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# Sugar-Based Surfactants for Pharmaceutical Protein Formulations

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## **Erklärung**

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 (in der Fassung der sechsten Änderungssatzung vom 16. August 2010) von Herrn Prof. Dr. Wolfgang Frieß betreut.

## **Ehrenwörtliche Versicherung**

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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# 1. Introduction

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## 1.1. Formulation Challenges with Biologicals

### 1.1.1. General Considerations

Unlike small chemical drugs (or New Chemical Entities (NCEs)), biologicals and especially protein pharmaceuticals form higher ordered structures. For the maximum efficacy and safety of the drug product it is mandatory to preserve this fragile system of interactions and arrangements until the drug is applied in the patient's body and arrives at the site of action (Porter 2001; Rosenberg 2006). In general, proteins are forming small specific and less hydrophobic surfaces. Usually the more hydrophobic amino acids are buried in the protein's core to reduce the contact area to water to a minimum. The more polar amino acids are predominant on the surface of the protein. This leads to strong hydrophobic interactions in the inner parts of the molecule and to an increased hydration at the surface. Only the target sites of e.g. antibodies contain a higher hydrophobic part at the surface (Ptitsyn 1987).

Proteins usually have only one specific target, e.g. receptors, antigens. This high specificity is their big advantage compared to NCEs, which often show side effects due to targeting multiple sites. NCEs act only partially specific. Opioids for example bind to several types of receptors. For some antipsychotic drugs their unspecificity makes them even more potent. But with more targets, the odds of a higher incidence of severe side effects are increasing.

Only proteins that are in the native state are active against their target (Weir et al. 2002). Furthermore, unfolded or aggregated protein molecules can in some cases lead to severe side effects. Different stresses can lead to chemical and physical denaturation of the protein. The next few examples should demonstrate the problems with handling pharmaceutical proteins as bulk substance or in the final dosage form. In the downstream process human Growth Hormone (hGH) aggregates can occur

within the filter during sterile filtration (Maa and Hsu 1998). During lyophilization various proteins form aggregates during the freezing process itself. E.g. Interleukin-1 receptor antagonist or tumor necrosis factor binding protein aggregate at the ice-liquid interface unless stabilized with surfactant (Chang et al. 1996b).

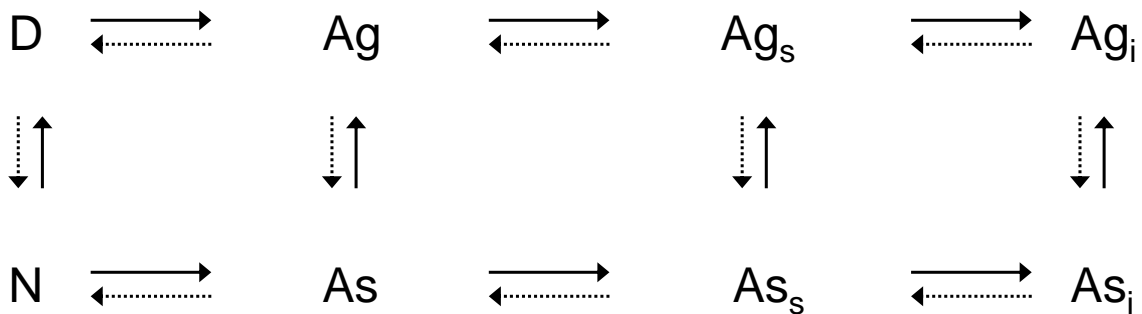
The oxidation of recombinant human monoclonal antibody HER2 is strongly enhanced in the presence iron ions derived from corrosive material during fill & finish processes (Lam et al. 1997).

Furthermore the storage and shipping temperature is an important factor for protein stability. Myofibrillar ATPase becomes more denatured at -5°C than at -20°C (Martino and Zaritzky 1989). This phenomenon is explained with recrystallization of the protein at higher temperatures in the frozen state (Williamson et al. 1999; Pham 2006; Fernandez et al. 2008).

Protein stability is not only an issue for content loss and hence a less efficient manufacturing process but may also lead to immunogenic side effects (Porter 2001; Patten and Schellekens 2003; Rosenberg 2006). Immunogenicity is a major concern especially when proteins are administered as multiple doses over prolonged periods (Patten and Schellekens 2003). The most prominent example for the breakdown of immune tolerance against a naturally occurring protein is probably the “erythropoietin (Epo) case”. The formation of non native like structures is related to the breakdown of immune tolerance against both the synthetic substance and Epo derived from the human body. This can be explained by the fact that the aggregated protein molecules form haptens for the immune system. The human body in turn is not able later on to differentiate the origin of the hapten that also lies somewhere on the protein surface. When the body tries to eliminate all Epo the consequence for the patient is a so called pure red cell aplasia (PRCA). The only therapeutic option for this adverse effect is a complete blood transfusion to remove antibodies from the patient (Casadevall et al. 2002; Rossert et al. 2005; Schellekens 2005; Schellekens and Jiskoot 2006). Thus, the formulator in pharmaceutical industry has to try to prevent the protein from all types of chemical and physical degradation, which are explained in this chapter.

### 1.1.2. Physical Instability

Physical denaturation describes the unfolding of the native protein structure. One can differentiate between partial and complete denaturation (Figure 1). A frequent consequence of denaturation is unfolding. But also native proteins can form associates (Brange 2000).



**Figure 1: Simplified model of proposed protein aggregation and association mechanism (Mahler et al. 2005)**

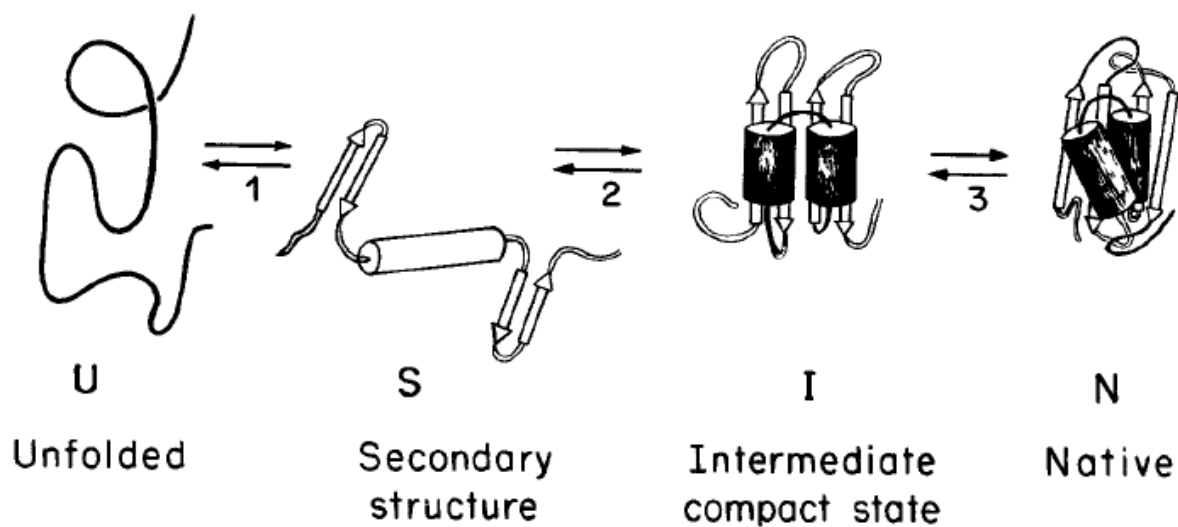
In Figure 1 N is the native state protein that can unfold to the denatured state D or form small associates As. As are oligomers and multimers whose monomer subunits have a preserved native structure. D or As can form aggregates Ag based on unfolded monomers. As can grow and build up large soluble As<sub>s</sub> or when solubility limits are exceeded can form insoluble associates As<sub>i</sub>. From smaller aggregates larger soluble Ag<sub>s</sub> and insoluble aggregates Ag<sub>i</sub> can be generated (Mahler et al. 2005).

