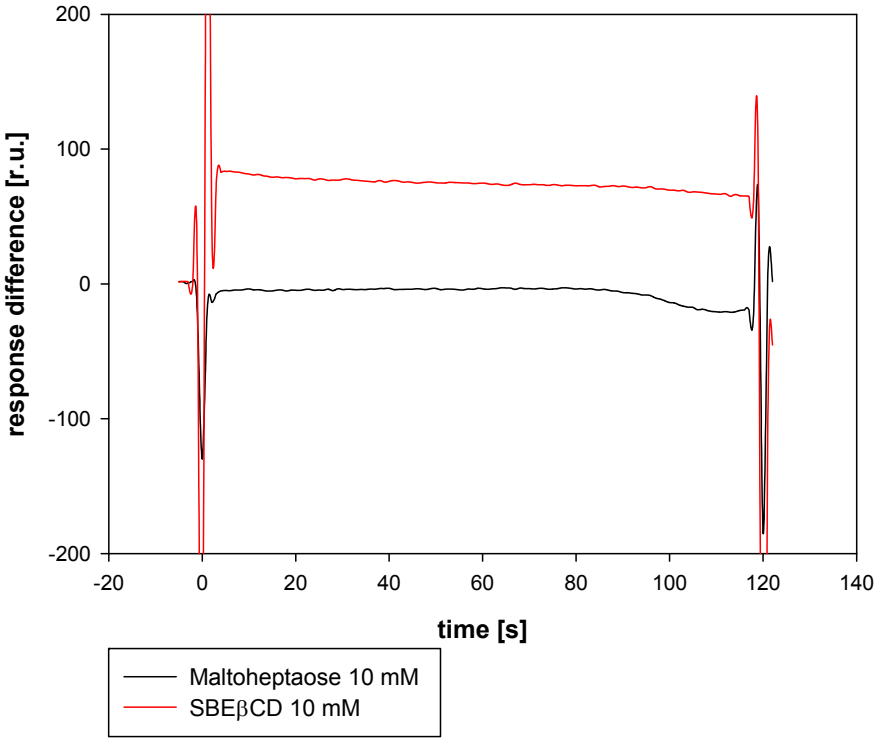

Figure 5.4: SPR-sensorgram depicting the interaction of 2.5 mM maltoheptaose with immobilized rh-GCSF. 10 mM SBEβCD was included into the measurements as internal standard in order to provide comparability to results obtained at different time points. Maltoheptaose and SBEβCD were injected for 120 s at a flow rate of 30 µL/min. The experiment was conducted at pH 4 in 10 mM sodium acetate buffer.

2.2 INFLUENCE OF IONIC INTERACTION

As previously described, SBEβCD showed the strongest binding to rh-GCSF. Interestingly, SBEβCD is the only ionic CD-derivative that was included into the binding studies. Therefore

it is conceivable, that the interaction between SBEβCD and rh-GCSF might be of purely ionic character, not involving any hydrophobic interaction due to the incorporation of protein residues into the CD-cavity. This explanation for binding is substantiated when considering the distribution of charges in the binding experiment. Interaction between SBEβCD and rh-GCSF was observed at pH 4 (Figure 5.2) which means that rh-GCSF, having a pI of 6.1, had a net positive charge[35] and SBEβCD, because of the very low pKa of the sulfonic acid groups, carries multiple negative charges at all physiologically tolerable pH-values. Therefore rh-GCSF and SBEβCD exhibit opposite net charges at pH 4 and ionic attraction could indeed account for the interaction at pH 4. Consequently, in order to evaluate whether ionic attraction is solely responsible for the interaction between SBEβCD and rh-GCSF, a control experiment was performed at pH 7. At this pH the pI of rh-GCSF is exceeded and the protein then carries a net negative charge while SBEβCD remains strongly negatively charged. Thus, at pH 7 repulsive ionic forces between SBEβCD and rh-GCSF occur. In Figure 5.5 it can be seen that nevertheless binding between SBEβCD and rh-GCSF could be observed indicating that SBEβCD-rh-GCSF interaction was not exclusively due to ionic interaction and the incorporation of hydrophobic protein residues into the CD-cavity likely took place.

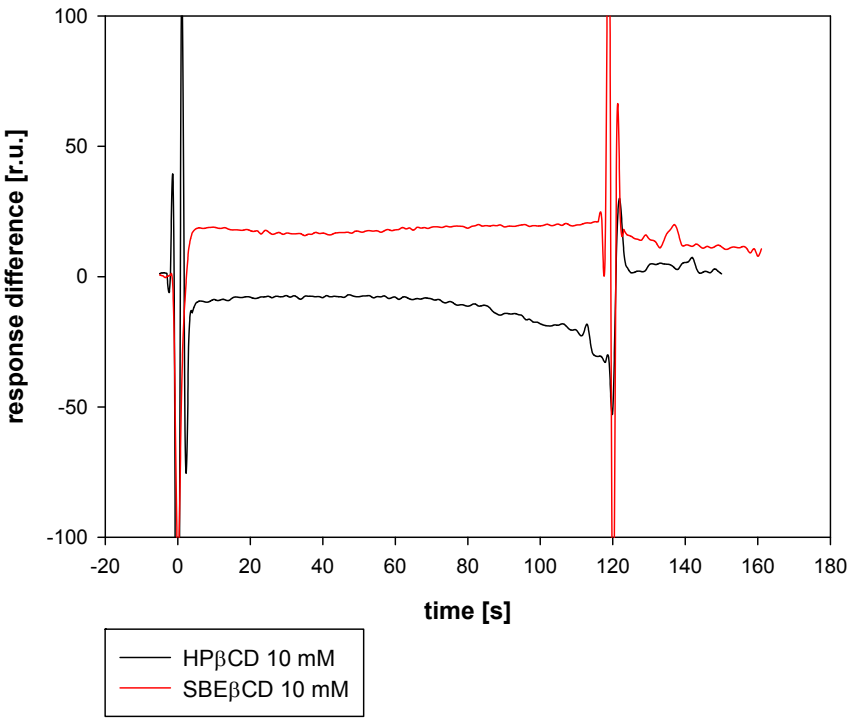

Figure 5.5: SPR-sensorgram showing the interaction of 10 mM SBEβCD and 10 mM HPβCD with immobilized rh-GCSF. HPβCD and SBEβCD were injected for 120 s at a flow rate of 30 µL/min. The experiment was conducted at pH 7 in a 10 mM sodium phosphate buffer in order to evaluate the effect of the charge of the binding partners on interaction.

However, the equilibrium plateau response observed when measuring the binding signal of a 10 mM SBEβCD solution to immobilized rh-GCSF is significantly smaller at pH 7 (approximately 20 RU) than that observed at pH 4 (see Figure 5.2, approximately 80 RU), indicating a smaller extent of interaction at neutral pH. A possible explanation for the pH

dependency of the binding process is that at opposite net charges ionic attraction leads to an accumulation of SBEβCD molecules in the vicinity of rh-GCSF and subsequent incorporation of protein residues into the CD-cavity is thereby statistically facilitated. Interestingly, as also observable from Figure 5.5, the binding of HPβCD to rh-GCSF is no longer observable at pH 7, although HPβCD is a neutral molecule and charge effects do not explain this change in the binding behavior. It is therefore speculated, that the protein undergoes structural changes at pH 7, which hinder the solvent accessibility of protein residues, which are available for inclusion into the CD-cavity at pH 4.

2.3 CONFIRMATION OF SPR RESULTS BY FLUORESCENCE SPECTROSCOPY

In order to further corroborate the results obtained by SPR, a direct titration of rh-GCSF with SBEβCD followed by intrinsic steady state fluorescence spectroscopy was carried out. The basis of this direct fluorescence spectroscopy titration is that almost all studies on the matter of protein-CD-interaction have identified aromatic amino acid residues as the main site of interaction with CDs, notably with derivatives of β-CD, whose cavity diameter allows a good fit of Phe, Tyr, His and Trp into the hydrophobic cavity[7,10-12,14,36]. Therefore changes in the microenvironment of hydrophobic amino acids on the protein occur which can be monitored by intrinsic steady state fluorescence spectroscopy. From titration curves, that are generated by plotting fluorescence parameters versus amount of compound added, binding affinities can be calculated[9,11]. Figure 5.6 shows the change in fluorescence intensity at the maximum emission wavelength of rh-GCSF upon the addition of SBEβCD.

The addition of SBEβCD leads to an increase in intrinsic steady-state fluorescence that reaches saturation after the addition of about 4 mM SBEβCD to the rh-GCSF solution (Figure 5.6). In order to avoid inner filter effects, the experiment was carried out in a very dilute solution containing only 1 µg/mL of rh-GCSF. The obtained data can be fitted to a simple one-site saturation binding model yielding an apparent dissociation constant of $3.65*10^{-4}$ mol/L. The value roughly lies in the same order of magnitude as the value obtained by SPR for the same system ($9.28*10^{-3}$mM), thereby confirming the weak, but reproducible interaction between SBEβCD and rh-GCSF. HPβCD in contrast did not lead to a measurable increase of intrinsic rh-GCSF fluorescence (data not shown), confirming the significantly weaker interaction of HPβCD and rh-GCSF that was already observed by SPR.

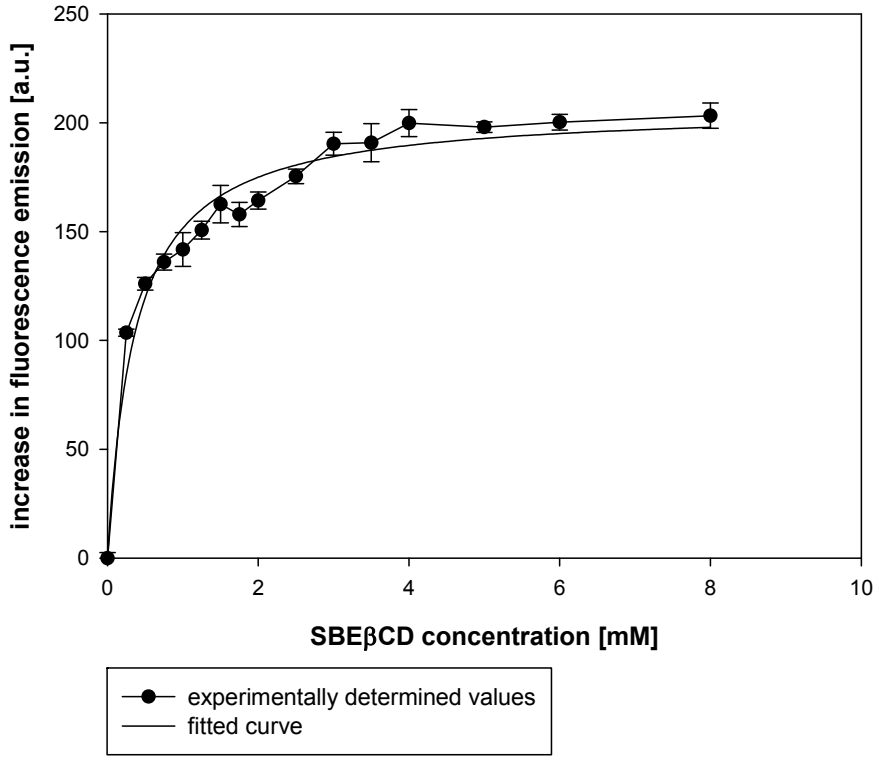

Figure 5.6: Titration of 1 µg/mL rh-GCSF with SBEβCD in 20 mM sodium phosphate buffer at pH 4 followed by intrinsic steady state fluorescence spectroscopy (excitation wavelength of 280 nm, emission wavelength of 337 nm). The data are fitted to a binding curve assuming a simple one site saturation binding model to yield an apparent dissociation constant of $3.65*10^{-4}$ mol/L.

2.4 CONFIRMATION OF SPR-RESULTS USING SURFACE ACOUSTIC WAVE SENSORS

As discussed earlier, one of the major obstacles for the evaluation of the interaction of cyclodextrin-derivatives and immobilized protein by SPR are the strong changes in the refractive index of the CD-bulk solution as compared to the respective buffer solution without CD. These “bulk effects” add to the change of the signal that is brought into proportion to the bound mass of the analyte (in this case the cyclodextrin derivatives in relatively high concentrations) as the actual binding signal[32]. In order to further confirm the results obtained by SPR, surface acoustic wave sensors, as a biosensor technique that is not sensitive to changes in the refractive index, were applied to monitor the interaction between rh-GCSF and SBEβCD.

As described in more detail in Chapter 2, surface acoustic wave sensors use piezoelectric materials to generate an acoustic wave. The amplitude and/or the velocity of the surface acoustic wave is strongly influenced by coupling to any medium contacting the surface[32]. In contrast to SPR, SAW sensors are sensitive to changes in mass, density, viscosity and acoustic coupling phenomena, but not to changes in the bulk refractive index, thereby providing useful complementary information to the SPR results. The instrument used for the experiments belongs to the class of Love-wave sensors, which are currently among the most sensitive acoustic sensors. The measurements were carried out on the commercially

available S-sens® K5 sensor, which uses five sensor elements on one sensor chip. The recorded phase shifts can be related to the bound mass of the analyte after discrimination from viscoelastic effects[37] as described in chapter 2.

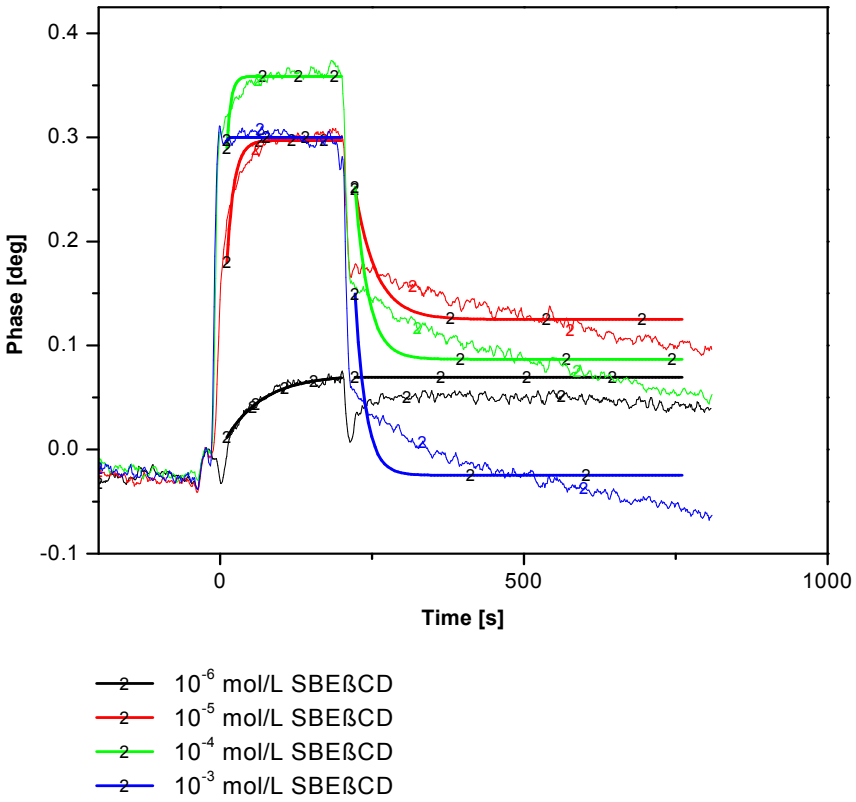

Figure 5.7: Time dependent phase signal as measured for the binding of increasing concentrations of SBEβCD to rh-GCSF which was immobilized to a S-sens K5 biosensor quartz chip surface. Kinetic evaluation of the association and dissociation process (fitted curves are represented by the straight lines) resulted in a K_d = 8.59*10-4 mol/L.

As shown in Figure 5.7, the time dependent phase signal was measured at different concentrations of SBEβCD and analysis of the association and dissociation kinetics was applied in order to determine the equilibrium binding constant. Figure 5.7 depicts the binding signals that were obtained after injection of four different concentrations of SBEβCD to rh-GCSF which was immobilized onto the surface of the quartz chip. A clear binding signal was observed even at SBEβCD-concentrations as low as 10^{-5} mol/L, thereby confirming the binding of SBEβCD to rh-GCSF even at rather low concentrations. When assuming a 1:1 Langmuir binding (in analogy to the analysis of the SPR data and the fluorescence titration data) a binding constant of $K_d = 8.59*10^{-4}$ mol/L is obtained. The value is between that determined by direct fluorescence titration ($3.65*10^{-4}$ mol/L) and that determined by SPR ($9.28*10^{-3}$ mol/L), yielding an overall satisfactory degree of agreement between the different methods of determination. One reason for a higher binding affinity as determined by SAW as compared to SPR could be the fact that in SPR no referencing to cells without immobilized protein was carried out and that, therefore, unspecific binding events could have added to the binding signal resulting in an overestimation of the binding affinity.

In addition to the quantitative evaluation of SBEβCD-binding to rh-GCSF by using the SAW-sensor, potential binding of HPβCD to rh-GCSF was also examined by SAW. It was found that no clear binding signal could be recorded after the injection of HPβCD to immobilized rh-GCSF (data not shown). This result is again in good agreement with SPR data (little binding, as previously discussed no quantification possible) and the fluorescence titration data (no interaction recorded at all).

2.5 SUMMARY OF EVALUATION OF SPR AS A METHOD FOR THE DESCRIPTION OF CD-PROTEIN BINDING

In summary, SPR was shown to be a suitable method to monitor the interaction between CD-derivatives and rh-GCSF. Using SPR it was possible to differentiate between CD-derivatives that showed binding to rh-GCSF (notably SBEβCD, weak binding also observed for α-CD and HPβCD) and others that did not exhibit any signs of binding to rh-GCSF (MβCD, HPγCD). A number of control experiments were performed in order to make sure that the obtained results actually describe the characteristic CD-protein interaction and that the results are not due to experimental artifacts. By shifting the pH during the binding experiment it was shown, that ionic interaction facilitates SBEβCD binding to rh-GCSF but that it is not the only driving force of the interaction. Since the linear sugar maltoheptaose, containing the same number and linkage of glucose molecules as β-CD-derivatives, lacks any signs of binding, it can be assumed that the presence of the hydrophobic CD-cavity is a necessary prerequisite for the interaction between β-CD-derivatives and rh-GCSF.

Unfortunately, quantification of the binding affinity was only possible if a strong and reproducible steady-state plateau response was achieved. For the weakly interacting CD-derivatives HPβCD and α-CD no such quantification of the binding affinity was possible. For the binding of SBEβCD to rh-GCSF a binding affinity was calculated by applying an analysis of the steady state binding response. The obtained value for the binding affinity was in reasonable agreement with literature values (Table 1.3 in Chapter 1) and also with two further methods for the determination of binding affinity, direct fluorescence based titration and the use of a surface acoustic wave sensor.

The information gathered on the solution binding between rh-GCSF and the different CD-derivatives in relation to accelerated stability studies will be discussed in the following section along with the results obtained for the binding between the two further model proteins and various CD-derivatives.

2.6 CD-INTERACTION WITH MAB, RH-GCSF AND RH-GH AS STUDIED BY SPR AND CORRELATION TO ACCELERATED STABILITY STUDIES

Based on the evaluation of SPR as a method to monitor the weak interaction of CD-derivatives and proteins using rh-GCSF as a model protein, binding between various CD-

derivatives and the two further model proteins, IgG and rh-GH, was also analyzed. Thereby it was intended to provide a data set that allows for a systematic correlation of the results from the accelerated stability studies to the results of the interaction analysis. The discussion of the binding properties of the IgG and rh-GH to CDs is confined to the two CD-derivatives that were also included in nearly all accelerated stability studies, HPβCD and SBEβCD. Table 5.1 gives a summary of the results on the investigation of interaction between HPβCD and SBEβCD to all three model proteins.

Table 5.1: Summary of the SPR-investigations on the interaction of HPβCD and SBEβCD with three different model proteins.

"Strong interaction" means that interaction was quantifiable by SPR, "significant interaction" means that a reproducible, concentration-dependent response was obtained by SPR and "weak interaction" means that the response signal was barely detectable and concentration-dependency was poor.

	IgG	**Rh-GCSF**	**Rh-GH**
HPβCD	No interaction	Weak interaction No quantification	Significant interaction
SBEβCD	Strong interaction K_d=1.47*10^3 mol/L	Strong interaction K_d=9.28*10^{-3} mol/L	Significant interaction

For the IgG comparably high immobilization efficiency was achieved with almost 10,000 RU remaining on the CM 5® -chip, thereby providing a high sensitivity for the monitoring of binding reactions. As with rh-GCSF, after the injection of different concentrations of HPβCD onto the immobilized IgG only a weak response could be observed. At lower concentrations of HPβCD (< 2.5 mM) no binding response at all was recorded (Figure 5.8). If observed, the weak binding response of HPβCD was poorly reproducible: repeated injections yielded differently low or no responses. Therefore it has to be assumed that HPβCD does not bind to the IgG in solution to a noteworthy extent. As with the binding of SBEβCD to rh-GCSF direct fluorescence based titration of the IgG with HPβCD was carried out as a confirmation of the SPR results. The results of the titration of the IgG with increasing HPβCD-concentrations are shown in Figure 5.9.

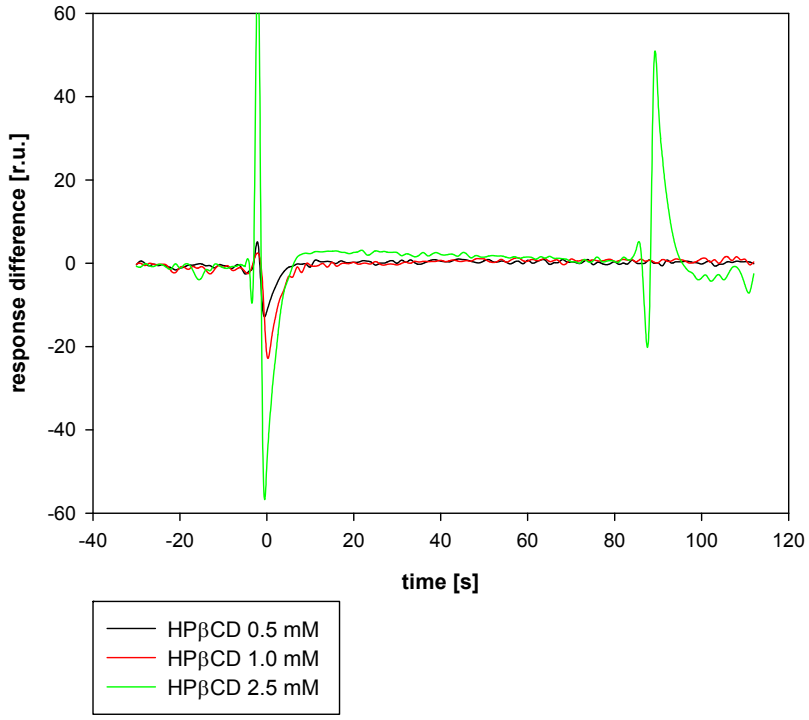

Figure 5.8: Representative sensorgram showing the SPR signal after injection of different HPβCD concentrations to an immobilized IgG. The experiments were carried out in 20 mM His buffer pH 5.8 and injection was carried out for 80 s at a flow rate of 20 µL/min.

When the spectra were corrected for dilution after the titration procedure, they almost perfectly overlaid. Neither shifts in the maximum emission wavelength nor significant changes in fluorescence emission intensity could be observed. Thus binding of HPβCD to the IgG could not be detected by this method further confirming the results obtained by SPR.

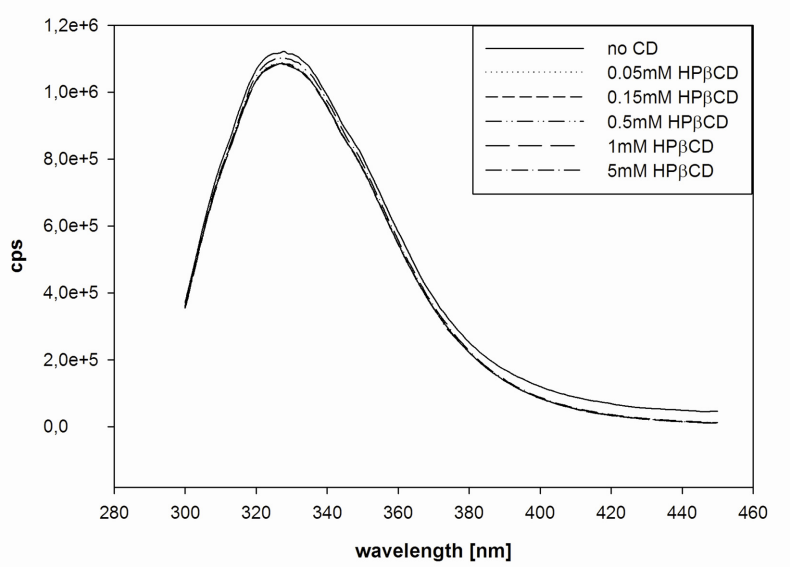

Figure 5.9: Titration of a 100 mM HPβCD stock-solution into a 0.24 mg/ml solution of the IgG in His buffer at pH 5.8 and monitoring of intrinsic steady-state fluorescence (excitation wavelength of 280 nm). All spectra were corrected for dilution.

In contrast to HPβCD, SBEβCD exhibited remarkable and well reproducible binding to the immobilized protein (Figure 5.10). As described for the binding of SBEβCD to rh-GCSF, the

steady state binding responses could be used for the determination of the binding affinity of SBEβCD to the IgG and an equilibrium binding constant of K_d = 1.47*10^3 mol/L assuming 1:1 binding-stoichiometry was obtained. The obtained value is in fair agreement with the value obtained for the binding of SBEβCD to rh-GCSF (9.28*10^{-3} mol/L). Also the IgG and SBEβCD were oppositely charged at the pH of the binding experiment, thereby presumably facilitating the binding reaction. In summary, although in terms of size and structure a very different protein, the IgG qualitatively and quantitatively exhibits the same binding behavior to HPβCD and SBEβCD as rh-GCSF: significant and quantifiable binding over a wide concentration range to the ionic SBEβCD and no or only minor binding to HPβCD.

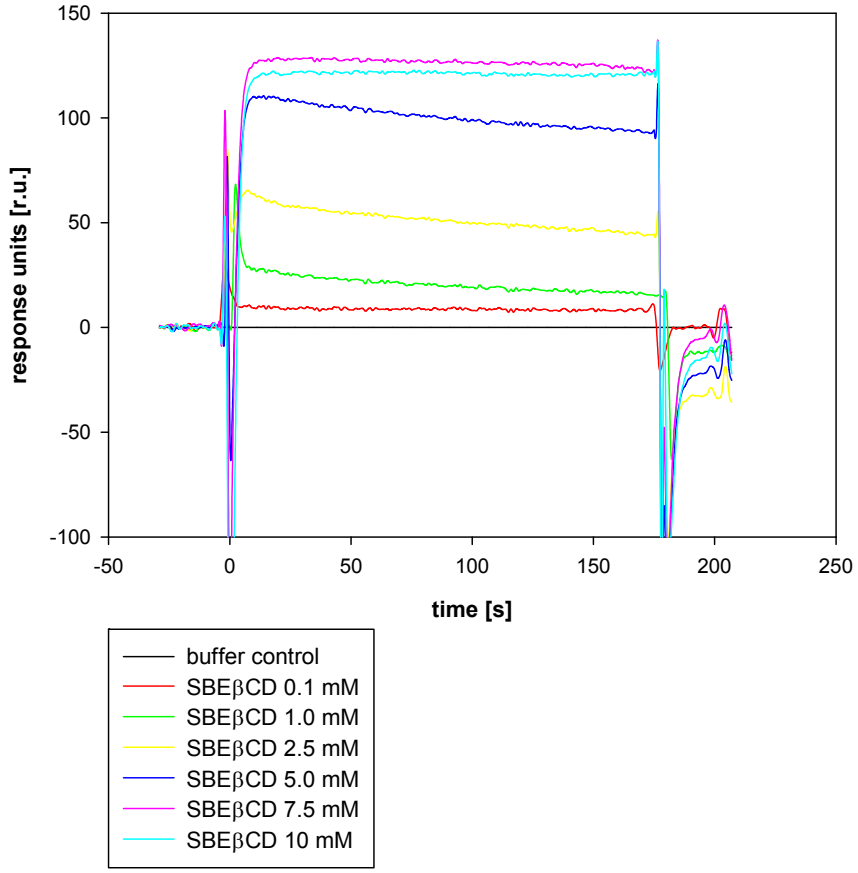

Figure 5.10: Sensorgram depicting the concentration-dependent interaction of SBEβCD with immobilized IgG. The experiments were carried out in 20 mM His buffer pH 5.8 at a flow rate of 20 µL/min. Equilibrium response analysis yielded a binding affinity of K_d=1.47*10^3 mol/L assuming 1:1 binding-stoichiometry.

In Chapters 3 and 4 of this thesis it was discussed that HPβCD was very efficient in inhibiting interfacial aggregation of both the IgG and of rh-GCSF (Table 5.2 summarizes important findings from the accelerated stability studies). During stress testing the findings obtained with rh-GCSF qualitatively very well agreed with the results obtained for the IgG. In formulations of both proteins, HPβCD well inhibited interfacial aggregation that was provoked by either agitation with exposure to the air-water interface or by F/T stress exposing the protein to the ice-water interface. At the same time HPβCD did not compromise the quiescent storage stability of rh-GCSF at elevated temperature. In contrast, the ionic CD-

derivative SBEβCD turned out to be detrimental to both the IgG and rh-GCSF stability in all three stress conditions. Therefore, although structurally very different proteins, rh-GCSF and the IgG qualitatively show very similar trends during accelerated stability testing in formulations containing HPβCD and SBEβCD.

Table 5.2: Summary of the effects of HPβCD and SBEβCD on aggregation of the three model proteins under accelerated stability conditions as discussed in Chapters 3 and 4.

+++ means complete stabilization, ++ means clear stabilization, + means weak stabilization, 0 means no effect and – means accelerated aggregation

Model protein	Stress condition	Effect of HPβCD	Effect of SBEβCD
IgG	Agitation	+++	-
	Freeze-thawing cycles	+++	n/a
	Elevated temperature	0	-
Rh-GCSF	Agitation	+++	-
	Freeze-thawing cycles	++	Concentration dependent + / -
	Elevated temperature	0	-
Rh-GH	Agitation	+++	+
	Freeze-thawing cycles	+++	++
	Elevated temperature	++	+++

However, no correlation can be established between binding of the two CD-derivatives to the IgG and rh-GCSF and the inhibition of aggregation. HPβCD, which was efficient in the inhibition of aggregation under a variety of conditions, was found not to bind to the IgG and to bind only very weakly to rh-GCSF. Therefore, it appears that other principles than direct binding in bulk solution govern the stabilization of rh-GH and the IgG by HPβCD. Since HPβCD was notably effective against interfacially-induced aggregation, it can be speculated that HPβCD exerts effects at interfaces comparable to those of non-ionic surfactants: competitive displacement of proteins from the interface and thereby the prevention of protein unfolding. Another possibility could be that the IgG and rh-GCSF exhibit a different conformation at the interface than in bulk solution with better accessibility of hydrophobic amino acid residues by CDs at the interface than in bulk solution. This would mean that

direct binding in the bulk solution as determined by SPR and fluorescence spectroscopy, in which the proteins maintain a rather native structure with little exposure of hydrophobic amino acids, does not correctly reflect potential binding at the interfaces, in which the tertiary structure of proteins is often significantly altered. Therefore, in order to understand whether binding between HPβCD and the two model proteins at the interface explains aggregation inhibition, it would be desirable to study in more detail the effects of HPβCD at interfaces.

In contrast to HPβCD, SBEβCD did exhibit significant binding to the IgG as well as to rh-GCSF. However, that leads to increased aggregation rates under almost all stress conditions.

This “inverse correlation” of binding and stabilizing behavior was unexpected, since binding of CDs to hydrophobic protein amino acids is generally regarded as prerequisite for the aggregation inhibition[14]. Instead of a correlation to the stabilization of the model proteins, a correlation to *de*stabilization was observed. A number of reasons could explain this unexpected relation. An effect of SBEβCD as ionic excipient on colloidal protein stability, due to the shielding of the repulsive forces between the protein molecules, might play a role. Furthermore, in Chapter 4 it was discussed that SBEβCD led to a very pronounced decrease of apparent T_m of rh-GCSF by more than 10°C. Apparently, the strong decrease of conformational stability of rh-GCSF could contribute to the undesired effects of SBEβCD. The observation of a strong decrease in T_m by the addition of a β-CD derivative is in agreement with studies by Cooper et al.[38] who found out, that protein thermodynamic stability can be strongly reduced by the addition of CDs due to preferential binding to the unfolded state, which consequently results in a shift of the folding equilibrium towards the more unfolded state. A different explanation of the detrimental effects of SBEβCD on rh-GCSF and IgG stability despite significant binding over a wide concentration range could be that SBEβCD-binding to rh-GCSF and the IgG does not involve the shielding of solvent-exposed hydrophobic protein residues. Therefore, aggregation could proceed via hydrophobic interaction despite the binding of SBEβCD. However, this explanation is in contrast to the results of the experiments discussed in the previous section, that lead to the conclusion that ionic interaction alone does not account for binding between the proteins and SBEβCD and that the linear sugar maltoheptaose did not exhibit any binding at all.

For rh-GH a very different relation between binding and stabilization during accelerated stability testing compared to the other two model proteins was observed. Literature results that reported the binding of HPβCD and SBEβCD to the partially unfolded state of rh-GH, such as observed at pH 2.5[11] or after partial chemical denaturation[9], could be confirmed by our experiments. Figure 5.11 shows the binding of HPβCD to immobilized rh-GH at a pH of 2.5 as an example.

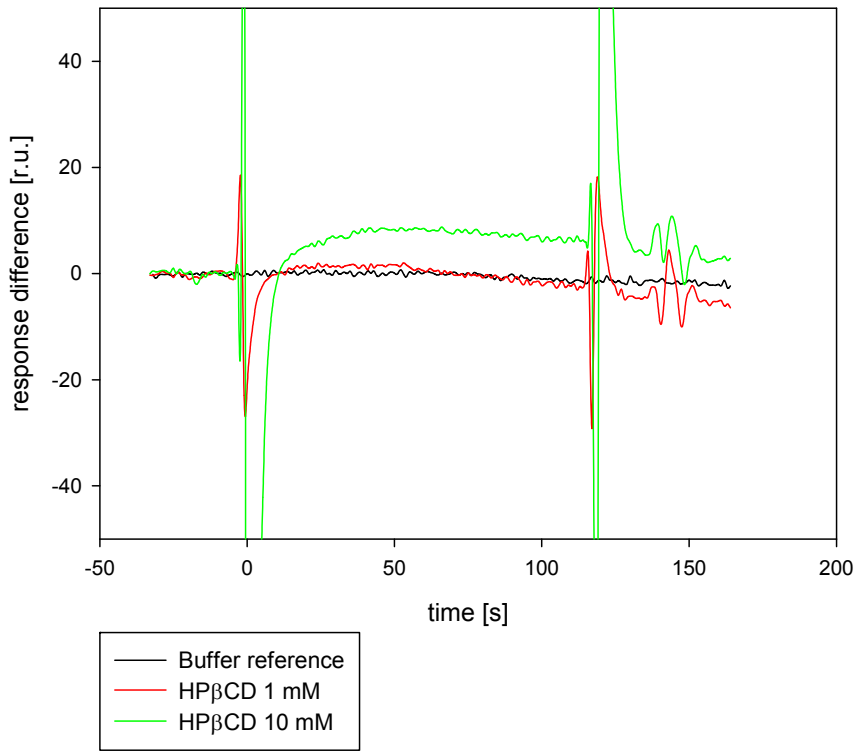

Figure 5.11: Representative sensorgram depicting the interaction of two concentrations of HPβCD with immobilized rh-GH at pH 2.5 in glycine buffer. The experiment was carried out at a flow rate of 10 µL/min for 120 s.

A clear binding response can be observed. However, despite clearly observable binding, a reliable quantification of the binding affinity could not be carried out, which might have been due to the comparably poor immobilization efficiency of rh-GH (only about 800 R.U remained on the chip). In addition, clear binding was also observed after the injection of SBEβCD to immobilized rh-GH.

However, in contrast to the results obtained with rh-GCSF and the mAb, both CD-derivatives well stabilized rh-GH against all tested stress conditions (see Table 5.2). Therefore, in the case of rh-GH it seems as if binding of the CD-derivatives to rh-GH actually also translates into aggregation inhibition. As discussed in previous chapters, this correlation could be due to rh-GH exhibiting unique structural properties with a high percentage of solvent-accessible aromatic amino acids that make binding sites for CD-derivatives unusually accessible. Hence, it appears that in the case of proteins which expose a significant amount of surface hydrophobicity and that contain a high percentage of aromatic amino acids binding of cyclodextrins actually also leads to aggregation inhibition. However, since most therapeutic proteins, such as mAbs, expose only little or no measurable surface hydrophobicity prior to aggregating, binding to CD-derivatives in solution is unlikely and therefore inhibition of aggregation due to the shielding of hydrophobic amino acids does not occur. Stabilization against aggregation that is triggered by the presence of interfaces seems to be a completely different situation and could be either due to the protein-displacement from the interface by CD-derivatives (as observed with non-ionic surfactants) or to binding at the interface when

the previously (in the bulk solution) buried hydrophobic amino acids of the protein core are exposed at the interface. Therefore the composition of the surface layers of mixed protein-CD solutions needs to be studied in order to understand the role of cyclodextrins at interfaces, which protein formulations can be exposed to. A detailed investigation of the behavior of CDs at the air-water interface and possible interactions of CDs and proteins at the interface will be discussed in the following chapter.

3 MASS SPECTROMETRY FOR THE DETECTION OF CYCLODEXTRIN-PROTEIN COMPLEXES

Mass spectrometry was applied to further evaluate the stoichiometry of binding between cyclodextrins and proteins. In order to simplify the analysis of the acquired binding data, a 1:1 binding-stoichiometry of protein-CD interaction was assumed in the previously described SPR and fluorescence spectroscopy studies as well as in studies performed by other authors[9,11]. However, there are several literature reports stating that also the formation of higher order complexes (two or more cyclodextrin molecules binding to one guest molecule) may occur[14,39]. The techniques employed so far in this thesis are limited with regard to the determination of the stoichiometry of protein-cyclodextrin binding. However, a further characterization of the stoichiometry of CD-protein interaction is highly important in order to obtain a complete picture of CD-protein interaction. Therefore electrospray-ionization mass spectrometry (ESI-MS) was chosen as an analytical method because of its widely published potential to preserve weak non-covalent bonds upon transferring them into the gas phase[40-46]. In addition to the determination of the binding stoichiometry it was intended to evaluate to which extent ESI-MS could generally serve as a reliable tool to rapidly detect binding of a greater number of combinations of cyclodextrins and proteins.

There are many publications reporting the reliable detection of host-guest complexes composed of cyclodextrins and amino acids, peptides and even proteins as binding partners using ESI-MS [6,27,47-50]. In these studies the detection of signals that correspond to the exact mass to charge ratio of the potential complexes was used as proof for the existence of the respective solution-phase complexes. As discussed in the introduction of this chapter, the incorporation of suitably sized residues into the cavity of cyclodextrins involves mainly hydrophobic interactions. Due to the “softness” of the electrospray ionization procedure it is believed that these complexes, based on hydrophobic interaction, also persist in the gas phase[31,45]. Thereby ESI-MS is regarded as a powerful tool for rapid and material-saving analysis of non-covalent CD-guest complexes.