As indicated by the dotted lines, the reaction back to a more native like state typically only occurs under special conditions like high pressure for the refolding of human Growth Hormone (St. John et al. 2001).

An important aspect of physical instability is the size of the associates or aggregates. Proteins can arrange in small units like dimers, trimers or tetramers, but also in larger oligomers or aggregate to large multimers. Although some protein pharmaceuticals have a native state that comprises monomers, dimers and hexamers, e.g. insulin (Manallack et al. 1985; Bhattacharyya and Das 1999) or glycogen phosphorylase (Paladini et al. 1994) that show enhanced stability or activity in an oligomeric state, for most pharmaceutically applied proteins the monomer is the only acceptable, stable, and active state.

The problem with bigger oligomers is that they can act as epitopes for the patient's immune system. This recognition can lead to acute immunogenicity or breakdown of the immune tolerance (see above) to exogenous substances (Schellekens 2005; Rosenberg 2006).

Often the first transition from N to D is going through a slightly unfolded state, the so-called molten globule state (Figure 2) (Ptitsyn 1987; Kumar et al. 1995; Bam et al. 1996). These intermediates are less compact than the native state, but show similar Stokes radii. The molten globule state is usually thermodynamically unstable and transforms into completely unfolded protein. But in some cases this partially unfolded protein can be preserved and can fold back to the native state by high pressure as applied for different proteins (Zhang et al. 1995; Bam et al. 1996).



**Figure 2: Proteins are folded via an Intermediate State (I), the molten globule state (N), into the native form. I is usually slightly bigger than N (from (Ptitsyn 1987)).**

### 1.1.3. Chemical Instability:

Chemical degradation of protein comprises a number of different processes.

**Deamidation** is the most prominent degradation pathway for proteins (Robinson and Robinson 2001) for example insulin is deamidated at Asn21 under acid and at AsnB3 under neutral pH conditions (Brange et al. 1992). For deamidation one of the two amino acids with amid functions, asparagine (Asn) or glutamine (Gln), must be present in the protein (Brange et al. 1992; Sasaoki et al. 1992; Shire 1996). Asn is

much more susceptible to deamidation than Gln. For both amino acids the mechanism leads via cyclic imide products to the oxidized acid, aspartic acid or glutamic acid or their respective iso-form (Robinson and Robinson 2001; Robinson and Robinson 2004).

The amino acids most likely to undergo **oxidation** are methionine, cysteine and histidine (Gu et al. 1991; Stadtman 1993; Nabuchi et al. 1995; Fransson et al. 1996; Li et al. 1996a; Zhao et al. 1997). Methionine can react to its sulfoxide or under stronger conditions to its sulfone (Manning et al. 1989). Oxidized cysteine is forming inter- and intramolecular disulfide bridges, under stronger conditions reactions to its sulfenic, sulfinic and finally sulfonic acids are possible, too (Florence 1980; Stadtman 1993). In this work Interleukin-11 and hGH are applied. Both proteins are susceptible to methionine oxidation (Pikal et al. 1991; Yokota et al. 2000).

When aspartic acid (Asp) is present in the protein, peptide bonds are eager to **hydrolyze** at the N-terminal and C-terminal adjacent to an Asp residue. This behaviour is favoured when the following amino acid is proline and glycine. In some cases hydrolysis of Asn is following the deamidation of Asp to Asn (Manning et al. 1989; Brange et al. 1992; Li et al. 1995; Reubsaet et al. 1998). RhIL11 is also cleaved by hydrolytic mechanisms between Asp133 and Pro134 under acidic conditions (Kenley and Warne 1994).

The potential for **racemization** is present in all amino acids but Gly. Asp and Glu racemize via cyclic imide intermediate formation. The rate of racemization is strongly structure dependent (Stephenson and Clarke 1989; Kimber and Hare 1992; Ritz and Schutz 1993; Luthra et al. 1994; Shahrokh et al. 1994). Shifts in pH and high temperatures are the cause of this degradation mechanism.

Cys, Ser, Phe, Thr and Lys are prone to  **$\beta$ -elimination**. This is a special pathway of racemisation, where the intermediate product is cleaved after conversion. The products, originating from elimination mechanisms will contribute to physical instability such as aggregation, adsorption or precipitation. For example recombinant human Macrophage colony stimulating factor is supposed to be  $\beta$ -eliminated under alkaline conditions (Nashef et al. 1977; Schrier et al. 1993). Insulin shows  $\beta$ -elimination after thiol-induced interchange (Costantino et al. 1994).

A consequence of  $\beta$ -elimination can be **disulfide scrambling**. Free thiol groups can be oxidized forming disulfide bridges. Insulin shows disulfide scrambling in the dried



state. This is even more pronounced when the lyophilizates showed higher residual moisture (Costantino et al. 1994; Kuwata et al. 1994; Shahrokh et al. 1994).

The formation of anhydrides from Asp and Glu is another possible degradation pathway where intramolecular bonds are formed. These reactions are strongly dependent on pH and are sometimes proposed to occur in the presence of formaldehyde, e.g. for vaccines (Schrier et al. 1993; Prestrelski et al. 1995; Schwendeman et al. 1995). **Non-disulfide cross-linking** (also called non-reducible cross-linking) is also a degradation pathway of Interleukin-2, that is increased by the presence of polysorbate 80 in the liquid protein formulation (Wang et al. 2008). This degradation mechanism, usually oxidative, can e.g. be thioether formation and is detectable via SDS-Page (Wang 1999).

A big number of therapeutic proteins, especially monoclonal antibodies show a specific level of glycosylation (Wang et al. 2005). **Deglycosilation** can impact thermal stability and function of the protein as shown for human Interferon- $\beta$  and phytase. Furthermore, the isoelectric point can be shifted due to this degradation and impair physical stability of a formulation (Runkel et al. 1998; Bagger et al. 2007a). Deglycosilation can occur pH triggered and lead to thermally unstable proteins that are prone to intracellular degradation (Wang et al. 1996; Dobson 2003).

In the presence of reducing sugars, such as glucose and fructose, proteins can undergo a degradation pathway called the **Maillard reaction**. This type of reaction is well known from food browning during baking. Products are usually yellowish to brownish and heterogeneous. A free amino group of an amino acid and a hemiacetal in the sugar are essential for this kind of reaction (Reyes et al. 1982). E.g. for human relaxin in the lyophilized state it is important to remove reducing sugars from the formulation as they significantly react with the protein (Li et al. 1996b).

Physical and chemical instability are usually affecting each other. On the one hand side aggregation can occur due to covalent linkage of two unfolded monomers (Muhammad et al. 2009) and on the other hand chemical reactions may be enhanced in the unfolded state (Kendrick et al. 1997).