However, the question to which extent non-covalent complexes detected in the gas-phase actually represent the binding properties in solution is extensively discussed in literature. For cyclodextrin-guest complexes this question was first raised in the mid-nineties by Cunniff and Vouros[51]. In their studies the detection of “false-positive” complexes was reported, i.e. complexes that were detected in the gas-phase but that were very unlikely to exist in solution. It was suggested that the β-CD-amino acid complexes detected by ESI-MS may not be inclusion complexes, but rather electrostatic adducts. It was assumed that electrostatic adducts with cyclodextrins occur whenever the potential binding partner carries an amine function which becomes positively charged in the gas phase. The positively charged amine residues are likely to interact with the electronegative oxygen atoms of the β-CD molecules in a manner comparable to unspecific complex formation with residual instrumental ammonium

contamination[51]. To corroborate their conclusion, Cunniff and Vouros compared the binding of β-CD to amino acids without aromatic residues (that do not fit into the β-CD cavity in solution and therefore do not form solution complexes) and found that every amino acid – regardless of its residues - yielded gas-phase adducts. Conversely, β-CD complexes with aromatic non-polar substances could not be detected, thereby resulting in false negatives.

Further detailed studies were conducted in order to elucidate the question whether the detected complexes are due to hydrophobic solution-phase interaction or only due to ion-dipole interaction after removal of the liquid phase and transfer into the gas-phase. Many of these studies used dissociation methods, which means that the complexes of interest were first isolated in the gas phase and then subsequently analyzed by dissociation procedures. Examples include collision-induced dissociation[50,52,53], heated capillary dissociation[47] and blackbody induced radiation dissociation (BIRD)[48]. However, the results remained ambivalent with studies stating that the gas-phase complexes are the product of electrostatic gas-phase artifacts only and others concluding that also hydrophobic solution-phase interaction contributes to the formation of the complexes[6,54,55].

Most of the cited studies on the issue of CD-guest-interaction involve low molecular weight guest molecules such as amino acids or small peptides. However, there is also one report on the interaction between CDs and a protein as detected by ESI-MS[27]. Cao et al. applied ion trap electrospray ionization mass spectrometry to investigate complex formation between β-cyclodextrin and hen egg white lysozyme. Guest exchanging experiments were performed in order to confirm that the detected complexes are not due to non-specific adducts. Since the experiments by Cao et al. were performed on the same kind of instrument as the studies discussed in the following sections (ion trap mass spectrometer), the published MS-parameters were used as a starting point for the described investigations. As pharmaceutically relevant model proteins IFNα2a and rh-GCSF were investigated. IFNα2a was not included into the studies discussed in the previous chapters of this thesis. However, the question whether it interacts with HPβCD and thereby leads to a stabilization of the protein was raised in earlier works[56] and is therefore of comparable mechanistic interest as the question whether CDs interact with the model proteins investigated in this thesis (rh-GCSF, IgG, rhGH).

In summary, it can be stated that there oftentimes is an electrostatic contribution when complexes are detected by ESI-MS. However, there are a number of thorough and detailed studies that point towards the possibility that hydrophobic solution-phase derived interaction also contributes to complexes that are detected by ESI-MS. Care has to be taken when interpreting the results of CD-complexation studies carried out by ESI-MS and therefore appropriate control experiments have to be conducted. In order to get an understanding of the influence of unspecific, false-positive complex formation, a number of control experiments were included into the studies performed, such as a systematic variation of the

molar ratios of the potential binding partners. In addition, detailed comparisons of the CD-binding behavior to the binding behavior of linear sugars were conducted as well as binding experiments with model amino acids and model non-basic amino acid derivatives.

3.1 ANALYSIS OF PURE CYCLODEXTRIN DERIVATIVES

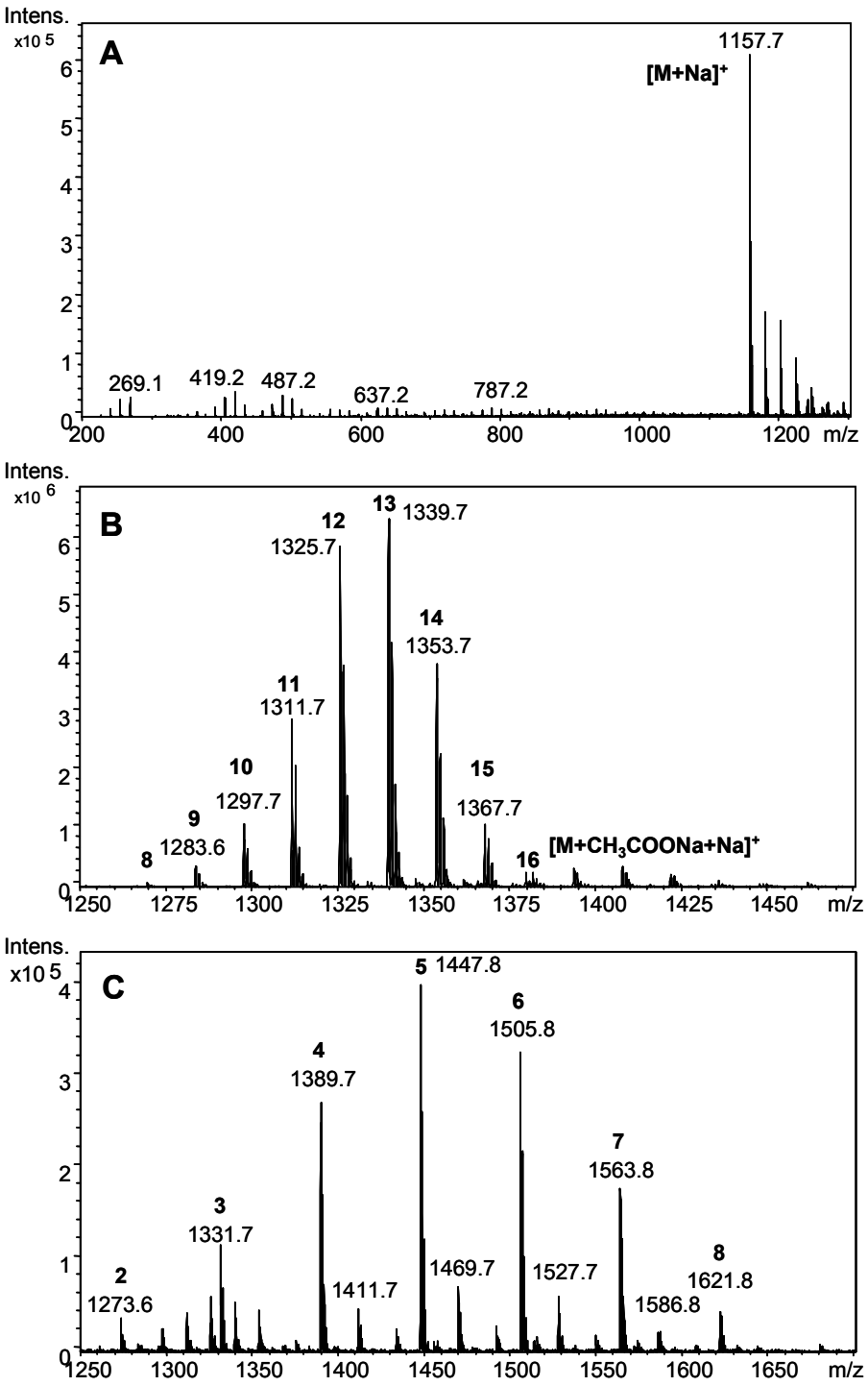

Figure 5.12: ESI-MS full scan spectra of native β-CD (A), Methyl-β-CD (B) and HPβCD (C). The bold numbers above the mass/charge ratios indicate the degree of substitution of the respective species. The spectra were recorded in positive ion mode from a 50:50 mixture of methanol and water containing 0.1 % glacial acetic acid.

Before analyzing potential complexes of proteins and cyclodextrins the isolated components were studied first. This proceeding is of importance notably for the substituted cyclodextrins. Depending on the manufacturer, the degree of substitution of CD-derivatives greatly varies and in order to later be able to identify complexes between CDs and guest molecules by their precise mass to charge ratio, it is necessary to first exactly determine the mass distribution of the cyclodextrin-derivatives alone. Pure CD-samples were best measured in positive ion mode (Figure 5.12). The mass determined for the native β-cyclodextrin species was in excellent accordance to values reported in literature (Figure 5.12 A) [54]. As visible form the broad distributions depicted in part B and C of the figure, CD-derivatives are chemically very heterogeneous products. The bold numbers above the respective mass/charge ratios indicate the degree of substitution of MβCD and HPβCD. It can be seen that highest relative abundance was obtained for the 13-fold substituted MβCD and for the 5-fold substituted HPβCD. The knowledge of this manufacturer-specific mass distribution pattern is crucial for identifying CD-guest complexes by their precise mass to charge ratio in the following experiments. The smaller peaks next to the main peaks representing the mass of the respective CD-derivative that are visible in Figure 5.12 correspond to the $M+Na^+$ ions. In the spectrum of MβCD (Figure 5.12 B) also the $[M+CH3COONa+Na]^+$ ions of several MβCD-derivatives could be identified.

3.2 CYCLODEXTRIN COMPLEXES WITH RH-GCSF AND RH-IFN-A2A

Having characterized the molecular mass distribution of the complex commercial CD-derivative products, potential interaction with model proteins was investigated. According to the procedure described by Cao et al.[57] measurements were carried out from an unbuffered aqueous solution of the proteins. In Figure 5.13 A the full scan spectrum of rh-IFNα2a is depicted. The highest relative abundance is observed for the 9-fold positively charged peak and the molar mass that can be derived from this spectrum (19254 g/mol) is in good accordance with literature values[58]. When using a 50/50-mixture of water and methanol the average charge state of IFNα2a is increased to higher values (+12-peak is dominating instead of +9-peak, spectrum not shown), indicating unfolding of the protein in the solvent[44]. As unfolding would distort measurement results, it was decided to perform all following experiments from aqueous solutions instead of from mixtures with Methanol.

When a ten-fold molar excess of the parent β-CD molecule is added to the solution of rh-IFNα2a, extra signals can be observed in the spectrum (Figure 5.13 B, signals are marked with a single dot). The mass difference between the extra peaks and the rh-IFNα2a peaks is exactly equal to the mass of a single β-CD molecule.

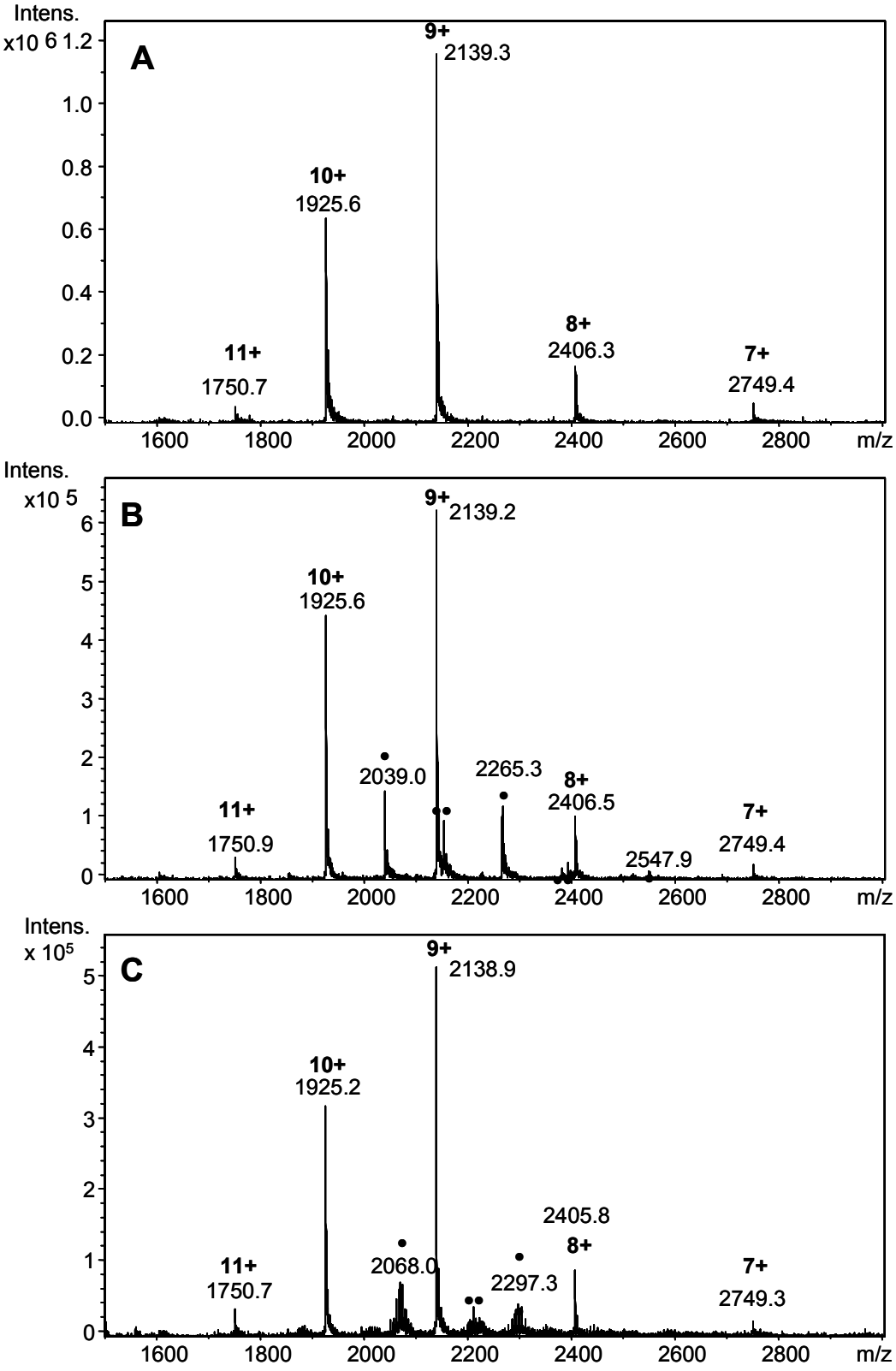

Figure 5.13: Full scan ESI-MS spectra of pure IFNα2a (A), an IFNα2a-mixture with native β-CD (B) and an IFNα2a mixture with HPβCD (C). Signals corresponding to 1:1 complexes are marked with a single dot above the respective peak, and 1:2-complex are marked with a double dot above the respective peak. The cyclodextrin molar excess was 10:1 and the experiments were performed from pure water.

In addition, weaker signals were observed whose mass difference to the signal of IFNα2a exactly corresponded to the mass of two β-CD molecules. Therefore 1:1 complexes and 1:2 complexes between rh-IFNα2a and native β-CD were detected when analyzing mixed

solutions of the two potential binding partners. Due to the significantly higher relative abundance of the 1:1 complex-signals as compared to the 1:2 complex-signals, it was assumed that a 1:1 binding stoichiometry between the native β-CD and IFNα2a is dominating.

When analyzing the spectra of mixed rh-IFNα2a-HPβCD solutions (Figure 5.13 C), extra peaks could be observed as well as compared to the pure rh-IFNα2a spectrum . As previously discussed, HPβCD is a chemically heterogeneous product and therefore not only isolated extra peaks, but a distribution of extra peaks is observed in the ESI-MS spectrum in the presence of HPβCD. Again, the mass difference to these extra-peaks can be clearly attributed to the added CD-derivative. A 1:1 binding stoichiometry was again dominating, but also peaks consisting of two HPβCD-molecules per rh-IFHα2a molecule were detected with a very weak intensity. Altogether the relative abundance of the complex signals was lower for the HPβCD-complexes than for the native β-CD complexes. However, this observation could not be related to a lower tendency of HPβCD to form complexes with rh-IFNα2a than the parent CD-molecule, but had to be attributed to the statistical fact that at the same molar amount of complexes in solution, the HPβCD-rh-IFNα2a complex signals were divided into a multitude of peaks corresponding to the different HPβCD-subspecies, whereas for the β-CD complexes only one distinct and therefore more intense peak resulted.

Effect of molar ratio and absolute concentration

In order to reduce the extent of unspecific “statistical” complex formation (due to the coincidental presence of a CD-molecule and an IFNα2a-molecule in the same droplet before solvent evaporation) and an accidental subsequent shift of the complexation equilibrium towards association, dilution series were carried out as suggested by literature[34,59,60]. The absolute concentration of the potential binding partners was lowered to half of the original concentration of 1 µg/mL and also the molar ratio between the binding partners was step wisely reduced until no more complexes could be detected. The reduction of the concentration of both binding partners did not effect complex formation (spectra not shown), but subsequent reduction of the molar excess of the “host” – binding partner (the CDs in this case) was efficient in discriminating between the different combinations of binding partners tested. Table 5.3 gives an overview on most of the tested combinations of binding partners including the control experiments that will be discussed in the following sections. In brackets, the minimum relative molar amount of the guest molecule compared to the host molecule is indicated at which a complex signal was still detectable. The absolute concentration of the guest molecule was kept constant at 1 µg/mL and the concentration of the host molecule was varied accordingly. It can be seen that when native β-CD is employed even at ten-fold excess compared to the concentration of IFNα2a, complexes were still detectable.

Table 5.3: Overview on the evaluation of complex formation between different cyclodextrins and potential guest molecules as well as on control experiments to elucidate the extent of unspecific gas-phase complex formation.

+ stands for weak but clearly detectable complexes (3 < signal to noise ratio <10); ++ stands for a signal to noise ratio > 10; +++ stands for a very strong complex signal with a relative abundance that exceeds that of the single components.

In brackets the minimum relative molar amount of the guest molecule compared to the host molecule is indicated at which a complex signal was still detectable. The absolute concentration of the guest molecule was kept constant at 1 µg/mL and the concentration of the host molecule was varied accordingly.

	"Host"							
"Guest"	**HPβCD**	**β-CD**	**α-CD**	**γ-CD**	**Maltoheptaose**	**Maltopentaose**	**Sucrose**	**Trehalose**
IFNα2a	**+** (2:1)	**+** (10:1)	**+** (1:1)	**+** (1:1)	**+** (1:1)	**+** (1:1)	**+** (1:10)	**+** (1:10)
Lysozyme	**+** (1:1)	**+** (1:1)	**+** (1:10)	**+** (1:1)	**+** (1:1)	**+** (1:1)	**+** (1:1)	**+** (1:1)
N-Acetyltryptophanamide	**++**	**+++** (10:1)	**+** (10:1)	**+++** (10:1)	**0**	**+**	**0**	**0**
Tryptophan	**+++**	**+++** (10:1)	**+++** (10:1)	**+++** (10:1)	**+++**	**+++**	**++**	**++**

For HPβCD complex formation was still detectable when measured at half the concentration of IFNα2a (molar ratio IFNα2a:HPβCD 2:1). However, all the complexes observed after reducing the molar excess of the CD-component to less than 10-fold were 1:1 complexes. Summing up these dilution experiments it can be concluded that the observed 1:1 complexes were specific (reflecting complexes from the solution phase), but that the 1:2 complexes of IFNα2a with the CDs were nonspecific since they quickly vanished after dilution.

Comparison of CD-derivatives

Comparing complexes detected for the various CD-derivatives tested, it can be seen from Table 5.3 that also α-CD and γ-CD - IFNα2a complexes were detected by ESI-MS. However, when using a lower molar concentration of these two CDs than the IFNα2a-concentration, complexes were no longer detectable, indicating that the formed complexes were not specific. HPβCD-complexes in contrast, were well detectable until, pointing towards a higher specificity.

Effect of solvent on complex detection

As pure water is an unusual solvent when dealing with protein formulation experiments, the binding experiments between IFNα2a and the different CDs were repeated from buffered solution in order to evaluate the effect of pure water. Experiments from an ammonium acetate buffered solution were well comparable those obtained from pure water (spectra not shown).

Rh-GCSF

Comparable binding experiments as with IFNα2a were also performed with rh-GCSF and the four CDs listed in Table 5.3. In order to keep the results and the amount of spectra clearly laid out, it is refrained from showing them here. In summary, rh-GCSF exhibited the identical binding behavior to HPβCD, β-CD, α-CD and γ-CD as IFNα2a: 1:1 binding stoichiometry dominated and 2:1 binding stoichiometry was only observed when a high molar excess of the CD-component was used.