## 1.2. Stabilizing Additives in Pharmaceutical Protein Formulation

There are several types of excipients applied to prevent physical degradation of pharmaceutical proteins. Surfactants are applied to prevent surface induced unfolding, as artificial chaperones to reverse unfolding and in some cases to prevent chemical denaturation. This group of excipients is discussed in detail in the paragraphs below. Other applied excipients are sugars and polyols, amino acids, buffer salts, polyethylene glycols (PEG), other polymers, metal ions.

### **1.2.1. Other Excipients than Surfactants**

**Sugars and polyols** act through the preferential exclusion mechanism, introduced by Timasheff et al. into the pharmaceutical field (Timasheff 1993). Preferential exclusion of the excipient from the protein leads to a stronger hydration of the protein, which in turn leads to a denser packing of the protein molecules to minimize exposure of hydrophobic protein parts at the surface. Furthermore, reduced protein surface due to preferential exclusion reduces the chemical potential and is thus less prone to oxidative processes (Kendrick et al. 1997). Sugars are also able to remove metal salts from the solution and may prevent metal ion catalyzed chemical degradation or act in other ways as antioxidant (Lam et al. 1997). In freeze-dried formulation, sugars are acting as water replacement by providing hydroxyl functions to the protein (Crowe et al. 1993a; Crowe et al. 1993b; Schuele et al. 2008). The tendency of in particular sucrose and trehalose to form amorphous cakes makes them ideal bulking agents in dried protein formulations (Arakawa et al. 1993; Chang et al. 1996a). Furthermore these disaccharides lead in many cases to solid systems with a glass transition temperature and relaxation rates high enough for effective storage stability. In numerous cases trehalose stabilizes slightly better than sucrose (Tanaka et al. 1991; Hora et al. 1992; te Booy et al. 1992; Pikal and Rigsbee 1997; Cleland et al. 2001; Maury et al. 2005). Reducing sugars should not be applied, as they have the tendency to react to Maillard-products with terminal amino groups as shown for human relaxin (Li et al. 1996b). The use of the sugar alcohol mannitol usually yields crystalline solid formulations (Akers 2002). Shorter polyols like glycerol can be added to freeze-dried formulations for the suppression of local, nanosecond relaxations and frequency shifting of collective vibrations that occur upon addition of diluents. Therefore, they increase protein stability although the glass transition

temperature is decreased (Cicerone et al. 2005).

**Amino acids** most probably stabilize due to preferential exclusion as shown for pig heart mitochondrial malate dehydrogenase similar to carbohydrates (Jensen et al. 1996). Furthermore, for some amino acid / protein systems the amino acids protect the protein from oxidation, e.g. histidine is preventing oxidation of papain (Kanazawa et al. 1994). Cysteine and (partially) methionine stabilize recombinant human Ciliary Neurotrophic Factor, and recombinant human Nerve Growth Factor (Knepp et al. 1996).

**Buffer salts** on the one hand are mandatory to provide and stabilize the pH-value that shows the best retention of the native state of the protein, but also the choice of buffer affects the stability of the formulation. For example, in the typical pH range phosphate buffer in freeze-dried formulations tends to solidify in two different salts which differ in their solubility. Thus the pH during freezing can change dramatically. If one takes a closer look on the phosphate buffer system during freezing, one can detect up to 13 eutectic temperatures with decreasing pH with decreasing temperature and thus changing the surface charge of the protein (van den Berg and Rose 1959; Orii and Morita 1977; Franks 1993; Gomez et al. 2001; Pikal-Cleland et al. 2002). For some buffer systems the position in the Hofmeister series or their tendency to salt out/salt in proteins influence the stability of liquid formulations (Bagger et al. 2007b; Le Brun et al. 2009). Especially for highly concentrated protein formulations the addition of the right type of buffer salt and the correct pH are of importance (Shire et al. 2004; Salinas et al. 2010). The desired pH value should be well away from the isoelectric point of the protein to keep the net surface charge of the protein high and thus reduce intermolecular attraction forces (Yang and Honig 1993; Le Brun et al. 2009; Salinas et al. 2010). Not only the salting out effects and the pH must be observed, but ionic strength plays a major role in protein stabilization, too. Not only the type of buffer salt must be correct, but also the salt concentration (Giancola et al. 1997; Hawe and Friess 2008; Salinas et al. 2010).

In the past **human serum albumin** (HSA) have been an alternative for non-ionic surfactants. When added in high amounts, the surfaces of container systems are covered with e.g. HSA. But due to problems arising from blood borne pathogens such as prionic agents, bovine and human Serum Albumins are not the excipients of choice anymore. Also compared to recombinant HSA non-ionic surfactants are a

safer alternative as HSA may lead to immunogenic reactions (Braun et al. 1997)

**Polyethylene glycol** (PEG) is used to precipitate or crystallize proteins (Prestrelski et al. 1993; Izutsu et al. 1995; Alden and Magnusson 1997). The phenomenon of precipitation of proteins in the presence of PEGs may be explained with the preferential hydration of PEGs (Timasheff 1992) or by the coverage of hydrophobic surfaces, but the problem finally remains not completely solved.

### **1.2.2. Surface Active Agents (Surfactants) in Parenteral Formulations of Protein Pharmaceuticals:**

Surfactants are commonly used in pharmaceutical protein formulations to compete for interfaces that might cause unfolding and aggregation of the API. These interfaces are mostly between the aqueous protein solution on the one side and on the other side container walls, stoppers (coated and uncoated), the ice-crystals (during freezing in lyophilization processes or while storing), filter materials, tubes and coatings of e.g. pumps, and of course mostly the air-water interface. Especially when these interfaces are rapidly changing, e.g. due to shaking and stirring, surfactants showed their stabilizing abilities (Mahler et al. 2005). In 16 out of 23 approved monoclonal antibody formulations in 2006 surfactants are added as excipients (Wang et al. 2006). In most cases polysorbates and poloxamers are the excipients of choice. The concentration range of the applied surfactants is usually between 0.01 and 3 mg/ml (Hawe et al.). Monoclonal antibodies are not the only proteins that can be stabilized by the addition of surfactants. The application of non-ionic surfactants is found ubiquitary in protein formulations and well described in literature. Enzymes and cytokines (Chang et al. 1996b), vaccines (Lang et al. 2007), fusion proteins (Chou et al. 2005), etc. can be stabilized with the addition of surface active substances.

#### **1.2.2.1. Physicochemical Aspects of Surfactants in Protein Formulations:**

There are several theories concerning the stabilizing effect of non-ionic surfactants in protein formulations. Postulated theories are surface competition on the one hand.

On the other hand stabilization of the native state due to preferential exclusion, formation of mixed micelles with native structured protein, as well as direct interactions with hydrophobic amino acids on the surface of the proteins are named as stabilizing mechanisms (Chang et al. 1996b; Bam et al. 1998). Surfactants are even proposed to act as artificial chaperones and to help to refold proteins that are already denatured (Bam et al. 1996). For some proteins complexes between hydrophobic cavities and the alkyl chains of the surfactants should be formed that are even more stable than the native protein (Giancola et al. 1997). Furthermore, it is described that with the use of higher molecular weight surfactants like poloxamers additional thermal stability is achieved by increasing the viscosity and hence molecular flexibility of the protein. The addition of 10 % poloxamer stabilized urease and IL-2 against shaking. This is way more than good applicable. The authors describe the stabilizing mechanism to increasing of the viscosity of the system (Wang and Johnston 1993). Preferential exclusion has also been discussed as potential mode of stabilization (Randolph and Jones 2002). But systems that show preferential exclusion typically increase the surface tension and especially for some proteins even preferential interaction demonstrated, e.g. fusion proteins and membrane proteins (Sukow et al. 1980; Takakuwa et al. 1999; Chou et al. 2005; Garidel et al. 2009). The theories of surface competition and the surfactant-protein interaction are further explained below.

#### **1.2.2.2. Surface competition**

Proteins as well as surfactants are surface active. The drawback of proteins in the presence of hydrophobic interfaces is their tendency to expose hydrophobic parts to this side through unfolding (Chang et al. 1996b; Baszkin et al. 2001; Kiese et al. 2008; McAuley et al. 2009). Nonionic surfactants have a higher surface pressure and are thus able to reduce the contact time of proteins to interfaces. Different approaches have been utilized to prove this competition directly, e.g. overflowing cylinder method (Eastoe and Dalton 2000; Bain 2008), atomic force microscopy (Mackie et al. 2001; Gunning et al. 2004; Woodward et al. 2009), equilibrium surface tension measurements or rheology measurements in a Langmuir trough (Pearson and Alexander 1968; Wu et al. 2006) or surface tension measurements using a

pressure-controlled pendant-drop surface balance (Wege et al. 2004). The mechanism is not always proposed to be the same. Some authors add direct interaction through charge changes, when ionic surfactants are applied to the interaction mechanisms. But the generally published opinion on the interfacial interaction is that the stronger surface active surfactant molecules displace protein molecules from the air-water interface (Wilde et al. 2002).

In many studies the impact of surfactants on aggregation of pharmaceutical proteins has been discussed. hGH stressed by agitation, which creates an artificial increase and renewal of the air-water interface, could be stabilized by surfactants (Katakam et al. 1995; Katakam and Banga 1997; Maa and Hsu 1997; Bam et al. 1998). Other examples include the stabilization of Interleukin 2 by polysorbates during shaking (Wang et al. 2008) Monoclonal antibodies are stabilized by surfactants (Levine et al. 1991; Mahler et al. 2005; Mahler et al. 2009) and stabilization of recombinant human Factor XIII is by the addition of PS 20 (Kreilgaard et al. 1998). Charman et al. used different approaches to induce unfolding (Charman et al. 1993). In all cases PS 20 acted as a moderate to strong stabilizer.

Further interfacial stress methods are related to the solid state. Repetitive freezing and thawing as a method of stress testing was published already in 1961 in a short Nature article on the stability of catalase (Shikama and Yamazaki 1961). During spray-drying interfaces change dramatically and here, too, surfactants proved their stabilizing ability at the interface. E.g. Maa et al. spray-dried hGH with and without surfactant (Maa et al. 1998). The amount of insoluble aggregates is dramatically decreased from 30 % to <1 % after the addition of 0.05 % PS 20.

#### **1.2.2.3. Molecular interactions between proteins and surfactants**

The most prominent interaction between proteins and surfactants is possibly the application of sodium dodecyl sulphate as denaturant in electrophoresis. In the SDS-PAGE sodium dodecyl sulphate is applied to completely unfold the protein. In this case the charged hydrophilic head of the surfactant is attached at first to the positively charged amino acids and then to the protein peptide backbone. This leads to a complete linearization of the protein. In a polyacrylamid gel this protein-

surfactant complexes can be separated by size as the net charge is mainly size dependent (Shapiro et al. 1967). With this technique separation of proteins only depending on the molecular weight of the protein is possible. Understandably this interaction has nothing to do with protein stabilization.

Furthermore, in structural analysis of proteins, detergents form mixed micelles together with membrane proteins. In this application the surfactants act as membrane replacement (Prive 2007; Lopez et al. 2009). At first the original cell membrane is disrupted by the addition of surfactants and mixed micelles result. After dialysis against surfactant solutions the phospholipids will be removed stepwise and the proteins are stabilized only due to the presence of surfactant micelles.

Another aspect of direct protein surfactant interaction is the fact that proteins which are present in the molten globule or unfolded state can be refolded easier when surfactants are added to the solution. In literature this mechanism is described for hGH and non-ionic surfactants (Bam et al. 1996; Bhattacharyya and Das 1999) whereas ionic surfactants would not assist refolding in the case of rhodanese (Tandon and Horowitz 1987). The terminology for substances that show this behaviour is artificial chaperone. Bam et al. found a ratio of less than 4 molecules polysorbate per molecule human Growth hormone as the best stabilizing ratio for protection against unfolding after interfacial stress. They explained this by coverage of the hydrophobic patches on the protein's surface by the surfactant's fatty acid chains (Bam et al. 1995; Bam et al. 1998) and hence a site specific interaction between the protein molecule and surfactant molecules.

#### **1.2.2.4. Surfactants in pharmaceutical products**

In

Table 1 a selected list of marketed formulations is given. The most frequently used surfactants are Polysorbate 20, Polysorbate 80 and Poloxamer 188 in a concentration range between 0.001% and 0.1%.

Product name	API	Manufacturer	Surfactant	Concentration
Aranesp	Darbepoetin alpha	Amgen	PS 80	0.005
Gonal-f	Follitropin alpha	EMD Serono	Poloxamer 188	0.01%
Herceptin	Trastazumab	Genentech	PS 20	0.06%
Humira	Adalimumab	Abbott	PS 80	0.1%
Lantus	Insulin glargin	Sanofi-Aventis	PS 20	0.02%
Lucentis	Ranibizumab	Genentech	PS 20	0.01%
Mircera	Methoxy-PEG- Epoetin beta	Roche	Poloxamer 188	0.01%
Neulasta	PEG- Filgastrim	Amgen	PS 20	0.0012%
Norditropin	Somatropin	NovoNordisk	Poloxamer 188	0.03%
Nutropin	Somatropin	Genentech	PS 20	0.02
Raptiva	Efalizumab	Genentech	PS 20	0.16%
Rebif	Interferon beta	EMD Serono	Poloxamer 188	0.012%
Reopro	Abciximab	Eli Lilly	PS 80	0.001%
Rituxan	Rituximab	Genentech	PS 80	0.07

**Table 1: List of selected pharmaceutical drug products and their surfactant content [respective full prescribing information]**

#### **1.2.2.5. Problems with Surfactants in Protein Formulation**

The number of approved surfactants in pharmaceutical formulations is limited. Most of the substances are non-ionic as their haemolytic activity is lower and cell rupture is less likely compared to ionic surfactants (Bonsall and Hunt 1971). Typically these non-ionic surfactants are based on PEG as their hydrophilic moiety. Autoxidation of the PEG residues is described for PS 20, PS 40 and PS 60 from Donbrow et al. (Donbrow et al. 1978) and similarly for PS 80 (Ha et al. 2002). Wang et al. also demonstrated a consequently negative impact on protein stability. Sorensen et al. mention that this problem generally occurs in biotechnology and bioanalytics (Jaeger



et al. 1994). They observed autoxidation in PS 20 and Triton-X (Jaeger et al. 1994). Ashani et al. found peroxide equivalents in Brij-35 and Triton-X (Ashani and Catravas 1980).

Apparently, this has also implication on protein stability and protein formulation. Recombinant human ciliary neurotrophic factor and recombinant human nerve growth factor are oxidized to a higher extend in the presence of PS 80 (Knepp et al. 1996). Recombinant human Granulocyte Colony Stimulating Factor is formulated with a very small content of PS 80 due to oxidation problems (Herman et al. 1996). Furthermore, it could be demonstrated that the occurrence of peroxide equivalents from the manufacturing process is related to a reduced long term stability of a monoclonal antibody and human protein relaxin (Nguyen et al. 1993; Lam et al. 1997). This effect is enhanced when protein formulations are stored at elevated temperatures.