3.3 CONTROL EXPERIMENTS USING LINEAR SUGARS

In order to further investigate the potential contribution of nonspecific binding to the signal of the cyclodextrin-protein peaks, comparisons to linear sugars were carried out. The linear sugars included maltoheptaose and maltopentaose as well as the disaccharides sucrose and trehalose.

Maltoheptaose was already employed as a negative control for the surface-plasmon resonance spectroscopy investigations discussed in the previous sections. The molecule is the linear analog of β-CD. Literature reports state that the equilibrium dissociation constants of maltoheptaose, e.g. for binding to fluorescent probes[61] or phenyl acetates[62], are significantly (2-3 orders of magnitude) smaller than those of its cyclic counterpart[63]. Although the linear sugar molecule possesses a turn of a dextrin helix and therefore exhibits some

micropolarity, it cannot form inclusion complexes in solution and the very weak interaction between maltoheptaose and guest molecules in solution is attributed to hydrogen bonds between the OH groups of the sugar and the guest molecule[34]. Maltopentaose is a shorter analog of maltoheptaose and should therefore possess similar properties.

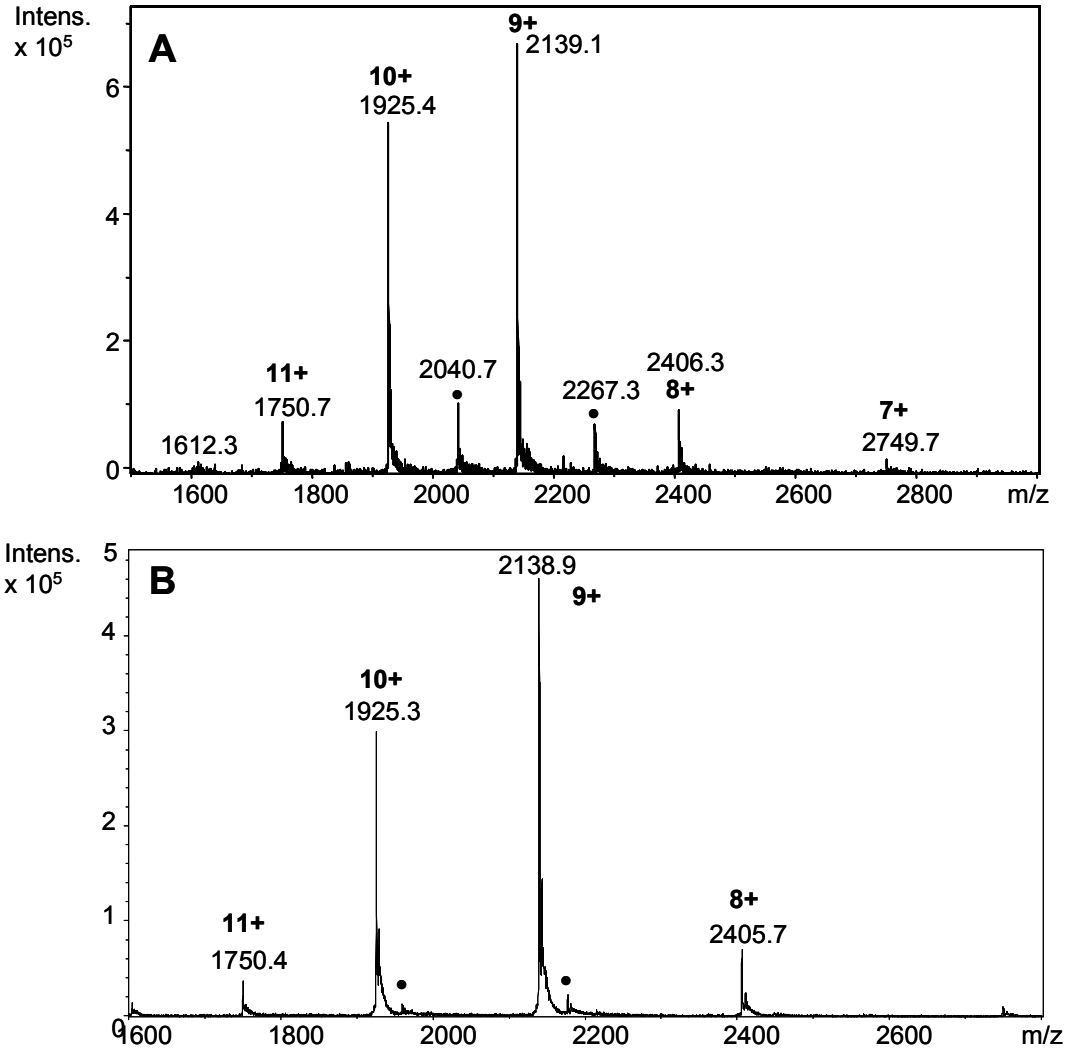

Figure 5.14: Full scan ESI-MS spectra of mixtures of IFNα2a and Maltoheptaose (A) and IFNα2a and sucrose (B). The solution in A was an unbuffered solution, B was sprayed from an ammonium acetate solution at pH 5. Carbohydrate molar excess was 10:1 in both experiments.

Figure 5.14 depicts ESI MS spectra of rh-IFNα2a with linear saccharides. In contrast to theoretical argumentation, from Figure 5.14 A it can be seen, that complexes between rh-IFNα2a and maltoheptaose could also be detected, despite its significantly weaker complexation potential as compared to β-CD. Thus, since maltoheptaose as the linear analog of β-CD without the hydrophobic cavity for the inclusion of guest compounds, shows complex signals in the presence of IFNα2a, it can be concluded that nonspecific binding contributes to complex formation between IFNα2a and cyclodextrins. However, in contrast to complex formation with β-CD and HPβCD, no complex signals were observed at sugar concentrations below the (molar) concentration of IFNα2a (Table 5.3) which means that, also in this case, the less specific binding is reflected by vanishing complex signals upon dilution,

whereas specific binding is preserved at low concentrations . For maltopentaose the identical behavior as for maltoheptaose was observed (Table 5.3).

As previously discussed, the disaccharides sucrose and trehalose belong to the group of preferentially excluded excipients. Therefore no interaction between rh-IFNα2a and the two disaccharides in solution takes place: repulsive forces between the protein backbone and the sugars dominate[64]. Nevertheless, as observable from Figure 5.14 B, showing a representative scan of IFN2a and sucrose, complex peaks between sucrose and rh-IFNα2a appear when sucrose is applied in a ten-fold molar excess. However, the intensity of the complex peaks is very weak, almost at the limit of detection. Upon reduction of the sugar concentration no more peaks can be observed (Table 5.3).

Summarizing, it was shown that at sufficiently high concentrations of the potential binding partner of the protein, complexes are formed due to unspecific interaction. However, it was also confirmed that a change in the concentration ratios of the two binding partners provides a good means to discriminate unspecific complex formation from more specific adducts.

3.4 CONTROL EXPERIMENTS USING AMINO ACIDS

In order to gain further understanding of the extent of unspecific contributions to the formation of complexes in the gas phase, further control experiments were performed. The focus of these experiments was on an estimation of the importance of basicity of the involved compounds.

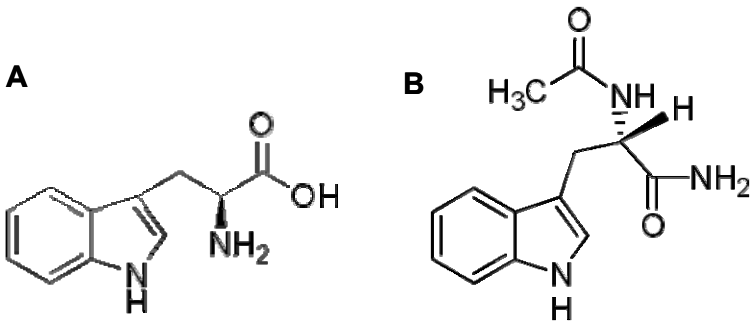

Figure 5.15: Chemical structures of the amino acid L-tryptophan (A) and its derivative N-acetyltryptophanamide that lacks the basic primary amino group.

Binding experiments with CDs and amino acids and monitoring by ESI-MS in order to elucidate the extent of unspecific binding were conducted earlier by Cunniff and Vouros[51]. Their studies were carried out with amino acids, which carry free amine functions and either aromatic or non-aromatic residues and both types of amino acids were found to form complexes with β-CD and its derivatives[51]. In search of model compounds that allow an investigation of the importance of basicity that even more resembled the actual situation in a protein, the amino acid L-tryptophan and its derivative N-acetyl-tryptophanamide were investigated (Figure 5.15). Since both compounds carry aromatic residues they should be expected to form inclusion complexes with β-CD and its derivatives in solution. However,

since L-Tryptophan also exhibits a free amine function, it is likely to additionally form electrostatic adducts with β-CDs. This complexing behavior is confirmed by Figure 5.16. Whereas L-Tryptophan forms intense complex peaks with all degrees of hydroxypropylated β-CD (Figure 5.16 A), complex peaks between the non-basic N-Acetyltryptophanamide and HPβCD can only be detected for the 5-fold substituted HPβCD molecule. Nevertheless, the signal is clearly detectable and its presence has to be attributed to specific hydrophobic interaction in solution.

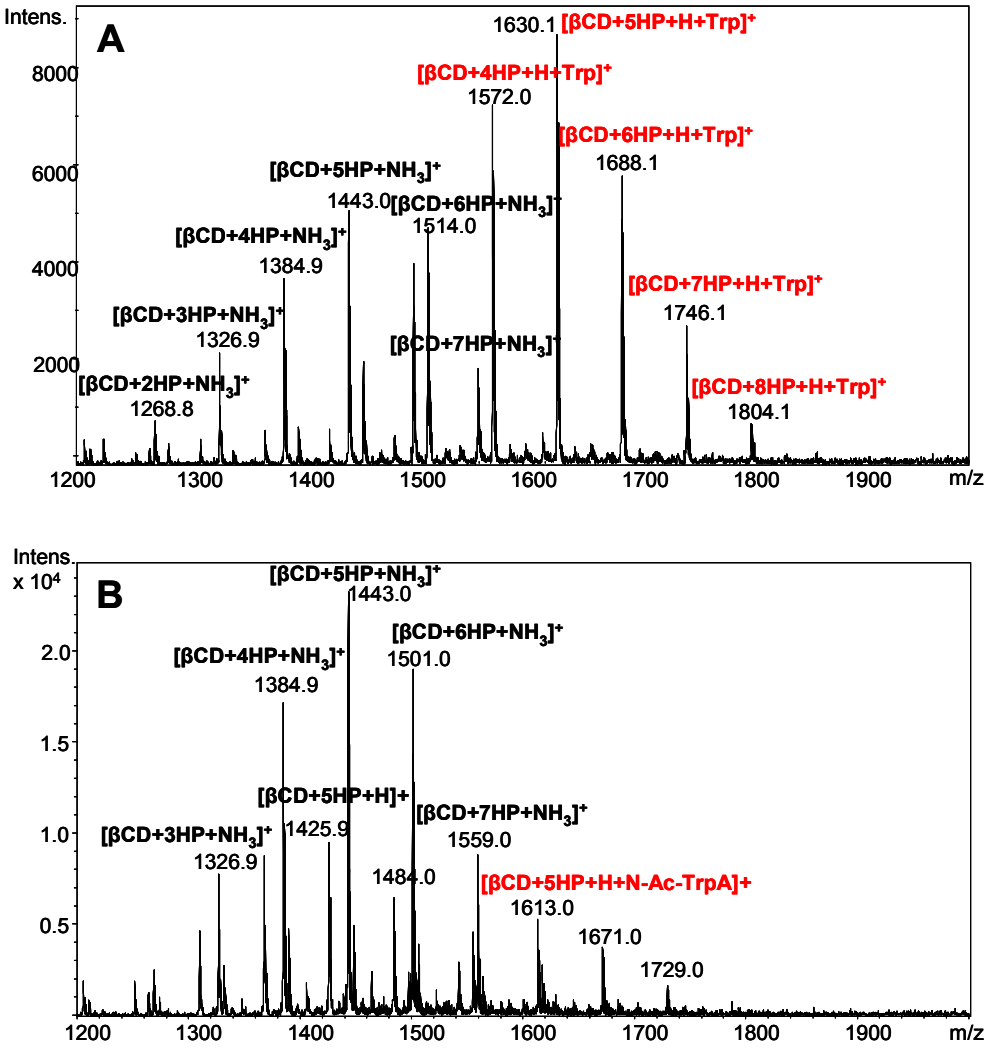

Figure 5.16: Full scan ESI-MS spectra of mixtures in a 1:10 molar ratio of HPβCD and the amino acid tryptophan (A) and of HPβCD and the amino acid derivative N-acetyltryptophanamide (B). The bold black labels represent HPβCD in varying degrees of substitution and the respective ammonium adducts. The bold red labels are attributed to complexes of HPβCD with either tryptophan (A) or N-acetyltryptophanamide (B).

The importance of basicity on the detected complexes is further highlighted by control experiments performed with maltoheptaose, which as a linear sugar likely does not form inclusion complexes in solution with the aromatic residues of the model compounds: For N-Acetyltryptophanamide no clear complex signal was obtained that could be attributed to maltoheptaose-N-Acetyltryptophanamide complexes (Figure 5.17 B). On the other hand, strong complex signals were obtained for the mixed solution of L-Tryptophan and

maltoheptaose (Figure 5.17 B). In turn, these complex peaks have to be regarded as purely unspecific.

In summary, the experiment demonstrates that basicity strongly contributes to unspecific binding, but that hydrophobic interaction can nevertheless be “hidden” behind the unspecific complex signals. The necessity for adequate control experiments is clearly highlighted.

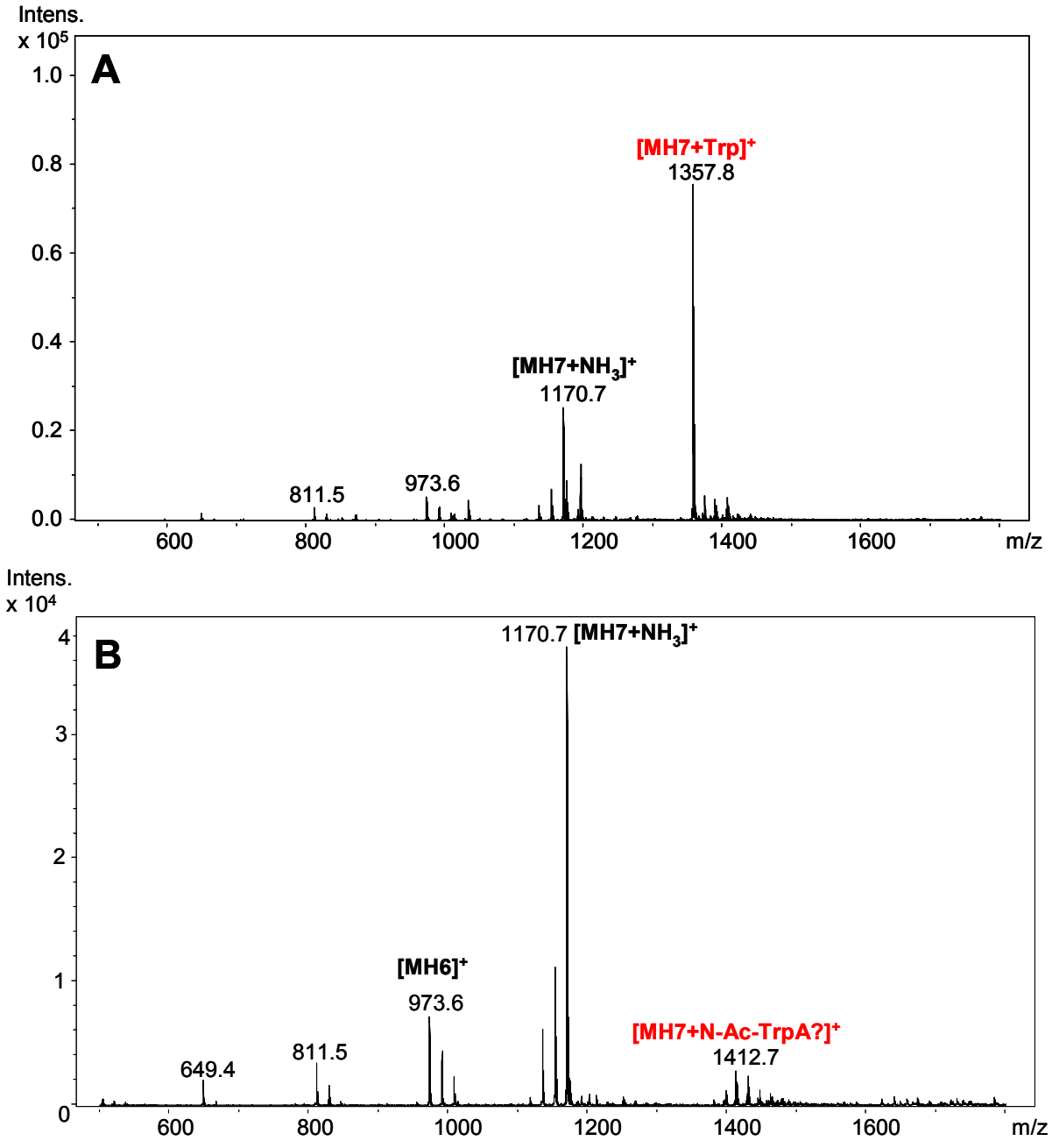

Figure 5.17: Full scan ESI-MS spectra of mixtures in a 1:10 molar ratio of maltoheptaose (MH7) and the amino acid tryptophan (A) and of maltoheptaose and the amino acid derivative N-acetyltryptophanamide (B). The bold black labels represent the ammonium adduct of maltoheptaose and its degradation product with one glucose residue less. The bold red labels are attributed to complexes of HPβCD with either tryptophan (A) or N-acetyltryptophanamide (B).

3.5 BINDING TO LYSOZYME

Finally, mixed solutions of cyclodextrins and a protein with documented affinity for cyclodextrins , lysozyme, was investigated[38,65,66]. By monitoring the binding behavior of a protein with a known affinity for CDs by ESI-MS, it was intended to draw parallels to the binding behavior of rh-GCSF and rh-IFNα2a to CDs as observed in the spectra that were previously discussed. Thereby, it was intended to further evaluate to which degree rh-IFNα2a and rh-GCSF bind to CDs in solution. As exemplarily demonstrated in Figure 5.18, 1:1-complexes between lysozyme and HPβCD could be clearly identified. When comparing the binding behavior (including the minimum molar ratios of the binding partners to form complexes with lysozyme) of lysozyme to that of IFNα2a, a very similar pattern was observed. Therefore, when taking into account that lysozyme was already demonstrated to bind to cyclodextrin-derivatives in solution, it can be assumed that also IFNα2a binds to CD-derivatives in solution to a very similar degree as lysozyme.

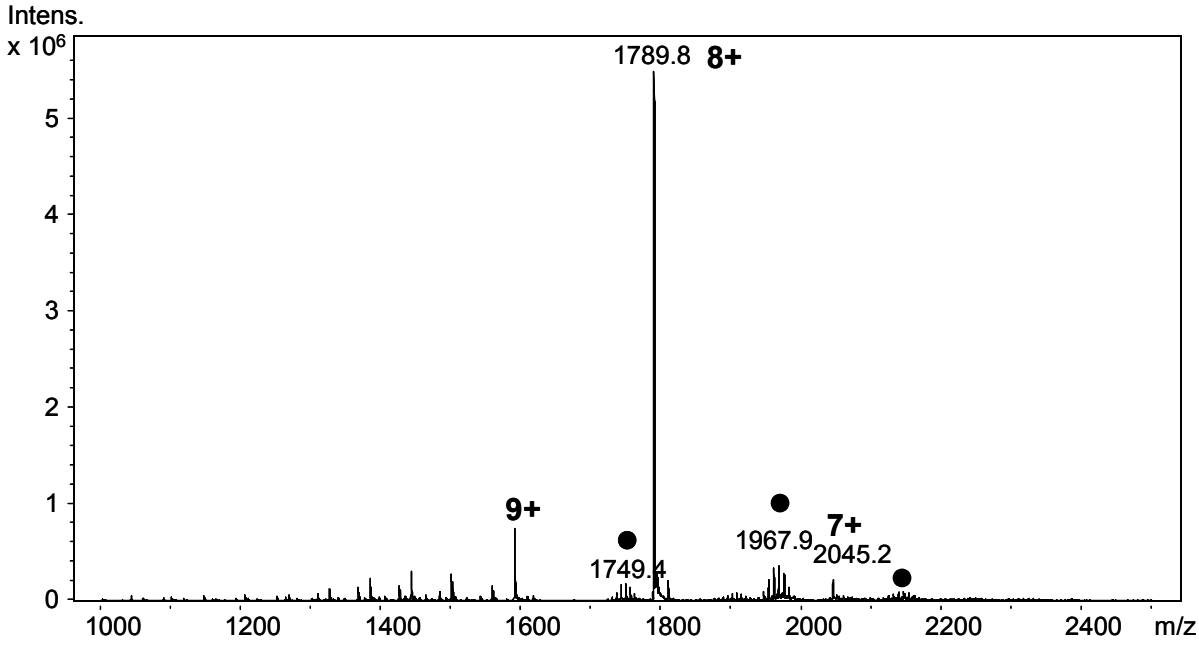

Figure 5.18: Full scan spectrum of a mixture of lysozyme and HPβCD in a 10:1 molar ratio. The bold labels indicate the respectively charged pure lysozyme peaks and the dots are attributed to the respective complexes of lysozyme and HPβCD.