For polysorbates autoxidation may additionally cause stronger hemolysis (Azaz et al. 1981). Additionally, some of the approved surfactants products are also attributed to show severe side effects like anaphylaxis, though in very low odd ratios (Attwood 1983).

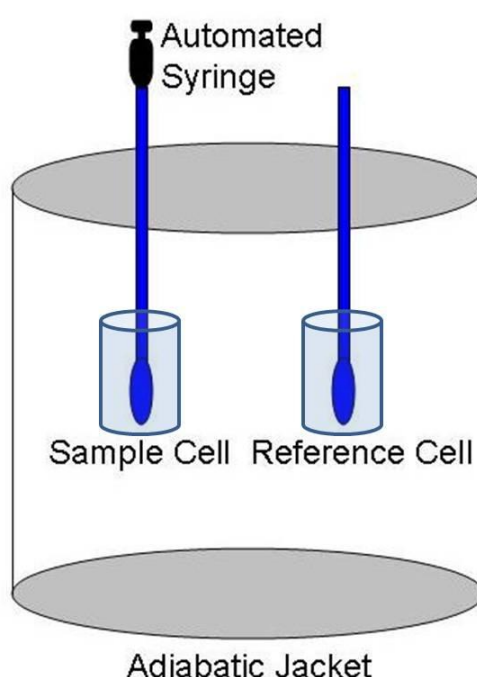
Consequently the term “dual effects” of surfactants on protein stability is stressed by a number of authors. The positive effect at the interfaces is accompanied by the slightly negative effects during storage. Suppliers of surfactants for protein formulation consequently offer special low oxidizing products purified by chromatography. But as the occurrence of H<sub>2</sub>O<sub>2</sub> equivalents is autologous one cannot completely suppress this phenomenon.

#### **1.2.2.6. Approaches to Understand Interaction of Proteins and Surfactants**

In order to understand the interaction between surfactants and proteins different analytical techniques can be applied. The three methods that were mainly utilized in this thesis are isothermal titration calorimetry (ITC), Two-Dimensional Nuclear Magnetic Resonance Spectroscopy (2D-NMR) and Flourescence Correlation Spectroscopy (FCS). These methods will be introduced in their respective chapters.

### 1.2.2.6.1. Isothermal Titration Calorimetry

ITC is one of the major tools for the determination of thermodynamics in protein science (Liang 2008). Especially protein binding energetics can be estimated using this technique (Leavitt and Freire 2001). An ITC machine in general is a microcalorimeter with an attached syringe that is capable of titrating small amounts of e.g. ligands into a sample cell. A general set-up is depicted in Figure 3. The sample cell contains the solution of interest and is attached to a syringe. The reference cell usually contains water or buffer.



**Figure 3: Sketch of a microcalorimeter set-up for ITC**

The main outcome of ITC-measurements is a binding isotherm depending on the amount of injected substance. Furthermore, ITC provides information on free energy of binding, enthalpy of binding and the heat capacity change in one single experiment. By testing at different temperatures the entropy of binding can be determined, too (Simon et al. 2002). Unfortunately evaluating binding isotherms in a single experiment is only feasible when strong interactions occur or when concentrations are extremely high (Chou et al. 2005). A good example for strong protein-surfactant interactions is the lysozyme-sodium dodecylsulfonate (SDS)-system where the anionic surfactants binds to the lysyl, histidyl, and arginyl amino

acid side chains and hence starts to unfold the protein to open more binding spots. In contrast for non-ionic surfactants the binding sites will be hydrophobic patches on the protein surface and no further binding occurs after these are saturated (Jones 1992). The interaction of SDS with proteins is probably the best studied and understood system and massively described in literature (Laemmli 1970; Hicks et al. 1992; Horowitz and Hua 1995; Keire and Fletcher 1996; D'Auria et al. 1997; Giancola et al. 1997; Gao and Wong 1998; Bhattacharyya and Das 1999; Nielsen et al. 2000; Hillgren et al. 2002; Levin et al. 2005; Nielsen et al. 2005a; Nielsen et al. 2005b; Bagger et al. 2007a; Nielsen et al. 2007a; Nielsen et al. 2007b; Andersen Kell et al. 2008; Otzen et al. 2008; Andersen Kell et al. 2009). Unfortunately, these interactions are not transferable into pharmaceutical formulation work, as SDS is too membrane solubilizing and hence hemolytic (Schott 1973; Lopez et al. 1998). Also the critical micelle concentration of pharmaceutically applied surfactants differ greatly from that of SDS. Hence, completely different amounts of monomeric surfactant interact with the protein in a completely different fashion and as the CMCs of the applied surfactants are much lower, a weaker interaction due to the pure lack of monomers will be detected.

To determine weak interactions between surfactants and proteins other approaches have been used (according to the publication by Otzen et al. (Andersen Kell et al. 2008)). By choosing two specific areas in the thermogram and by determining their shift towards higher concentrations binding stoichiometry can be calculated. If the x-axis of these thermograms is displayed in a protein to surfactant ratio all peaks should be similar in its maximum if there is some kind of specific binding area on the protein. Another way of displaying the data is putting only the surfactant concentration on the x-axis to prove purely surfactant-related events in a protein environment (Nielsen et al. 2005a). This way all graphs should show a similar shape with binding enthalpies and onset of micellization proportionally increasing to protein concentration (Andersen Kell et al. 2008). The interaction between pharmaceutical nonionic surfactants and nonspecific binding proteins is rather weak. In comparison, the binding of an antibody to its antigen reveals two magnitudes higher enthalpies (Pierce et al. 1999). Theories whether surfactants act as artificial chaperones also exist for the system rhodanese and insulin with Brij 35 (Bhattacharyya and Das 1999) or hGH and polysorbate (Bam et al. 1996). But for an active folding process, the

measured enthalpies by ITC seem very low. Contrary to this theory is the often found result of strong entropic driven reactions. But chaperones and their backfolding should reduce and not increase the entropy of a system as a higher ordered state will be achieved through this repair mechanism.

#### **1.2.2.6.2. Two-dimensional NMR spectroscopy**

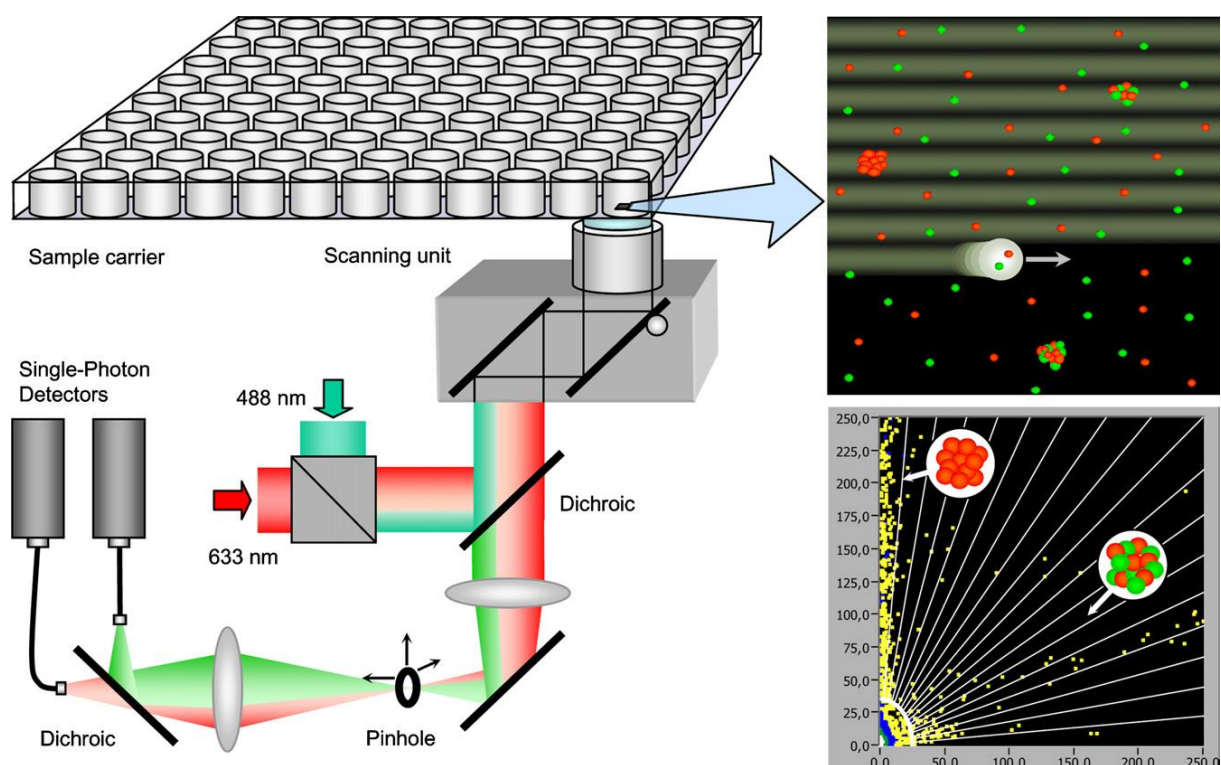
Another way of probing the molecular interactions between proteins and surfactants is Nuclear Magnetic Resonance (NMR) spectroscopy. For example Ulvenlund et al. (Sjoegren et al. 2005) investigated the interactions between polypeptides and alkylglycosides. They applied 2D-NMR spectroscopy to prove the interaction of oligopeptides comprising only one or very few different amino acids. With the help of two dimensional Nuclear Overhauser Effect spectroscopy (NOESY) cross-peaks arising from spins that are spatially close can be seen, but the signals must not be coupled via molecular bonds like in correlated NMR spectroscopy. Furthermore, it is possible to suppress such signals to be sure that no spatially close scalar (from the same molecule) signals disturb the NOESY cross-peak (Gawrisch et al. 2002). For NOE the average maximum distance between two spins is about 5.5 Å as the cross-relaxation signal intensity is dependent by the power of 6 to the distance between the coupled spin signals (Gawrisch et al. 2002). Matilainen et al. (Matilainen et al. 2008) used NOESY to determine the interaction between cyclodextrins and glucagon. Nakanishi et al. studied the same excipients in combination with the Alzheimer's disease related  $\beta$ -amiloid (Qin et al. 2002). Both groups found that the hydrophobic amino acids were packed into the cavity of the cyclic oligosaccharides. Wong et al. (Wymore and Wong 1999) applied To our knowledge the interaction between pharmaceutically applied proteins and surfactants has not yet been studied by NOESY.

#### **1.2.2.6.3. Fluorescence Correlation Spectroscopy**

Fluorescence Correlation Spectroscopy (FCS) is a technique firstly described by Magde et al. in 1972 (Magde et al. 1972). As a proof of principle they determined the

binding constant of ethidium bromide to DNA strands (Elson and Magde 1974; Magde et al. 1974). With the technical development of FCS single molecule tracking was possible due to the invention of confocal fluorescent microscopes and hence smaller detection volumes (Krichevsky and Bonnet 2002).

Further inventions led to scanning FCS systems, where the focus of the microscope moves through the sample cell (Figure 4) to increase the chances of detecting larger protein aggregates with diffusion times that would not be detectable with the regular apparatus. This method is called scanning for intensively fluorescent targets or SIFT (Giese et al. 2005; Levin et al. 2005). In the SIFT set-up following excitation with two different laser lines, two different fluorophores (“green” and “red”) can be analyzed simultaneously in the same focal volume in a confocal setup with single molecule sensitivity. The laser focus is moved through the sample by an optical scanning unit. Whenever an aggregate carrying multiple fluorescent labels passes the focus this results in a short burst of high fluorescence intensity. Individual aggregates pass the focus at different points in time and can be analysed in regard to labelling ratio. Today FCS is also heavily used in lead optimization as a convenient tool for high-throughput screening (Auer et al. 1998) and is also applicable in living cells (Bacia et al. 2006).



**Figure 4: Principle of aggregation analysis by dual-colour SIFT (from Giese, Bader et al. 2005)**

The interaction of proteins with micelles should be investigated using FCS by determining diffusion rates and calculating theoretical particle sizes. For this purpose typically fluorescent labeling of the protein is required which raises the functional question of potential effects of the label itself in the protein properties. To determine the diffusion rates of monomers and bigger oligomers in parallel a labeling degree of 1:1 was desired. This allows the fluorescence detector to find as bright particles (in this case large aggregates) as possible without being overloaded. Further sample properties like counts per particle (cpp) describing the brightness of the fluorescent conjugate and the total intensity of the sample could be determined. The cpp would represent the brightness of a fluorescent labeled protein. The inclusion complex of one or more protein molecules in a micellar “carrier” could result in an increased diffusion time detected in the focus of the apparatus. The big advantage of FCS over Photon Correlation Spectroscopy (PCS) is its lower detection limit and the smaller sample volume required for measurements. In theory a sample volume of a few fl=10<sup>-15</sup>l would be sufficient for measurement (Krichevsky and Bonnet 2002). As the resulting signal fluctuation is derived from Rayleigh scattering the signal intensity of PCS is strongly dependent on the particle radius as described in equation 1:

$$I = I_0 \frac{1 + \cos^2 \theta}{2R^2} \left( \frac{2\pi}{\lambda} \right)^4 \left( \frac{n^2 - 1}{n^2 + 2} \right)^2 \left( \frac{d}{2} \right)^6 \quad (1)$$

where  $I$  is the signal intensity with the wavelength  $\lambda$ ,  $I_0$  is the intensity of the laser source,  $\theta$  is the angle of the scattered light,  $R$  is the distance from the particle,  $n$  is the refractive index of the particle, and  $d/2$  is the particle radius. Hence the presence of big aggregates will suppress signals from smaller particles by sheer intensity (for further details see (Gun'ko et al. 2003)). This dependence is not as strong for FCS as the received signal is derived from the presence time in the focus. A bigger particle in the focus is not necessarily brighter as the amount of labeled protein is only a 0.1 %. So the possibility of aggregate formation with not labeled proteins is higher than with labeled ones.

With the help of different lasers and different fluorescent dyes it is furthermore possible to distinguish between different species and types of interactions in solution (Bieschke et al. 2000). In case of lipid bilayers the interaction between proteins and surface active substances could already be demonstrated (Takakuwa et al. 1999).

Chattopadhyay et al. used a similar experimental set-up to investigate the addition of the amino acid arginine in formulations on protein stability (Ghosh et al. 2009). For micellar systems in a pharmaceutical environment the interaction has not yet been determined.