3.6 SUMMARY AND CONCLUSIONS

The ESI-MS experiments discussed in this chapter showed that a 1:1 binding stoichiometry dominates binding between CD-derivatives and proteins. Higher order complexes were also observed in a few cases. However, their existence was very likely attributed to the high molar excess of the CD-derivatives in these cases and resulting gas phase artifacts.

Unspecific binding between CD-derivatives and the proteins under investigation certainly also contributed to the complex signals as evidenced e.g. by the binding of non-cyclic oligosaccharides. However, by systematic variation of the molar ratios of the binding partners

it seems possible to distinguish between purely unspecific and specific interaction that also takes place in solution. Control experiments performed with low molecular weight model compounds pointed out, that basicity of the binding partners played an important role in the formation of unspecific gas-phase artifacts.

Summarizing, ion trap ESI-MS was shown to be a valuable method for the determination of the stoichiometry of CD-protein interaction. However, when trying to unambiguously clarify whether CD-protein interaction in solution takes place (and to which extent), other methods such as surface-plasmon resonance spectroscopy are preferred.

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CHAPTER 6

MECHANISTIC STUDIES ON THE INTERFACIAL BEHAVIOR OF CYCLODEXTRINS

1 INTRODUCTION

In Chapters 3 and 4 of this thesis it was demonstrated that cyclodextrins are promising excipients for the prevention of protein aggregation at surfaces, as provoked for example by agitation of the protein solutions leading to exposure of the protein to the air-water interface. In contrast to the other CD-derivatives tested, HPβCD did also not negatively influence the stability during quiescent storage at elevated temperature of the investigated proteins, a phenomenon frequently observed with other excipients that stabilize proteins at interfaces, such as non-ionic surfactants. In addition, HPβCD possesses a favorable toxicological profile as excipient for parenteral administration considering that very low concentrations of HPβCD were sufficient for complete stabilization against aggregation (2.5 mM) and that doses as high as 6 – 8 g of HPβCD are administered to patients in approved parenteral products[1]. Therefore HPβCD was identified as the most promising CD-derivative for use in protein formulations.

The stabilizing effects of HPβCD with regard to surface-induced aggregation discussed in Chapters 3 and 4 qualitatively resemble those observed for polysorbate 80. CDs, like non-ionic surfactants, are known to potentially bind to hydrophobic parts of proteins and most often the potency of CD-derivatives to suppress protein aggregation is ascribed to their ability to incorporate hydrophobic protein residues in their interior cavity[1-2]. Some CD-derivatives are also reported to be surface-active like polysorbates. Thus, as already discussed at the beginning of Chapter 5, the overall hypothesis for the mechanistic investigations in Chapters 5 and 6 of this thesis was that HPβCD functions in manners analogous to non-ionic surfactants in mAb-formulations. Therefore the mechanistic approach of the experiments discussed in these two chapters is intended to identify or exclude the potential mechanisms by which CDs stabilize the IgG against aggregation at the air-water-interface.

For the model proteins IgG and rh-GCSF binding to cyclodextrin-derivatives in bulk solution could be excluded by the results discussed in the previous Chapter 5. It was shown that those cyclodextrins that well inhibited surface-induced aggregation of the two model proteins, showed no or only very minor binding to the proteins. Even more, SBEβCD, the derivative that in most experiments completely failed to inhibit protein aggregation at the air-water interface or even accelerated aggregation, was shown to bind to rh-GCSF as well as the IgG in solution over a wide concentration range. Obviously no correlation between binding in the bulk solution and stabilization could be established. Therefore the first part of the hypothesis

to be tested, CDs potentially stabilizing proteins by binding to exposed hydrophobic parts on the protein surface in the bulk solution, can be regarded as experimentally disproved.

Hence the experiments in the present chapter focus on the second part of the hypothesis, cyclodextrins acting at the surface like non-ionic surfactants, i.e. by displacing the protein due to competition at the air-water interface.

For polysorbate-protein mixtures, many studies are available linking protein stabilization against surface-induced aggregation to the mechanism of competition at the air-water interface[3-9]. In addition, detailed mechanistic studies are available on mixed polysorbate 80 – protein model systems that allow a thorough understanding of the adsorption processes at the air-water interface since the studies were carried out with a variety of different techniques that are capable to monitor adsorption processes at the air-water interface[9-11]. For example, adsorption of polysorbate 80 in the presence of the large protein recombinant Factor VIII (280 kDa) was studied using a Wilhelmy Plate tensiometer[9]. It was found that the steady state interfacial behavior was entirely governed by surfactant adsorption, when the mixture contained more than 18 ppm polysorbate 80. Although the equilibrium surface tension of the polysorbate-protein mixture had nearly identical equilibrium values as the pure polysorbate 80 solution, the rate of adsorption to the interface was significantly faster in the mixture. Furthermore, it could be shown that also solution binding of polysorbate 80 to recombinant Factor VIII influences the adsorption behavior of the mixture. Another study investigated the rheological, structural and mechanical properties of mixed adsorption layers comprised of bovine serum albumin (BSA) and polysorbate 80 at the air/water interface[10]. Not only tensiometry was applied but also shear rheometry and ellipsometry, assessing e.g. the adsorption layer thickness, were used for these investigations. The study confirmed competitive adsorption between BSA and polysorbate 80 with almost complete displacement of the protein at high polysorbate 80 concentrations. However, it was demonstrated that some BSA-molecules remained in the subsurface layer, slightly influencing the absolute values of the surface tension as well as average adsorption rates as determined by ellipsometry.

However, no such studies are available neither for monoclonal antibodies nor for rh-GCSF or rh-GH. Polysorbate 80 was frequently reported to stabilize monoclonal antibodies[12-13] and stabilization by competition at the air-water interface was implicitly assumed, however never directly demonstrated. Other studies investigated binding between different subtypes of mAbs and polysorbate 80 in the bulk solution as a possible explanation for aggregation inhibition[14-15]. However, no binding was observed and therefore it was concluded that other mechanisms hold responsible for aggregation inhibition of monoclonal antibodies by polysorbate 80. One study[16], that actually did investigate mixed polysorbate 80-IgG solutions, even concluded that polysorbate 80 only formed mixed surface layers with the IgG but does

not completely displace it from the surface, quite in contrast to another non-ionic surfactant investigated in that same study, Cremophor EL®. However, the results of the investigation may be challenged, since, for example, the surface tension of pure water for injection was determined as 53.2 mN/m, which might point towards some experimental inadequatenesses of the study.

Hence, before testing the hypothesis that HPβCD stabilizes proteins against interfacial aggregation in manners comparable to non-ionic surfactants, it first had to be demonstrated that non-ionic surfactants (polysorbate 80 throughout the investigations of this thesis) did actually stabilize the investigated proteins by competition at the air-water interface.

For cyclodextrins a lot less hints are available in literature that competition at the air-water interface with proteins could take place. First of all, a number of studies indicate that cyclodextrins, and most notably also HPβCD, which our investigations are focused on, are actually surface-active, which is a necessary prerequisite for competition at the interface[17-21]. Surface activity of MβCD and HPβCD was reported to strongly depend on the degree of substitution[18,20-21]. For instance for HPβCD values between 69 mN/m and 52 mN/m are reported for degrees of substitution ranging from 2.5 to 11.3, respectively. Since HPβCD (Cavasol®) used for our experiments has an average degree of substitution of about 5-6 (refer to the ESI-MS results in the previous Chapter 5) surface activity can be assumed as well. In addition to cyclodextrins being reported as surface active there are also two studies that relate the inhibition of surface-induced aggregation by HPβCD to the surface-activity of the excipient[17,20]. The effectiveness of HPβCD in reducing interfacially induced precipitation of porcine growth hormone was ascribed to the surface activity of HPβCD and it was speculated that the mechanism was analogous to that proposed for the stabilization by polysorbate 20[17]. 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. However, apart from these rather speculative explanations, no sound studies are available that examine in detail the composition of surface layers of mixed cyclodextrin-protein formulations.

It was decided to concentrate the detailed mechanistic investigations on two model systems. The monoclonal antibody, representing the currently most widespread class of therapeutic molecules, was chosen as a model-protein. HPβCD, which was identified as the most promising CD-derivative for IgG-stabilization and polysorbate 80, representing a standard non-ionic surfactant were investigated as stabilizers, allowing the comparison to studies discussed in the earlier chapters of this thesis.

In order to obtain a comprehensive picture of the situation at the air-water interface, adsorption and surface rheological parameters were characterized by tensiometry (using a Wilhelmy plate, drop profile analysis and maximum bubble pressure method) as well as dilational and shear rheometry.

In brief, the main objectives to be addressed in this chapter were to

- characterize the adsorption kinetics of polysorbate 80, HPβCD and the IgG as well as their respective mixtures over a wide range of concentrations at short time scales as well as during equilibrium adsorption in order to elucidate the surface layer composition of the formulations.
- describe the surface rheological properties of polysorbate 80, HPβCD and the IgG as well as their respective mixtures to supplement the information obtained by tensiometry.
- confirm the assumption that polysorbate 80 competitively displaces the IgG at sufficiently high concentrations
- compare the adsorption behavior of polysorbate 80 in the presence of the IgG to that of HPβCD in the presence of the IgG and draw mechanistic conclusions on the stabilization behavior of mAb-formulations by HPβCD and understand why HPβCD can even be superior to polysorbate 80 for the inhibition of aggregation of the IgG at the air-water interface.

2 EXPLORATORY EXPERIMENTS USING A WILHELMY PLATE INSTRUMENT

Initial surface tension measurements were carried out using a simple Wilhelmy plate instrument, in order to obtain basic information about the behavior of CDs at the air-water interface in absence and presence of a mAb. In addition, first comparisons to polysorbate 80 were carried out. As described in the materials and methods section of this thesis the surface-tension recordings were conducted for only 240s which is by far shorter than the time that is necessary for establishing an equilibrium condition of the mAb at the interface (> 12h, data not shown). However, since it was intended to relate the observed surface-tension to the results of agitation studies, in which constant renewal of the interface takes place, it seemed reasonable to choose short measurement times that better reflect the situation of the agitation-experiment than equilibrium data. The 240 s interval was chosen because it provided the shortest period of time in which it was possible to achieve acceptably reproducible results on the instrument.

Table 6.1: Surface tension of different mAb-formulations as determined by the Wilhelmy-Plate-method after 240 s of measurement time.

Formulation	σ [mN/m] buffer	σ [mN/m] with mAb
His 20mM no excipient	73.00 ± 0.51	61.53 ± 0.19
HPβCD 0.25mM	66.51 ± 0.46	62.25 ± 0.42
HPβCD 2.5mM	61.74 ± 0.29	59.15 ± 0.72
HPβCD 25mM	59.32 ± 0.34	57.70 ± 0.72
Polysorbate 80 0.04%	37.42 ± 0.44	38.76 ± 0.14
Polysorbate 80 0.004%	43.96 ± 0.86	43.35 ± 0.18

Table 6.1 summarizes surface tension values of different formulations with and without mAb. HPβCD lowers the surface tension indicating an accumulation at the interface (Table 6.1). From the results it can be roughly concluded that saturation of the interface with HPβCD is reached at a concentration of about 2.5 mM as the decrease of the surface-tension with increasing HPβCD-concentrations starts to level out. This concentration coincides with the minimum concentration needed for complete protection of the mAb against agitation-induced aggregation, as described in detail in Chapter 3.

As expected, the surface tension measurements clearly show that the mAb accumulates at the air-water interface (reflected by a decrease of the surface tension by 11.47 mN/m). Since both, HPβCD and the IgG, concentrate at the air-water-interface, competition between the two molecules at the interface can potentially occur. However, it is yet unclear whether HPβCD can actually displace the mAb from the interface. This uncertainty is further substantiated by the fact that the surface tension of the solutions containing both the IgG and HPβCD (at ≥ 2.5 mM, i.e. after saturation of the interface) is only slightly lower (≤ 3.83 mN/m)

than the surface tension of the solutions containing either one of the single components. If both components concentrated at the interface to the same extent as in absence of the respective other component the decrease of the surface tension would be more pronounced. However, from this data it cannot be determined which component actually dominates the surface layer. Further experimental techniques will be necessary to clarify this question.

For polysorbate 80-IgG solutions the situation is less unambiguous since the surface-tension of the mixture matches almost exactly that of the pure polysorbate 80 solution which can be taken as a hint that at both polysorbate 80 concentrations investigated the surfactant dominates the surfaces of the mixture. Interestingly, polysorbate 80 even at the lowest concentration tested caused a more pronounced decrease of surface tension than HPβCD did even in its highest concentration. However, as discussed in Chapter 3, this lower concentration of polysorbate 80 was not sufficient to completely stabilize the IgG against aggregation-induced agitation. Thus the extent of surface tension decrease alone cannot be used as an explanation of the stability of a formulation against aggregation at the air-water-interface. Also, the surface tension increment alone might not directly correlate with inhibition of protein adsorption to the air-water interface, especially when taking into account the time-scale (very short) at which the air-water-interface is renewed during agitation. Presumably, mass-transfer limitations due to the relatively low polysorbate 80 concentration compared to that of HPβCD could result in slower equilibration kinetics of polysorbate 80 vs. HPβCD at newly formed air-water interfaces. In order to clarify this question, further investigations of the adsorption behavior on a shorter time scale than in this experimental set-up are needed, reflecting time scales encountered during agitation.

In brief, the following conclusions can be drawn from the surface-tension measurements using the Wilhelmy-plate technique:

- HPβCD accumulates at the air-water interface, but it is clearly less surface-active than polysorbate 80.
- The data does not clarify whether HPβCD actually competes with the IgG for adsorption at the interface and which component dominates the interface of an IgG-HPβCD mixture.
- The surface tension increment alone does not seem to explain the capability of an excipient to inhibit aggregation at the air-water interface.
- The surface tension after very short adsorption times is difficult to be recorded by this technique.
- Further experimental techniques are required to characterize the behavior of HPβCD at the air-water interface in absence and presence of the IgG.

3 MAXIMUM BUBBLE PRESSURE EXPERIMENTS AT SHORT ADSORPTION TIME SCALES

In the discussion of aggregation at the air-water interface, it is usually assumed that by agitation a constant "renewal" of the air-water interface takes place[12,22-24], although this assumption has never been directly validated. In this context renewal refers to a mechanical destruction of the surface and not only to expansion and compression of the surface layer. Therefore, when trying to understand the effects of polysorbate 80 and HPβCD on agitation-induced mAb aggregation, it is an obvious step to select an analytical technique that allows monitoring the surface-tension of the formulations in their actual concentrations as employed in the agitation experiments (which was provided by the Wilhelmy plate experiments) as soon as possible after the formation of the surface (which was not possible using the Wilhelmy plate method). Also, since mass-transfer limitations of polysorbate 80 and resulting slower equilibration kinetics were discussed as a possible reason for polysorbate 80 (0.004 %) to stabilize the mAb less effectively than HPβCD (although the drop in surface tension after 240 s is a lot more pronounced in the polysorbate 80 solution than for the HPβCD solution) analyzing surface tension on a short time scale appears to be a promising approach to explain the good stabilizing properties of HPβCD.

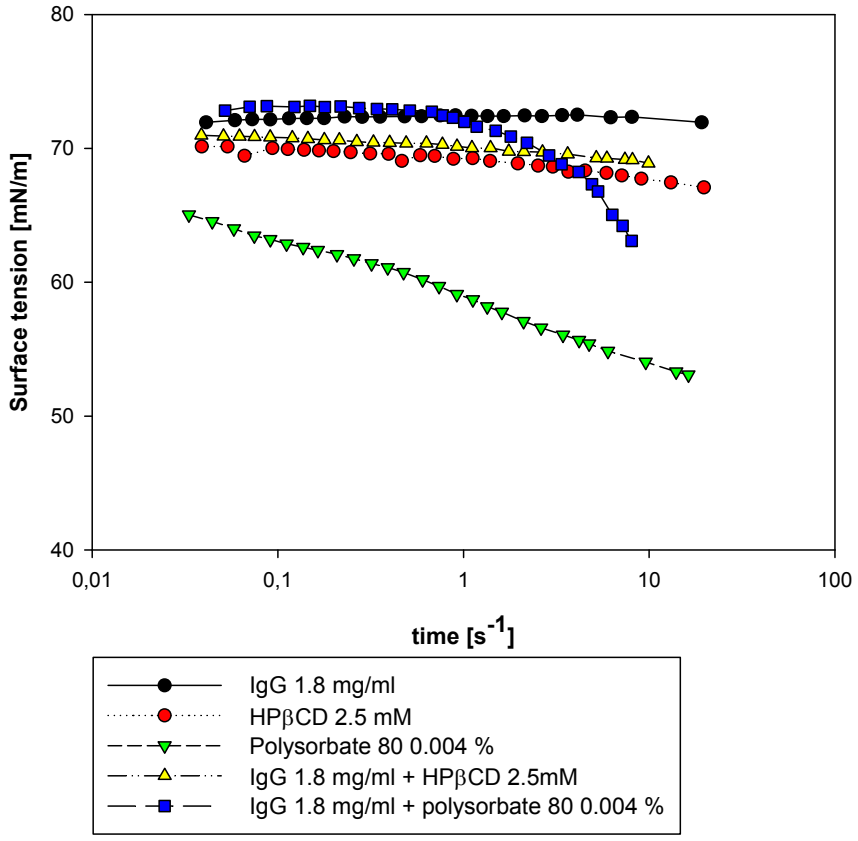

Figure 6.1: Dynamic surface tension of solutions of polysorbate 80, HPβCD and the IgG as well as their respective mixtures (in the identical concentrations as in the agitation experiments described in Chapter 3) as determined by the maximum bubble pressure technique.

The maximum bubble pressure method (MBPM) is frequently employed for the study of surface tensions over a wide surface lifetime range from milliseconds to several seconds[25-30].

Therefore the MBPM provides a valuable tool to monitor the adsorption of polysorbate 80 and HPβCD to newly formed surfaces in the presence and absence of the mAb. Figure 6.1 shows the dynamic surface tension of HPβCD, polysorbate 80 and the IgG in histidin buffer in the concentrations that were also used in the agitation study in Chapter 3. It can be seen that polysorbate 80 in the absence of the IgG lowers the surface tension much faster and to a higher degree than HPβCD. Even at the first value that was recorded (33 ms) the surface tension of the polysorbate 80 solution is already substantially decreased compared to the surface tension of the pure His buffer (between 72.6 mN/m and 73.4 mN/m depending on the experiment, data not shown). This is an indication that the de novo surface is very rapidly occupied by polysorbate 80 when employed at this concentration ($3*10^{-5}$ mol/L = 0.004 %)). In contrast, HPβCD only leads to a very slight decrease of surface tension during the experiment. However, the surface tension values also start at values slightly below that of the pure His buffer and also slightly below the value recorded for the IgG in absence of any excipients, but the differences are so small, that they can not be regarded as significant considering the experimental error.