#### **1.2.2.6.4. Electron Paramagnetic Resonance Spectroscopy**

Electron paramagnetic resonance is a nondestructive experiment where the surfactant micelles are spiked with a spin label. The spectroscopy reveals the ratio of hindered to freely rotating label and gives a statement on the monomeric amount of label in the sample. From the calculation of free micelles, free surfactant molecules and the protein concentrations, binding numbers can be extrapolated. Sukow et al. found out that Triton X in higher concentrations leads to higher binding numbers and correlated this with conformational changes of the protein (Sukow et al. 1980). Randolph et al. tried to calculate binding numbers of Brij and Tween to the proteins hGH and interferon gamma. (Bam et al. 1995). The drawback of this method is the spin labeling that might interfere with the formation of micelles and alter the experimental environment.

#### **1.2.2.6.5. Viscometry**

The surface behavior of surfactant films or protein films that are spiked with surfactants can be studied using viscosity measurements. Pearson et al. in 1968 used a system of surface tension, surface viscometry and surface potential to determine the partitioning of different proteins in cationic surfactant films (Pearson 1968). This experiment gives insight into the interfacial behavior of proteins and their competition for interfaces with surfactants. McAuley et al. used interfacial rheometry by means of an oscillating de Nouy ring (Pearson 1968; McAuley et al. 2009). Different ratios of protein to surfactant are equilibrated and shear elasticity module as well as shear viscosity are tested. After viscoelastic protein films were formed or if the surfactants showed higher surface pressure no film would be formed. This experiment can only be performed once, as due to the shear stress, the films will be

destroyed. This explains the drawback of this experimental set-up. It only gives insight into static systems, where the protein and the surfactant form films at an air-water or oil-water interface. In reality the partition of proteins and surfactants during shaking and generation of new interfaces is of interest and molecular size may have more impact in dynamic systems.

To overcome the problem of static systems, an overflowing cylinder (OFC) can be used. Unfortunately up to today this has not been applied in a pharmaceutical relevant system (Van Kalsbeek and Prins 1999; Eastoe and Dalton 2000; Bain 2008). A resistance plate and flow straightener generate plug flow beneath the free surface of the OFC so that it overflows uniformly on all sides. The outcome of the measurement is the difference between flow velocities at the surface and within the liquid stream. The rate of expansion of the surface and thus the interfacial flow rate is characterized by the help of dynamic surface tension measurements with a Wilhelmy plate or by ellipsometry (Manning-Benson et al. 1997). The dynamic surface tension is only dependent on surface activity and neither on the flow rate over the cylinder, nor on instrumental parameters.

#### **1.2.2.6.6. Small angle X-ray scattering**

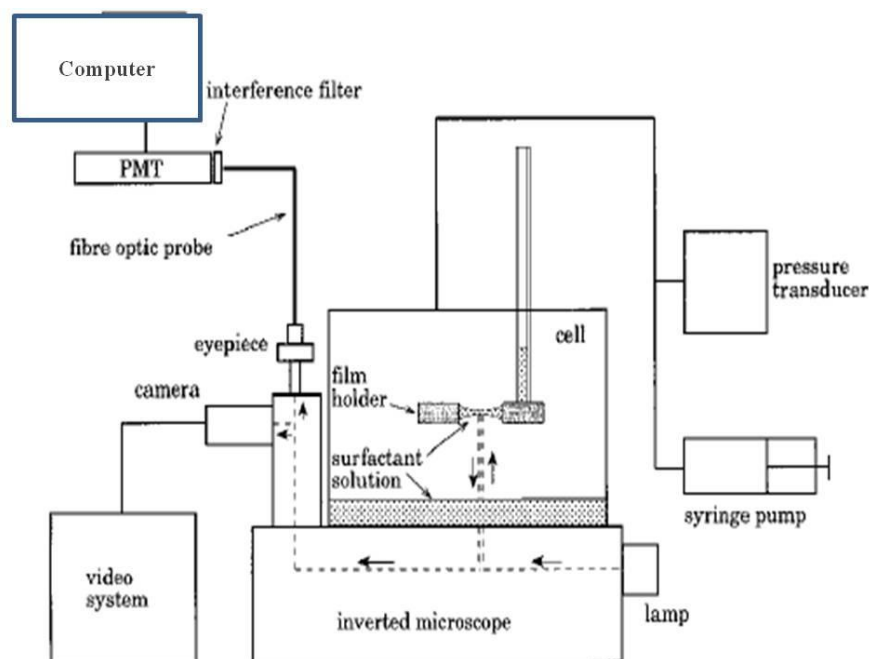
Small angle X-ray scattering can give insight into the three dimensional structure of proteins and surfactant micelles. When a binding number is already calculated the shape of the protein-surfactant complex can be derived from the data. This experimental set-up is yet only been used for model systems. But Otzen et al. could show the pathway of denaturation of bovine acyl-coenzyme-A-binding protein by SDS over several unfolded states and different kind of micelles, respectively. A similar mechanism could be proven for *Humicola insolens* Cutinase by Westh et al. (Nielsen et al. 2005b; Andersen Kell et al. 2009).

#### **1.2.2.6.7. Surface tension measurements**

Similar to surface viscometry, surface tension measurements in a classical Wilhemy set-up can give insight into the partitioning of surfactants into protein films and vice



versa. It is typically utilized in a static mode (Zourab et al. 1983; Wu et al. 2006; McAuley et al. 2009). Dynamic approaches are the use of a Langmuir trough or the use of thin-film balances (see Figure 5) (Bergeron et al. 1996; Sedev et al. 1999).



**Figure 5: Schematic picture of a thin film balance from (Bergeron et al. 1996)**

Another way of performing dynamic surface tension measurements is the (axisymmetric) drop shape analysis (Hansen and Rødsrud 1991; Chen et al. 1998). Maximum bubble pressure has also been tested to analyze the interaction between proteins and surfactants (Fainerman and Miller 1998). All these techniques require optical analysis of the formulations. With the help of these methods dilatational surface phenomena can be studied comparable to the overflowing cylinder.

#### **1.2.2.6.8. Atomic force microscopy**

Atomic force microscopy presents another tool to study protein surfactant interactions. The elasticity of films and foams can be determined with the help of highly sensitive cantilevers. Woodward et al. determined the displacement of milk proteins from surface by the addition of nonionic surfactants (Woodward et al. 2009). Furthermore, Wilde et al. found out that the displacement of proteins by surfactants in foams is strongly dependent on the charge of the polar headgroups of the surfactants

(Wilde et al. 2002). The additional information of atomic force microscopy is the image of the film with a high resolution. Surface pressure and surface tension can also be studied comparable to a de Nouy Ring for the air-water interface as well as for liquid-liquid interfaces (Goddard 2002).

#### **1.2.2.6.9. Equilibrium Dialysis**

The experimental set-up for equilibrium dialysis is rather simple. Protein and surfactant are placed in a donator separated from the acceptor by a semipermeable membrane with a molecular weight cut-off smaller than the proteins molecular weight. After incubating for longer time periods the surfactant concentration is quantified for the dialysate as well as for the protein side. Binding numbers can be calculated from the amount of protein in the cell and the surplus of surfactant in the protein solution. Sukow performed studies on the binding of the nonionic surfactant Triton X to rabbit and bovine serum albumin (Sukow et al. 1980; Sukow and Bailey 1981). Two important drawbacks of this method are that it is very time consuming and that the dialysis membrane might hamper big surfactant associates from migrating to the buffer side. Furthermore, during the long time period instability of the protein may substantiate.