Interestingly, in the presence of the IgG the adsorption of polysorbate 80 exhibits a lag phase of about 1 s before a measurable decay of the surface tension can be observed. Thus it can be concluded that polysorbate 80 diffusion to the surface is slowed down in the presence of the IgG. This lag phase can be interpreted as a hint for a decrease of the effective concentration of free polysorbate 80 that is available for adsorption to the interface. The observation of a lag phase could be interpreted in a way that initial binding to the IgG occurs and only after dissociation from the loose complex polysorbate 80 can reach the surface. This behavior is further confirmed when monitoring the adsorption process at the same IgG-concentration but at lower polysorbate 80 concentrations (Figure 6.2). In the presence of the IgG the lag time for surface adsorption of polysorbate 80 was even extended and once adsorption had started the rate became slower with decreasing polysorbate 80 concentrations. The respective solutions without IgG did not exhibit this behavior and adsorption had already started with first recorded surface tension values.

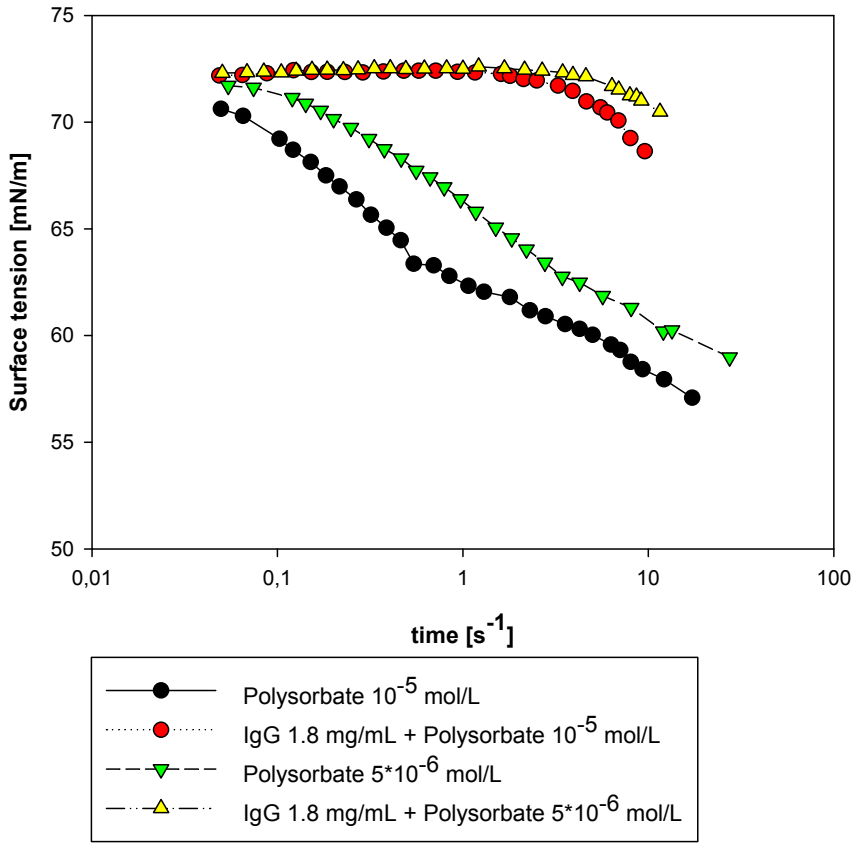

Figure 6.2: Dynamic surface tension of solutions of pure polysorbate 80 and IgG-polysorbate 80 mixtures as determined by the maximum bubble pressure technique.

In summary, also in the presence of the IgG, polysorbate 80 coverage of the surface occurs faster and to a greater extent than HPβCD coverage in the presence of the IgG. Therefore explanations such as limitations in mass transfer of polysorbate due to its low concentration that were supposed to account for the relatively good stabilization behavior of HPβCD (although its surface tension decrease is significantly smaller than of polysorbate 80) do not apply. Perhaps the postulated constant renewal of the interface does not take place and molecules that are once adsorbed remain at the surface for a longer time than expected and agitation processes mainly cause deformation of the surface but not necessarily a complete destruction. From this data only few hints at all are obtained on the stabilization mechanism of HPβCD, notably no hints on the original question whether a displacement mechanism of the IgG applies or not. Therefore different experimental approaches have to be chosen in order to reveal the surface adsorption behavior of the IgG-formulations in presence of HPβCD and polysorbate 80.

In summary, the following main conclusions can be drawn from the experiments using the maximum bubble pressure method:

- Polysorbate 80 (even in the lower concentration tested) adsorbs faster to the air-water interface than HPβCD.

- Therefore it can be assumed that HPβCD does not cover newly formed air-water interfaces (as encountered during agitation processes) faster than polysorbate 80.
- Other principles than fast surface coverage explain the good stabilizing properties of HPβCD during agitation.

4 SURFACE TENSIOMETRY BY DROP PROFILE ANALYSIS

The experiments carried out using Wilhelmy plate tensiometry and the maximum bubble pressure method allowed only some insight into the behavior of HPβCD at the air-water interface. It could be shown by Wilhelmy plate tensiometry that HPβCD does possess some surface activity. However, it still remained unclear whether HPβCD leads to a competitive displacement of the mAb from the interface in a manner comparable to polysorbate 80 or whether different mechanisms account for the stabilization of the IgG. Hints for the assumed competition of polysorbate 80 and the mAb at the air-water interface were obtained, but the study still lacked details such as the minimum concentration needed for displacement to take place. In order to clarify these questions, a wider experimental approach was chosen. The basic idea was to investigate in more diluted solutions than in the actual formulations in order to create conditions under which the adsorption processes and possible competition mechanisms occur at a slower time scale which can actually be studied in detail by the available methods. By making the adsorption behavior visible at lower concentrations it was intended to gain detailed mechanistic information on the adsorption behavior that then allows to draw conclusions about the actual formulations by extrapolating to higher concentrations and hence faster adsorption rates to the interface.

Before studying the mixtures of HPβCD, polysorbate 80 and the IgG every single component was investigated in absence of other components besides histidin buffer at pH 5.8 that was also employed in the formulation studies. Subsequently, the respective mixtures were investigated and the adsorption profiles of the mixtures were then compared to those of the isolated components. Surface tensions and, as discussed in the following section, also surface rheological parameters were investigated using drop profile tensiometry. Compared to ring tensiometry it has the advantage that no further interface (e.g. the platinum-water interface in the Wilhelmy-plate instruments) is introduced into the investigated system: it is a contactless method and results in a higher accuracy compared to contact methods, such as ring or plate tensiometry[31].

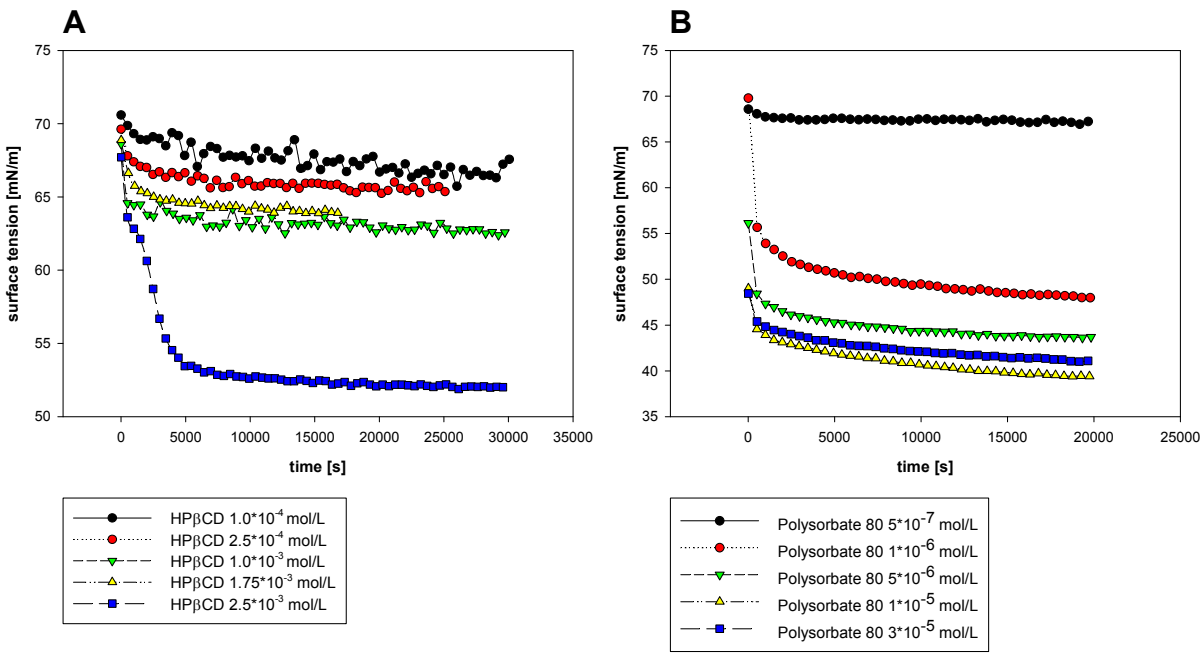

Figure 6.3: Dynamic surface tension of increasing concentrations of HPβCD (A) and of polysorbate 80 (B) as determined by drop profile analysis on a PAT 1 – instrument.

In Figure 6.3 A the adsorption kinetics of increasing concentrations of HPβCD in histidin buffered solution are shown. Again, it is confirmed that the molecule possesses some (comparably weak) surface activity as can seen from the drop in surface tension with increasing HPβCD-concentrations. Equilibration at the air-water interface is rather slow with equilibrium condition never attained earlier than 20,000 s (roughly five and a half hours). At the lower concentrations of HPβCD (≤ 1.75 mM) the equilibrium surface-tension never falls below 60 mN/m and it seems as if the isotherm reaches a plateau at about 1.75 mM. However, at an HPβCD concentration of 2.5 mM a sharp drop of surface tension can be noticed compared to the lower concentrations and equilibrium surface tension reaches values of approximately 52 mN/m. At higher concentrations of HPβCD than 2.5 mM the surface tension remains at comparably low values and in some repetitions of the experiment at 2.5 mM the drop in surface tension occurred at a later time point (> 10000 s) of the experiment than in the demonstrated case (data not shown). All equilibrium surface tensions that were observed in this experiment lay in the same range as the values that were determined earlier by different methods, as published in literature[18-21]. It is unclear why this sharp drop in surface tension occurs at values of about 2.5 mM HPβCD. One explanation could be the tendency of cyclodextrins to self-associate in solution at higher concentrations[32-33]. A tendency towards association in solution was reported for concentrations beyond 0.5 % (which is equal to about 3.5 mM HPβCD)[32]. Therefore the investigated concentration of 2.5 mM is at the threshold for the formation of self-associates. Considering that these associates are reported to collapse easily under the effect of shaking, temperature or sonication, it becomes clear why the surface tension drop occurred at varying time points of the experiment and in one case was even reversed during the experiment (data not shown).

The adsorption profile of polysorbate 80 that is shown in Figure 6.3 B is characterized by almost no surface activity of polysorbate 80 at the lowest concentration investigated ($1*10^{-7}$ mol/L) and an already fast adsorption and a clearly decreased equilibrium surface tension of 47.95 mN/m at twice that lowest concentration, indicating significant surface activity. The lowest equilibrium surface tension is achieved at $1*10^{-5}$ mol/L. At concentrations above $1*10^{-5}$ mol/L the surface tension increases again. Therefore at $1*10^{-5}$ mol/L a kink point in the surface tension isotherm (Figure 6.5 A, the dark brown curve which includes additional concentrations that were left out in Figure 6.3 for the sake of clarity) can be observed that indicates that the CMC is reached at that point. Due to the chemically heterogeneous nature of polysorbate 80 and the different kinds of buffers employed for the investigations reported in literature, the CMC-values for polysorbate 80 that are reported in literature vary significantly but the $1*10^{-5}$ mol/L determined for the present system are in the (lower) range of reported values[5,10,34-35].

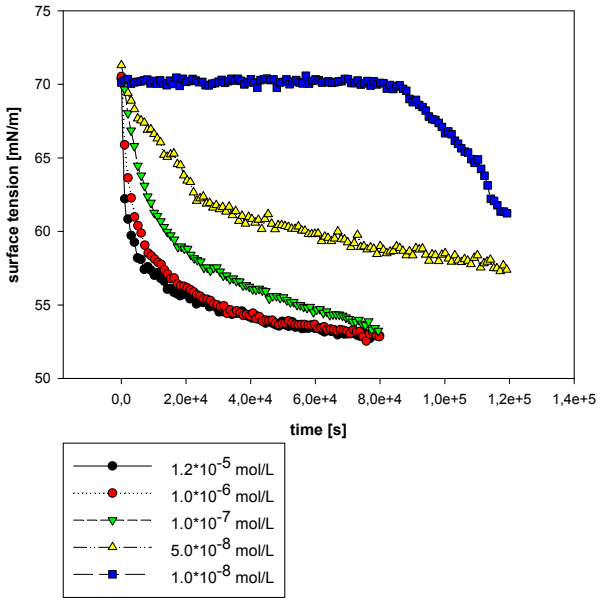

Figure 6.4: Dynamic surface tension of increasing concentrations of the IgG as followed by drop profile tensiometry.

In Figure 6.4 the dynamic surface tension of the IgG at different concentrations is shown. It can be seen that at the lowest investigated concentration ($1*10^{-8}$ mol/L) a long induction period precedes measurable adsorption to the air-water interface. The long induction period observed for the IgG (approximately 80,000 s which corresponds to 22 h), as compared to the induction period of other proteins such as lysozyme as determined by the same method at comparable concentrations (about 10,000 s according to [36]) can be explained by the large size of the IgG-molecule (146 kDa as determined by MALDI mass spectrometry in contrast to 14.3 kDa for Lysozyme as determined by ESI-MS, see Chapter 5). Because of the large molecular weight of the IgG its diffusion coefficient is small and diffusion to the subsurface from which adsorption to the air-water-interface takes place occurs only slowly[37]. The induction period also depends on the structural stability of the investigated molecule. In order

to produce measurable surface pressure after the adsorption, the protein also has to partially unfold at the interface. More flexible, non-globular proteins such as β-casein partially unfold faster and therefore show shorter induction periods[36,38]. However, the observed adsorption profile shows differences to the published adsorption profile of another IgG[37]. Whereas for the IgG investigated in our studies equilibrium surface tension reaches a steady value of about 53 mN/m beginning at concentrations of $1*10^{-7}$ mol/L, the published results reveal a saturation of the interface at concentrations as high as $2*10^{-5}$ mol/L also at about 53 mN/m. Lower concentrations of the published IgG achieve only higher equilibrium surface tensions and therefore smaller degrees of adsorption to the air-water interface, probably due to a lower hydrophobicity of that IgG. Such differences also point out, that the rather small differences in IgG-structure can lead to strong variations in the surface activity of IgGs, which is reflected in the very variable propensity of IgGs to aggregate due to exposure to the air-water interface[12-13,23].

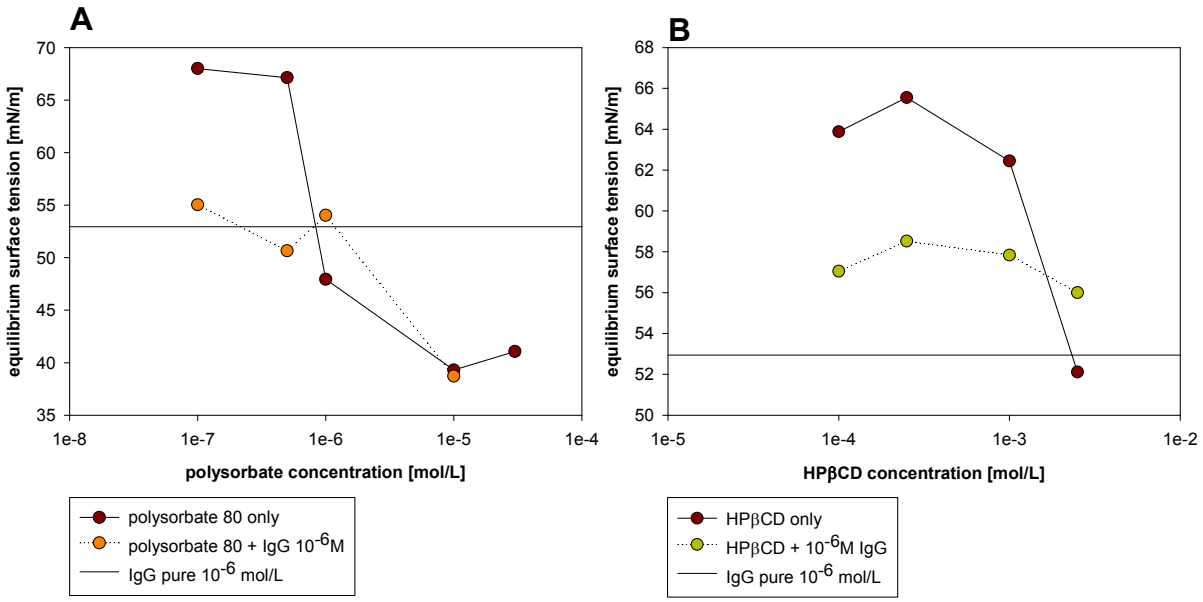

Figure 6.5: Surface tension isotherms of pure polysorbate 80 solutions and IgG-polysorbate 80 mixtures (A) as well as surface tension isotherms of pure HPβCD-solutions and IgG-HPβCD mixtures (B) as determined by drop profile tensiometry. The solid straight line indicates the surface tension of a $1*10^{-6}$ mol/L IgG-solution.

For the analysis of the mixed solutions of the IgG with polysorbate 80 or HPβCD, a constant mAb-concentration of $1*10^{-6}$ mol/L was chosen. The concentration is a compromise between a reasonable time to achieve equilibrium conditions (80,000 s) and not too fast initial adsorption that would obscure mechanistic observations by quickly cramming the interface with several adsorption layers of protein. The steady-state equilibrium surface tension of the IgG in absence of any excipients besides histidin buffer at pH 5.8 is indicated by a straight horizontal line in Figure 6.5 for comparison to the surface tension of the IgG-polysorbate 80 as well as the IgG-HPβCD mixtures. For the sake of comparison the surface tension

isotherms of the pure polysorbate 80-solution and the pure HPβCD-solution are also included into Figure 6.5.

As observable from Figure 6.5 A, at low concentrations of the surfactant 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 do not lower the surface tension of the mixture in the concentration range from $1*10^{-7}$ mol/L to $1*10^{-6}$ mol/L. These findings indicate the dominating contribution of the IgG to the composition of the adsorption layer of the mixture in this concentration range.

However, when the polysorbate 80 concentration is further increased to $1*10^{-5}$ mol/L the surface tension of the mixed solution of the IgG and polysorbate 80 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 $1*10^{-5}$ mol/L polysorbate 80, the surface layer is predominantly composed of polysorbate 80. The concentration at which polysorbate 80 alone determines the surface tension of the mixture coincides with the CMC of the pure polysorbate 80 solution as discussed already above. Therefore, further evidence, in addition to the data from the Wilhelmy plate measurements, for the competitive displacement of the mAb from the air-water interface at sufficiently high concentrations of polysorbate 80, is obtained.

For the mixed IgG-HPβCD solution a very different surface-tension isotherm than for the IgG-polysorbate 80 system is obtained, as shown in Figure 6.5 B. No matter how high the HPβCD-concentration is increased, the surface tension of the mixture does not significantly change. Moreover, the surface tension of the IgG-HPβCD solutions is higher than the surface-tension of the pure IgG, even at the lowest HPβCD-concentrations. Therefore, competitive displacement from the interface of the mAb by HPβCD, as observed with polysorbate 80, seems unlikely, since in that case the surface tension should have more closely approached the values of the pure HPβCD solution. It is also unlikely that both components have adsorbed to the interface at the same time, because if both components had concentrated at the interface to the same extent as in absence of the respective other component, the surface tension of the mixture should be lower than that of any of the single components. When keeping in mind that HPβCD could possibly interact with the IgG – not in the native IgG state, as shown in Chapter 5, but potentially in its partially unfolded conformation as occurring at the air-water-interface – the hydrophobicity of the IgG and therefore its tendency to adsorb to the air-water interface could be reduced by the presence of HPβCD. This reduced surface activity due to a loss of hydrophobicity could be reflected by the observed lower equilibrium surface-tensions. In addition, it is interesting that even the lowest investigated HPβCD-concentration leads to an increase of the equilibrium surface

tension of the mixture. In order to get deeper insight into these adsorption processes further experimental approaches are necessary. Surface rheology studies, carried out either by surface dilational rheology or by interfacial shear rheology will notably help to determine which component of the mixture is dominating the interface at which bulk solution composition. The techniques will possibly also help to determine if interface-specific complexes between the IgG and HPβCD since surface rheological studies are capable of detecting complexes in the surface layer, even if their amounts are small[39].

The main findings from the surface tensiometry measurements using drop profile analysis are summarized:

- Drop profile analysis tensiometry of dilute solutions at equilibrium adsorption times allowed good insight into the adsorption behavior of the IgG to the air-water interface in presence and absence of polysorbate 80 and HPβCD.
- Equilibrium surface tension measurements indicated that at high polysorbate 80 concentrations (roughly starting at the CMC) the air-water interface of mixtures of the IgG and polysorbate 80 is dominated by the non-ionic surfactant.
- In the presence of HPβCD the equilibrium surface tension of the IgG-cyclodextrin mixtures slightly increases as compared to the pure IgG, but it remains unclear which component dominates the interface of the mixture.

5 SURFACE DILATIONAL RHEOLOGY

The drop profile analysis method is a useful and accurate analytical tool to determine interfacial tensions as demonstrated in the previous section. Further instrumental progress in drop profile analysis technology lead to the possibility to perform harmonic area oscillations. Harmonic area oscillations at low frequency as a tool to perform surface dilational rheology studies were applied for the following investigations with the aim to assess the composition of the interfacial layer at the air-water interface. In addition to these studies, complementary experiments by interfacial shear rheology using a torsion pendulum rheometer are discussed in the next section.

For the surface dilational experiments seven oscillation frequencies were analyzed: 0.005 Hz, 0.01 Hz, 0.02 Hz, 0.04 Hz, 0.08 Hz, 0.1 Hz and 0.14 Hz. For the sake of clarity only four of these frequencies are shown in Figure 6.6 and in the following two Figures only one single oscillation frequency is displayed which, however, does not affect the conclusions that will be drawn from these Figures. Figure 6.6 shows the viscoelastic properties of the pure IgG solution as determined by surface dilational rheology. With increasing oscillation frequencies the observed viscosity and elasticity values decrease following the usual profile of proteins during such experiments[40]. As expectable, surface elasticity values are rather high as compared to the more flexible random coil protein β-casein described in literature[39,41]. The local minimum in the elasticity curve shown in Figure 6.6 A and B can likely

be attributed to conformational changes of the protein at the interface and not to differences in the adsorbed amount of protein at $1*10^{-7}$ mol/L[41].

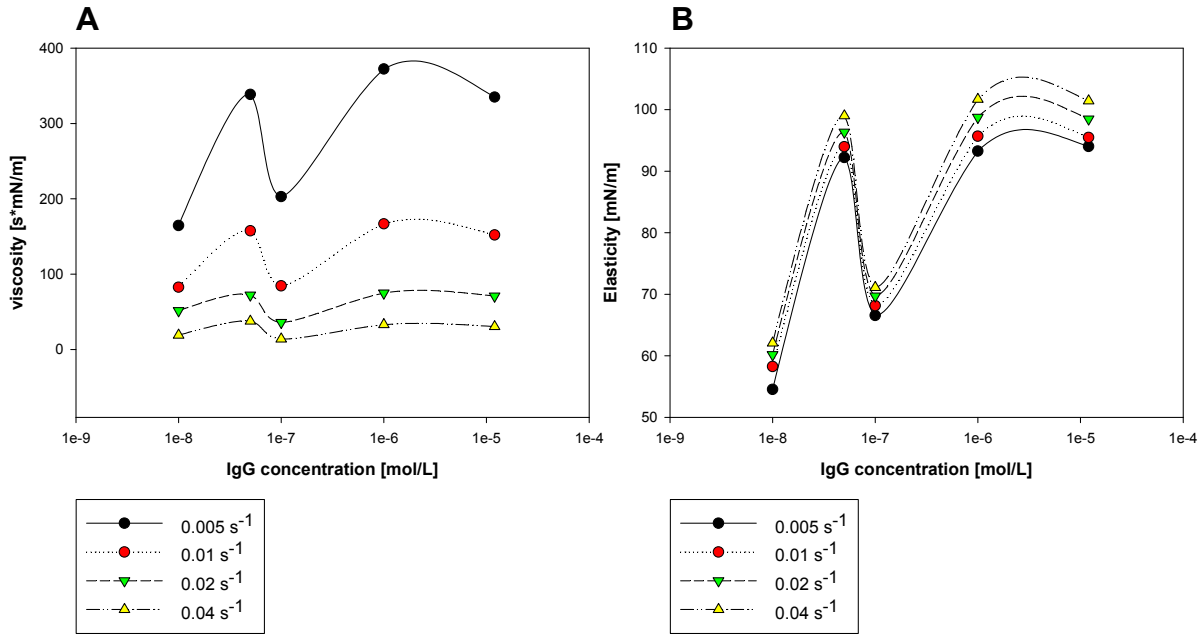

Figure 6.6: IgG viscosity vs. concentration (A) and IgG elasticity vs. concentration (B) at different oscillation frequencies at an IgG concentration of $1*10^{-6}$ mol/L in His buffer at pH 5.8, as determined by surface dilational rheology performed on a PAT 1 instrument.

The dilational viscosity values of the mixed IgG-polysorbate and IgG-HPβCD systems are presented in Figure 6.7. In order to keep the figure clear only the values obtained at an oscillation frequency of 0.005 s^{-1} are displayed. Further investigated frequencies showed the same trend. For comparison, the figure includes the viscosity value of the pure IgG solution, as discussed in Figure 6.6, at an oscillation frequency of 0.005 s^{-1} as a straight horizontal line. In Figure 6.7 A it can be seen that the viscosity profile of the pure polysorbate 80 solution has a distinct maximum at $1*10^{-5}$ mol/L, coinciding with the CMC of the surfactant. At all other investigated concentrations the viscosity values are significantly lower in a range of roughly 50-100 $s*mN*m^{-1}$. At low concentrations of polysorbate 80 the surface viscosity values of the IgG-polysorbate mixture are close to those that are observed for the pure protein depicted in Figure 6.6 (and the straight line in Figure 6.7). An increase of the polysorbate concentration from $1*10^{-7}$ mol/L to $5*10^{-7}$ mol/L does not alter the viscosity of the mixture, indicating that at these polysorbate concentrations the surface layers of the mixture are dominated by the pure IgG. A gradual decrease of the surface viscosity is observed beginning at a polysorbate 80 concentration of $1*10^{-6}$ mol/L and at a concentration of $1*10^{-6}$mol/L the surface layer exhibits viscosity values that are close to the values observed for the pure polysorbate solution, far below the values that can be expected for surface layers containing the IgG. Thus, in agreement with the results presented above, it is confirmed that polysorbate 80 displaces the IgG from the surface. Interestingly, a change of the surface layer properties towards the surfactant is already detected at a surfactant concentration of

$1*10^{-6}$ mol/L. This concentration lies in an order of magnitude lower than that determined by the equilibrium surface tension measurements that are displayed in Figure 6.5, indicating a higher sensitivity of the dilational rheology experiments.

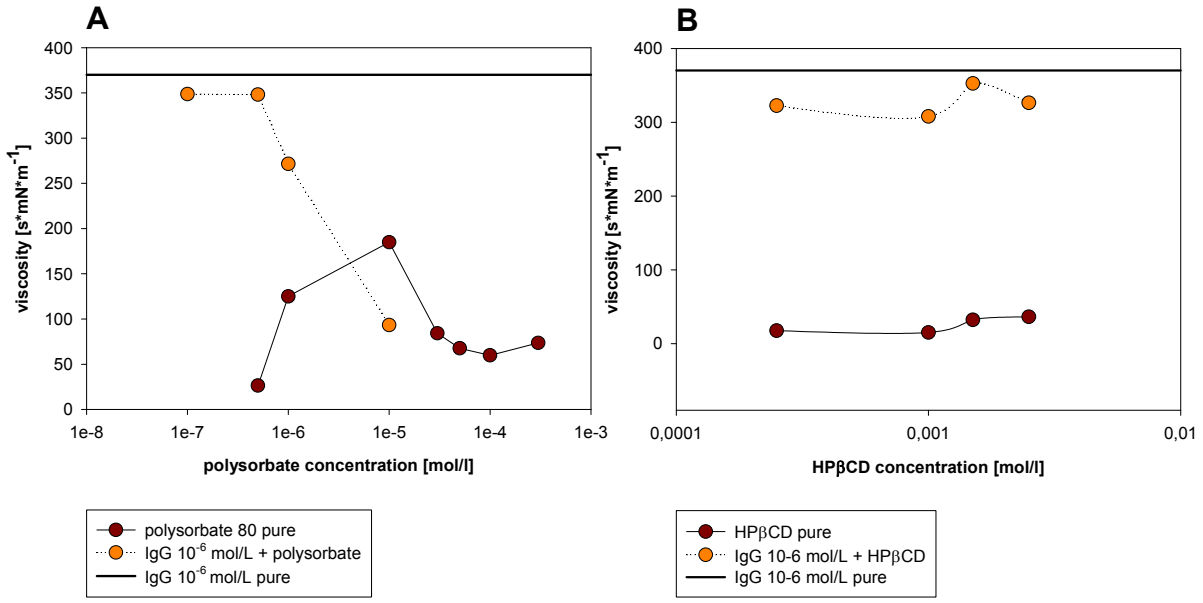

Figure 6.7: Dilational viscosity values of pure and mixed IgG and polysorbate solutions (A) and of pure and mixed IgG and HPβCD solutions (B), measured at an oscillation frequency of 0.005 s^{-1}. The straight line indicates the surface viscosity value of a pure IgG solution at the same oscillation frequency.

The solutions containing HPβCD show a completely different behavior (Figure 6.7 B). The viscosity values of the pure HPβCD solution are very low, never exceeding 50 s*mN*m^{-1}. In contrast, all viscosity values of the HPβCD-IgG mixture are very close to those observed for the pure IgG-solution. Therefore at none of the tested HPβCD concentrations, the rheological properties of the surface layer are significantly different from those of the pure IgG solution. Thus, the IgG is not displaced from the air-water interface even at the highest HPβCD-concentrations tested (2.5 mM which was effective at completely inhibiting mAb-aggregation during the agitation studies as discussed in Chapter 3 at even higher mAb-concentration than for these studies).

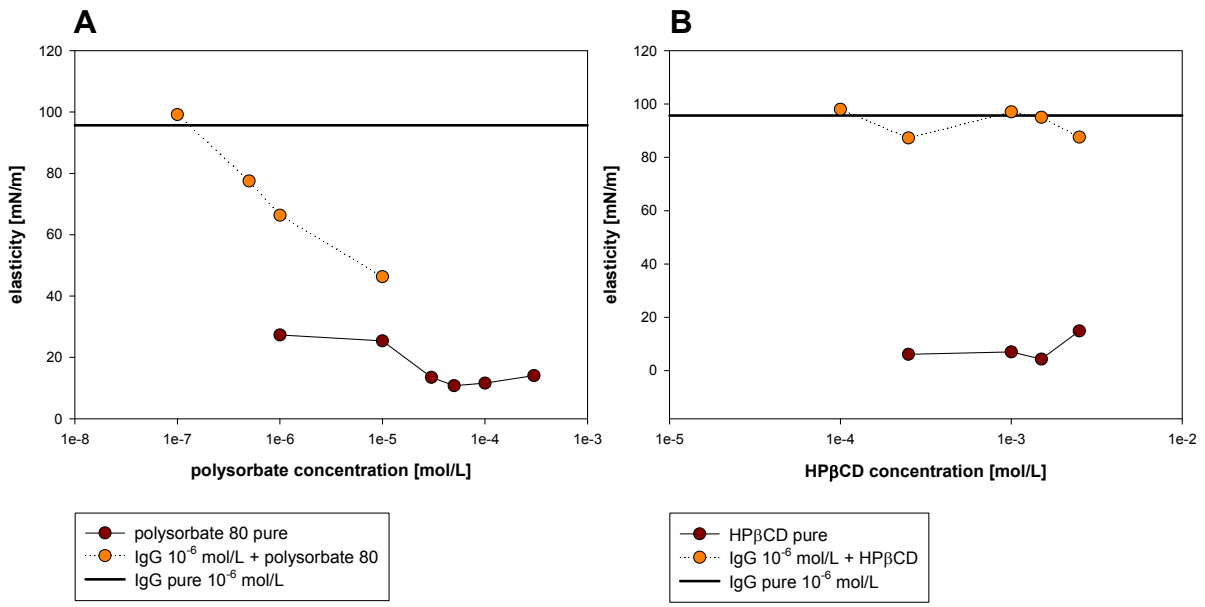

Figure 6.8: Dilational elasticity values of pure and mixed IgG and polysorbate solutions (A) and of pure and mixed IgG and HPβCD solutions (B), measured at an oscillation frequency of 0.005 s^{-1}. The straight line indicates the surface viscosity value of a pure IgG solution at the same oscillation frequency.

The dilational elasticity values that were simultaneously determined (Figure 6.8) can be understood in the same way. In Figure 6.8 A the surface elasticity values of the polysorbate 80-IgG mixture begin to already decrease at a polysorbate concentration of $5*10^{-7}$ mol/L, indicating that already at these low surfactant concentrations displacement of the IgG from the air-water-interface is beginning. It is conceivable that at these surfactant concentrations gaps that are temporarily available during the oscillation process (and the rapid expansion of the drop surface) are filled by small amounts of polysorbate 80 molecules in accordance with the orogenic displacement model[38]. In contrast to the conclusions drawn from the viscosity values discussed in the previous section, complete displacement of the mAb from the air-water interface at a polysorbate concentration of $1*10^{-5}$ mol/L seems not yet to be achieved, but residual amounts of the IgG can still be found in the air-water interface.

The trends observed from the dilational viscosity measurements for mixed HPβCD-IgG solutions are confirmed by the analysis of the dilational elasticity results (Figure 6.8 B). No alterations of the rheological properties of the surface layers can be observed at any of the HPβCD-concentrations in the mixture. Again, the elasticity values correspond very closely to the values of the pure IgG at every HPβCD-concentration. Therefore, once again it is confirmed that HPβCD does not reduce the amount of IgG that is adsorbed to the interface. No competitive displacement at the air-water interface can be observed.

It can be summarized that the dilational rheology experiments confirm the displacement of the IgG by the non-ionic surfactant polysorbate 80. In contrast, the mixed solutions of the IgG and HPβCD do not reveal any hints for such a displacement since the rheological surface characteristics at all HPβCD-concentrations are dominated by the IgG. However, when combining the finding of the reduced surface activity of the IgG in presence of HPβCD, as

observed by drop profile analysis and discussed in the previous section, and the fact that the IgG stays at the interface, as determined by the dilational rheology studies, it can be speculated that HPβCD interacts with the IgG at the surface in a way that does not lead to a displacement of the IgG from the surface but that reduces the hydrophobicity of the protein due to complexation and the shielding of hydrophobic protein residues.

The key conclusions of the dilational surface rheology experiments are:

- Dilational surface rheology was demonstrated to be a useful tool to assess the composition of the interfacial layer at the air-water interface.
- It was demonstrated that at high polysorbate 80-concentrations (slightly below the CMC) the IgG is displaced from the air-water interface by the non-ionic surfactant.
- In contrast, it was proven that the IgG remains at the air-water interface in the presence of even high concentrations of HPβCD.
- Taking into account the increase of the equilibrium surface tension as observed by drop profile tensiometry of the IgG in the presence of HPβCD, a loss of hydrophobicity (as the major driving force for adsorption to the air-water interface) of the IgG in the presence of HPβCD can be concluded. In turn it can be speculated that HPβCD interacts with the IgG at the surface (and only there) in a way that reduces the hydrophobicity of the protein due to complexation and the shielding of hydrophobic protein residues.

6 INTERFACIAL SHEAR RHEOLOGY

For a further confirmation of the observed trends surface shear rheological studies were conducted. Surface shear rheology is a sensitive technique to monitor structural changes occurring in the adsorption layer and therefore helps to understand the contributions of each bulk solution compound to the properties of the surface layer[42-43]. It is reported that globular proteins rapidly form dense network structures at the air-water interface and that even small amounts of non-ionic surfactants are effective at destroying these network structures, thereby rendering surface shear rheology a very sensitive technology to study mixtures of non-ionic surfactants and globular proteins[44-45]. The measurements were carried out using a torsion pendulum rheometer that is described in more detail in Chapter 2.

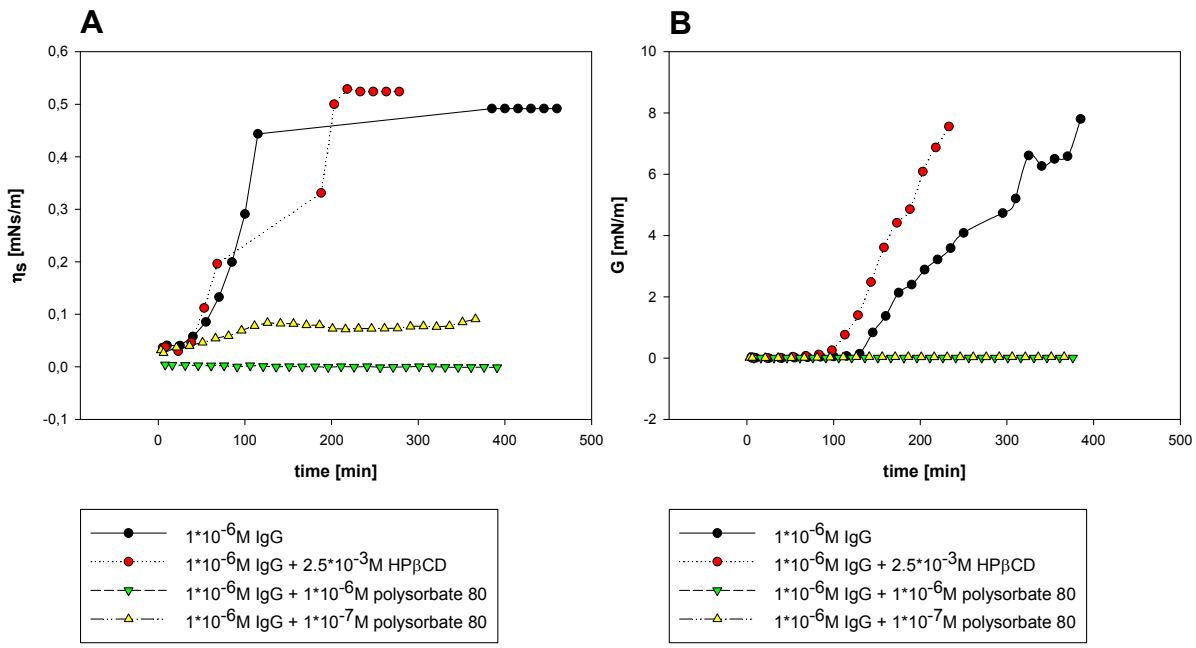

Figure 6.9: Dependence of the surface shear viscosity η_s (A) and elasticity G (B) on the adsorption time of the pure IgG solution, IgG-polysorbate 80mixtures and IgG-HPβCD mixtures at a constant IgG concentration of $1*10^{-6}$ mol/L as measured by interfacial shear rheology.

The surface shear viscosity η_s and the surface shear elasticity G of the pure IgG as well as of its respective mixtures with polysorbate 80 and HPβCD in dependence of the adsorption time are shown in Figure 6.9. In can be seen that after approximately 300 min adsorption time a very dense network structure is formed by the IgG, as the maximum elasticity and viscosity values that can be recorded by the torsion pendulum rheometer are reached, indicating very strong damping of the oscillation at this point and the pendulum gets “stuck” in the surface layer. A further increase of the surface viscosity and elasticity can not be followed by this technique. As observable from Figure 6.4 the IgG has not even reached its equilibrium adsorption value after this time. Upon the addition of $1*10^{-7}$ mol/L polysorbate 80 the network structure is strongly damaged as visible by very low surface viscosity value and no detectable surface elasticity. A further increase of the polysorbate 80 concentration leads to a complete loss of detectable surface structure and the surface is then entirely dominated by polysorbate 80. Observations are in good agreement with literature, similar behavior was described e.g. for a BSA/polysorbate 80 mixture[10] or mixed β-casein/C_{12}DMPO surface layers[39]. In contrast to the dilational rheology results discussed in the previous section, even lower concentrations of polysorbate 80 concentrations were found to be sufficient for a complete displacement of the IgG from the air-water interface, underlining the high sensitivity of surface shear rheology.

The following conclusions can be drawn from the interfacial shear rheology experiments:

- In contrast to polysorbate 80 HPβCD does not inhibit the formation of an IgG surface network structure, since the increase of viscosity in dependence of the adsorption

time is almost identical to that of the pure IgG and the increase of the surface shear elasticity is even slightly accelerated in the presence of HPβCD.

- Considering that interfacial shear rheology is a very sensitive technique that is capable of detecting even small amounts of low molecular weight compounds that adsorb to the surface in competition to globular proteins, it is further confirmed that HPβCD does not compete with the IgG for the occupation of the air-water interface, even at a high molar excess in the bulk solution.

7 SUMMARY AND CONCLUSIONS

An investigation of the interfacial adsorption behavior and the rheological surface characteristics of the IgG in absence and presence of both HPβCD and polysorbate 80 demonstrated that – in contrast to the postulated assumption – HPβCD does not stabilize the IgG against aggregation at the air-water interface in a manner comparable to non-ionic surfactants.

Initial surface tension measurements demonstrated that HPβCD indeed possesses some surface activity. However, compared to the non-ionic surfactant polysorbate 80 it is only weakly surface-active. Thereby, a competition for occupation of the interface might be possible, but from the results obtained by the Wilhelmy plate method a clear conclusion regarding the composition of the surface layer of mixed IgG-HPβCD solutions could not be drawn. Therefore further, more detailed experiments were conducted.

In order to clarify whether a particularly fast occupation of the air-water interface immediately after its formation by HPβCD (despite its moderate surface activity) could explain the stabilization of the IgG by the CD, maximum bubble pressure measurements were carried out. However, it was found that polysorbate 80 at low concentrations that was less effective regarding the inhibition of agitation-induced aggregation than the tested HPβCD-concentration, adsorb to the air-water interface much faster than HPβCD, excluding fast adsorption to de novo interfaces as an explanation for the aggregation inhibition by HPβCD.

The adsorption characteristics of the IgG, HPβCD and polysorbate 80 were studied in detail by drop profile analysis technology. First the individual components of the solutions were investigated and subsequently the respective mixtures. The equilibrium surface-tension values clearly demonstrated that after exceeding the CMC of polysorbate 80 the non-ionic surfactant dominates the surface of the mixture. Displacement of an IgG from the surface at high polysorbate 80 concentrations was demonstrated for the first time in literature. However, for HPβCD such behavior could not be proven. The equilibrium surface tensions of all the investigated mixtures (at different HPβCD concentrations) had roughly the same value, which was slightly higher than that of the pure IgG.

Surface shear and dilational rheology experiments further confirmed that polysorbate 80 displaced the IgG from the surface. Due to the higher sensitivity of the rheological studies it

was found that also polysorbate concentrations distinctly below the CMC could be sufficient for displacement of the IgG from the air-water interface. The rheological studies also clearly demonstrated that the IgG remained at the surface to full extent no matter how high HPβCD concentrations were present in the bulk solution. Therefore HPβCD does not competitively displace the IgG from the air-water interface and it is not acting like a weak non-ionic surfactant – an assumption that was expressed in literature and served as hypothesis to be tested for our experiments. However, it can be speculated, that due to its moderate surface activity HPβCD does reach the surface layers and interacts there (and only there, since no interaction between HPβCD and the native IgG in solution could be observed in Chapter 5) with the partially unfolded IgG. This interaction could lead to a lower hydrophobicity of the IgG, which was confirmed by the increased equilibrium surface tension values in our experiments.

The overall effects of excipients on protein aggregation during agitation can be related to a theory for how these compounds influence protein interactions with and their stability at the air-water interface. Because sugars like sucrose are excluded from the protein-water interface they can be expected to increase the surface-tension σ_{pw} at the protein-water interface[46-48]. The surface-tension at the air-water-interface (σ_{aw}) also increases with increasing concentrations of the low-molecular-weight sugars and sugar-alcohols. In contrast, the interfacial tension at the air-protein interface (σ_{ap}) is expected to be independent of the concentration of excipients in the bulk liquid[49]. As described in more detail by Mahler et al.[49] when taking into account the relation between the contact angle, the adhesion energy of the protein per unit area of the protein-air interface and the free energy of protein unfolding on the air-water interface ($\Delta G_{N\rightarrow U}$) it can be concluded that the $\Delta G_{N\rightarrow U}$ will decrease with increasing sucrose concentrations. As a consequence the protein, although more stable in the bulk due to preferential exclusion, will become less stable upon adsorption to the air-water interface. On the other hand this relation means that the excipients that lower the surface tension at both the air-water interface and the protein-water interface (decreasing both σ_{aw} and σ_{pw} and thereby increasing the contact angle) result in less spreading and a more stable protein at the surface[49]. However non-ionic surfactants and cyclodextrins, such as MβCD (but not HPβCD) in this case, may at the same time accelerate aggregation rates in the bulk.

In general a compromise has to be made in protein formulation: excluded excipients such as sucrose, trehalose, sorbitol and mannitol, which were employed for this work, are expected to stabilize the protein in the bulk, but clearly destabilize it at interfaces, whereas surfactants such as polysorbate 80 and some cyclodextrins may destabilize the protein in the bulk. For HPβCD stabilization at the interface could be achieved but thermodynamic stability in the

bulk was not affected. This property makes HPβCD a very promising excipient for protein formulation.

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CHAPTER 7

FINAL SUMMARY AND CONCLUSIONS

The general aim of this thesis was to investigate the role of cyclodextrins in the inhibition of therapeutic protein aggregation. The objective of the work originated in early studies that pointed towards cyclodextrins being capable of inhibiting protein aggregation under various stress conditions[1-5]. However, only few systematic studies on therapeutically relevant proteins have been described in literature so far. Understanding the underlying mechanisms by which cyclodextrins stabilize proteins against aggregation, thereby allowing for a more rational application of cyclodextrins in protein formulation, was additional motivation for the research work of this thesis. Finally, the need for alternatives to non-ionic surfactants as excipients in protein formulation accounted for studies in this thesis.

The work is presented in two major parts: First, the stabilizing effect of different cyclodextrins on the aggregation propensity of three structurally different, pharmaceutically relevant model proteins (an IgG, rh-GCSF and rh-GH) was investigated using different stress conditions. In the second part of the thesis, a detailed investigation of the underlying mechanisms of action that contribute to the inhibition of protein aggregation by cyclodextrins is presented.

In Chapter 1 a general introduction into the control of protein aggregation in liquid protein formulations is given. A focus of the discussion is put on state of the art excipients that can be used for protein stabilization and the limitations of these excipients. Whereas sugars and sugar alcohols are valuable excipients to increase protein stability in the bulk solution, they may lead to an increased susceptibility to surface-induced protein aggregation[6-7]. In contrast, non-ionic surfactants like polysorbate 80 are very efficient in inhibiting surface-induced aggregation, but lead to increased aggregation rates during quiescent long-term storage of protein formulations, in part due to residual as well as in-situ forming peroxides[8-12]. Reviewing literature, the potential of cyclodextrins as stabilizers against surface-induced aggregation becomes apparent[2,5]. Often the potency of cyclodextrins to suppress protein aggregation is ascribed to their ability to accommodate suitably sized, hydrophobic, solvent-exposed amino acid residues into the hydrophobic core of the cyclodextrins[3,13-17].

The materials and methods applied throughout this thesis are described ***in Chapter 2.***

In Chapter 3 the effects of different cyclodextrin-derivatives on the aggregation behavior of an IgG monoclonal antibody under various stress conditions, such as agitation, freeze-thaw and thermal stress, were described and HPβCD was evaluated as potential alternative to non-ionic surfactants as a stabilizer against interfacial stress in protein formulations.

It was demonstrated that HPβCD is well suited to inhibit aggregation of the IgG at the air-water interface. In contrast to other cyclodextrin-derivatives, HPβCD did not negatively affect

IgG stability during quiescent incubation at elevated temperature (60°C). Therefore, HPβCD was rendered the most promising CD-derivative for IgG stabilization and the further investigations on the effect of cyclodextrins on IgG-stability were focused on the HPβCD-IgG system. Most importantly, remarkably low, and therefore presumably non-toxic, concentrations of HPβCD (≥2.5 mM) were found to be sufficient for complete inhibition of agitation-induced IgG-aggregation. The same low concentration of HPβCD was also shown to be sufficient for the complete inhibition of IgG-aggregation induced by repeated freeze-thaw cycles. Therefore it was concluded that HPβCD is generally suitable to inhibit aggregation of the IgG triggered by the presence of interfaces.

In order to benchmark the potential of HPβCD as protein stabilizer, HPβCD was compared to state of the art protein stabilizers. Compared to polysorbate 80, HPβCD was found to provide comparable and even superior stabilizing properties as non-ionic surfactants for the prevention of surface-induced IgG aggregation. Comparing HPβCD to low molecular weight sugars and sugar alcohols as commonly applied protein stabilizers, such as sucrose, trehalose, sorbitol and mannitol, the sugars even promoted agitation-induced IgG-aggregation. Although chemically also classified as sugar, the cyclic oligosaccharide HPβCD thus showed a completely different effect on IgG-aggregation than the low-molecular sugars. Hence, the preferential exclusion mechanism which describes the stabilizing effect of low-molecular weight sugars on proteins, does not explain protein stabilization by HPβCD.

In order to further evaluate HPβCD as a formulation alternative to polysorbate 80, a long term storage stability study was conducted for six months at 4°C, 25°C and 40°C. It was demonstrated that HPβCD did not exert negative effects on IgG-stability upon quiescent storage. In contrast, formulations containing polysorbate 80 exhibited a distinctly decreased IgG stability. Summarizing, the application of HPβCD offers the potential to stabilize protein pharmaceuticals against interfacial stress without a destabilization upon quiescent storage, as it is encountered using non-ionic surfactants. The observed trends were further confirmed at higher antibody concentrations (50 mg/mL), representing therapeutically relevant mAb concentrations. A reduction of agitation-induced aggregation by HPβCD (and polysorbate 80) was also observed at high IgG-concentration; however, a complete inhibition of agitation-induced aggregation could not be achieved. A formulation containing both, HPβCD and sucrose, was demonstrated to preserve the advantages of both excipients and thus offer optimum stabilization both during agitation and during quiescent storage. The studies on the IgG showed that the only ionic CD-derivates included into the studies, sulfobutylether-CD-derivatives, are generally not suitable for mAb-formulation. No matter which degree of substitution and which ring size (either β-CD- or γ-CD-derivatives) was tested, IgG-aggregation rates were increased in presence of the sulfobutylether-CD-derivatives. Observations were further confirmed by the second model protein investigated, rh-GCSF.

Conclusions are discussed in ***Chapter 4***, in which the effects of cyclodextrins on the stability of two further model proteins, rh-GCSF and rh-GH are discussed. In the studies on rh-GCSF it was shown that, as with the IgG, SBEβCD turned out to be detrimental to protein stability during agitation, repeated freeze-thaw cycles and quiescent storage studies.

Furthermore in Chapter 4 it was discussed that the effect of cyclodextrins on aggregation of rh-GCSF, although the protein is structurally very different from the IgG, are generally in good qualitative agreement with the effects on the IgG. HPβCD was again demonstrated to well inhibit agitation and freeze-thaw induced aggregation at concentrations ≥2.5 mM. Also, HPβCD did not negatively influence rh-GCSF stability during quiescent storage at 50°C whereas formulations containing polysorbate 80 exhibited dramatically increased amounts of aggregates after storage. Therefore, the results obtained with rh-GSCF confirm, that HPβCD could serve as a valuable alternative to non-ionic surfactants in protein formulation.

The third model protein investigated in this thesis, rh-GH, showed a different aggregation profile in the presence of cyclodextrins. With only few exceptions, rh-GH was stabilized against aggregation by all CD-derivatives under all stressing conditions. In addition to HPβCD, also the ionic SBEβCD reduced aggregation of rh-GH. This difference of rh-GH as compared to the other two model proteins was attributed to a structural particularity of rh-GH. The protein contains an unusually high percentage of aromatic amino acids of which some are exposed even in the protein's native state and binding between rh-GH and different CD-derivatives had been described in literature[3]. Therefore binding between the CD-derivatives and rh-GH was assumed as common stabilizing principle under all stressing conditions whereas the effects of the CDs on the stabilization of rh-GCSF and the IgG appeared to depend more on the applied stressing condition.

Altogether the stability studies on the three model proteins point out that so far in protein formulation a compromise had to be struck: excluded excipients like sucrose and trehalose stabilize proteins in the bulk solution, but clearly destabilize them at interfaces whereas surfactants such as polysorbate 80 and some cyclodextrins may destabilize proteins in the bulk. With HPβCD, it has been demonstrated in this work that the inhibition of interfacial aggregation is feasible without a reduction of protein stability in bulk solution. This property renders HPβCD a very promising excipient for protein formulations.

To elucidate the mechanism of stabilization, binding between CD-derivatives and the three model proteins in bulk solution was investigated as discussed in ***Chapter 5.*** In search of a sensitive analytical technique to monitor the weak and transient cyclodextrin-protein interactions, surface-plasmon resonance spectroscopy was evaluated using rh-GCSF as a model protein. Control experiments were carried out in order to exclude that unspecific binding signals between the immobilized proteins and cyclodextrins are recorded by the method. Comparisons of the binding of cyclodextrins to rh-GCSF to the binding of linear

sugars such as maltoheptaose, serving as negative control, demonstrated that the presence of the hydrophobic CD-cavity is a necessary prerequisite for the observation of interactions between β-cyclodextrins and rh-GCSF. By performing the binding experiments at varying pH levels, simple ionic attraction between charged cyclodextrins and the immobilized protein was excluded as exclusive driving force for binding. Observations were confirmed by applying two further orthogonal experimental methods, intrinsic fluorescence based titration and surface acoustic wave sensors.

Binding experiments were carried out with different cyclodextrin-derivatives and all three model proteins. For rh-GH correlation between binding in bulk solution and stabilization against interfacial aggregation was observed, confirming literature trends. However, for the other two model proteins, the IgG and rh-GCSF, no correlation between binding and the inhibition of aggregation could be established. HPβCD, which was efficient in the inhibition of protein aggregation, was found not to bind to the IgG and to bind only very weakly to rh-GCSF. In contrast SBEβCD, which was detrimental for the stability of rh-GCSF and the IgG, showed clear binding to both model proteins. Therefore, other principles than direct binding in bulk solution (where the proteins maintain a rather native conformation with little exposure of hydrophobic amino acid residues) govern the stabilization of the IgG and rh-GCSF by HPβCD. Since stabilization of the two model proteins mainly occurred against surface-induced aggregation, the behavior of HPβCD at the air-water interface was studied in more detail in Chapter 6. The binding studies using surface-plasmon resonance spectroscopy were complemented by ESI-MS data on the stoichiometry of interaction. It was demonstrated that a 1:1-binding stoichiometry dominates binding between β-cyclodextrins-derivatives, such as HPβCD, and proteins. It was shown that in order to derive sound conclusions from cyclodextrin binding experiments by ESI-MS appropriate control experiments have to be carried out.

In ***Chapter 6*** mechanisms of protein stabilization against surface-induced aggregation were further investigated. Detailed studies on the interfacial adsorption behavior at different time scales as well as investigations on the rheological surface characteristics of the IgG, both in absence and presence of HPβCD and polysorbate 80, were discussed. The aim of the investigations was to evaluate to which extent HPβCD stabilizes the IgG in the same manner as non-ionic surfactants, namely by competitive displacement of the protein from the air-water interface thereby inhibiting unfolding and subsequent aggregation of the protein after adsorption to the interface. Surface tension measurements demonstrated that HPβCD possesses only weak surface-activity as compared to polysorbate 80. Also, it was shown that HPβCD does not adsorb faster to the interface than polysorbate 80 which could have been of importance during agitation processes. Using the drop profile analysis technology it was shown that after exceeding the CMC of polysorbate 80 the surface of mixed IgG-

polysorbate 80 solutions was dominated by polysorbate 80, indicating displacement of the IgG by polysorbate 80. In contrast, in the presence of HPβCD no hints for displacement of the protein from the interface could be obtained. However, a moderate increase of the equilibrium surface tension of the IgG in presence of HPβCD points towards a lower hydrophobicity of the IgG in the presence of HPβCD. This observation points towards the occurrence of binding between HPβCD and the IgG only at the interface, where the conformation of the protein is expected to be different from that in bulk solution, but not in the bulk solution. Surface shear and dilational rheology experiments further confirmed that polysorbate 80 displaces the IgG from the interface whereas the IgG remains at the interface even in the presence of high concentrations of HPβCD.

Further mechanistic investigations on the role HPβCD in the prevention of surface-induced IgG aggregation should directly address interaction between the cyclodextrin and the protein in the interfacial layer at the air-water interface in order to verify the hypothesis derived from this work.

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PRESENTATIONS AND PUBLICATIONS ASSOCIATED WITH THIS THESIS

ARTICLES

T. Serno, J. Carpenter, T. Randolph, G. Winter
Inhibition of agitation-induced aggregation of an IgG-antibody by Hydroxypropyl-β-cyclodextrin
Journal of Pharmaceutical Sciences. Volume 99, Issue 3, Date: March 2010, Pages: 1193-1206

T. Serno, R. Lang, S. Gottschalk, G. Winter
Convenient separation of GCSF using Asymmetrical Flow Field-Flow Fractionation
Application Note submitted to Wyatt Technology Corporation, June 2007

POSTER PRESENTATIONS

T. Serno, J. Carpenter, R.Miller, G. Winter
The role of Hydroxypropyl-β-cyclodextrin in the prevention of monoclonal antibody aggregation
7th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Valetta, Malta, March, 8th-11th 2008

T. Serno, J. Carpenter, T. Randolph, G. Winter
Cyclodextrins as alternative to non-ionic surfactants in monoclonal antibody formulation
„Science to Market“ conference, Hannover, Germany, October 6th – 7th, 2009

T. Serno, J. Carpenter, G. Winter
Cyclodextrins as stabilizing excipients for monoclonal antibody formulations: evaluation of effects and mechanisms and comparison to other excipients
AAPS National Biotechnology Conference, Toronto, Ontario, Canada, June 22-25, 2008

T. Serno, J. Carpenter, E. Pittenauer, G. Allmaier, G. Winter
Interaction of various cyclodextrin-derivatives with rh–GCSF
2007 Colorado Protein Stability Conference, Breckenridge, CO, USA, July, 19th – 21st 2007

S. Herrmann, A. Youssef, T. Serno, G. Winter
Increased thermal stability of IFNα2a by HPβCD was detected by FTIR but not by microcalorimetry

6th World Meeting on Pharmaceutics, Biopharmaceutics, and Pharmaceutical Technology, Barcelona, Spain, April, 6th-10th 2008

ORAL PRESENTATION

T. Serno, J. Carpenter, G. Winter

Cyclodextrins inhibit surface-induced aggregation of a monoclonal antibody

Invited short lecture, 6th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Barcelona, Spain, April, 6th-10th 2008

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