#### **1.2.2.6.10. Fluorescence Spectroscopy**

Fluorescence spectroscopy has been used in different ways to determine protein-surfactant interactions. One approach is to measure the quenching of the intrinsic fluorescence of the proteins' aromatic amino acids. Unfortunately the sensitivity of this method is not very high and only limited statements on mechanisms can be made (Hillgren et al. 2002; Nielsen et al. 2005a). An advantage of this method that it enables to study kinetics using high throughput machines (Andersen Kell et al. 2009). Total internal reflection fluorescence is utilized when adsorption phenomena take place. As for example human Growth hormone is adsorbed on different surfaces (Buijs et al. 1998). The removal of fluorescent dyes like Nile red or Bis-ANS from hydrophobic patches of the protein's surface can also be studied (Bam et al. 1996).

The dyes could also be used as markers for unfolding due to the addition of surfactants (Hawe et al.). The addition of pyrene to protein solutions was used to determine shifts in CMC and hence binding numbers by Westh et al. (Andersen Kell et al. 2008).

The drawback of all extrinsic set-ups is the addition of a very hydrophobic substance to the sample environment and hence an alteration of the system (Hawe et al.).

#### **1.2.2.6.11. Other**

There are further methods in biophysics and biochemistry that could possibly be applied on pharmaceutical protein surfactant systems like Pulsed-field-gradient spin-echo (PGSE)-NMR (Hillgren et al. 2002),  $^1\text{H}$ -NMR together with  $^{13}\text{C}$ -NMR (Gawrisch et al. 2002), Brewster angle microscopy (Mackie et al. 2001) surface plasmon resonance spectroscopy, mass spectrometry, dynamic scanning calorimetry although often discussed as tricky (Katakam et al. 1995; D'Auria et al. 1997), optical reflectometry (Sun and Tilton 2001), and  $^{14}\text{C}$  in situ radio tracing (Baszkin et al. 2001) that are not explained in deeper detail.

### **1.3. Objective of this thesis**

Surfactants are the excipients of choice to reduce interface induced stress to pharmaceutical proteins. The PEG based surfactants that are approved today pose a problem due to their oxidizing behavior. One objective is to test new excipients that are comparable to the gold standard polysorbate. The surfactants should be comparable with respect to lowering of the surface tension, CMC in absolute and/or molar ratios, hemolytic activity, solubility, and other general physico-chemical properties. On the other hand they should show some advantages over the polysorbates:

**No PEG residue** or other **oxidation** related moieties

**Higher purity** to assure better batch to batch reproducibility

**Easy** and **cheap** to synthesize

If possible, derived purely from **renewable resource**

Most important, the alternative surfactants have to provide protein stabilization properties similar or better than the polysorbates. Different stress methods are applied to different pharmaceutical proteins and the extent of aggregation and oxidation is studied.

As stated in the introductory part it is still unclear by which mechanism surfactants are able to stabilize protein pharmaceuticals. Hence the interaction on a molecular level is studied in cooperation with partners via with Isothermal Titration Calorimetry (ITC), Nuclear Overhauser Enhancement Magnetic Resonance Spectroscopy (NOESY) and two dimensional Fluorescence Correlation Spectroscopy (2D-FCS).

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## 2. Physicochemical characterization of technical mixtures of sugar based surfactants

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### 2.1. Introduction

Surfactants can be classified into four groups based upon the charge of the polar headgroup of the surfactants (

Table 2). First scientific classifications were offered by Bancroft et al. almost 100 years ago (Bancroft 1912). One may also group surfactants by their hydrophilic-lipophilic balance value (HLB-value). This classification is still in use for now more than 50 years (Griffin 1949), although not completely accurate (Pasquali et al. 2008). Furthermore surfactants can be classified by their tendency to form either W/O or O/W emulsions (hence W/O or O/W-emulsifier).

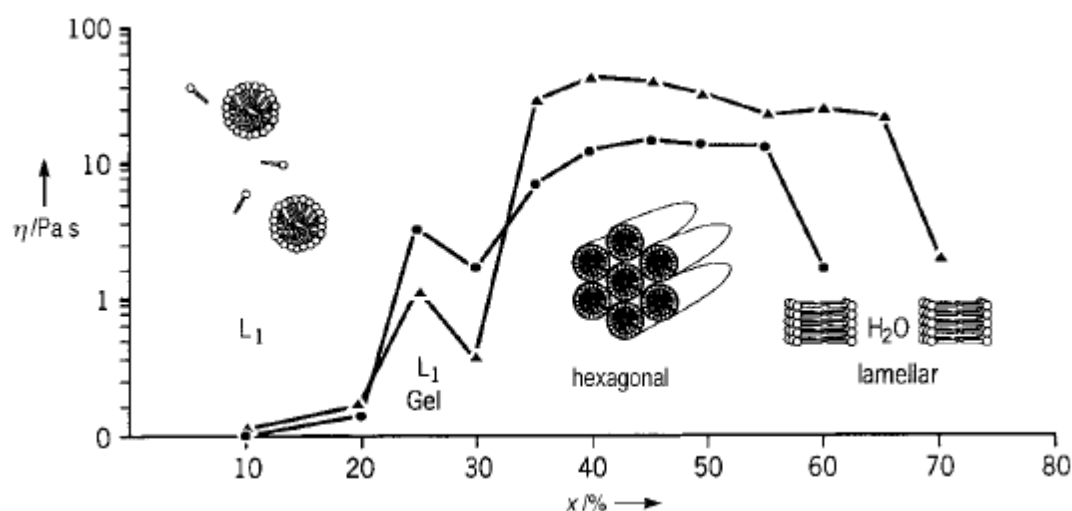
Class of surfactant	Examples
Cationic surfactants	Quats
Anionic surfactants	SDS, SLS
Zwitterionic surfactants	Phosphatidylcholine
Nonionic surfactants	Polysorbates, PEG fatty acid esters

**Table 2: Surfactant classification by charge of the polar headgroup**

Cationic surfactants are widely used as cleansing vehicles in the petrochemical industry or as preservatives (Attwood 1983; von Rybinski and Hill 1998). Anionic surfactants are commonly used in biochemistry and their most prominent example is sodium dodecyl sulphate (SDS), ubiquitarily used in gel electrophoresis (Shapiro et al. 1967). In some cases anionic surfactants are used in semisolid or liquid oral

formulations. Zwitterionic surfactants could theoretically be used in protein formulation although their surface activities are rather low compared to nonionic surfactants. But usually they are applied in pharmaceutical industry in early formulation for solubilization of poorly soluble drugs. Zwitterionic surfactants can be used as transport vesicles e.g. liposomes as they tend to form bigger associates (Schurtenberger et al. 1985). In pharmaceutical industry probably the main application of charged surfactants can be found in topical formulations such as creams or ointments as classical emulsifiers.

In this work all tested substances belong to the class of nonionic surfactants. Although not charged, the HLB values of these substances are also rather high as complete water solubility is mandatory for later use in aqueous parenteral solutions. Nonionic surfactants usually show a low critical micelle concentration (CMC) in water, as their tendency for self-association is high. This is commonly explained by the lack of shielding charges around the nonpolar parts of the molecule and the resulting low solubility in water (Rulison 1995; Patist et al. 2000).

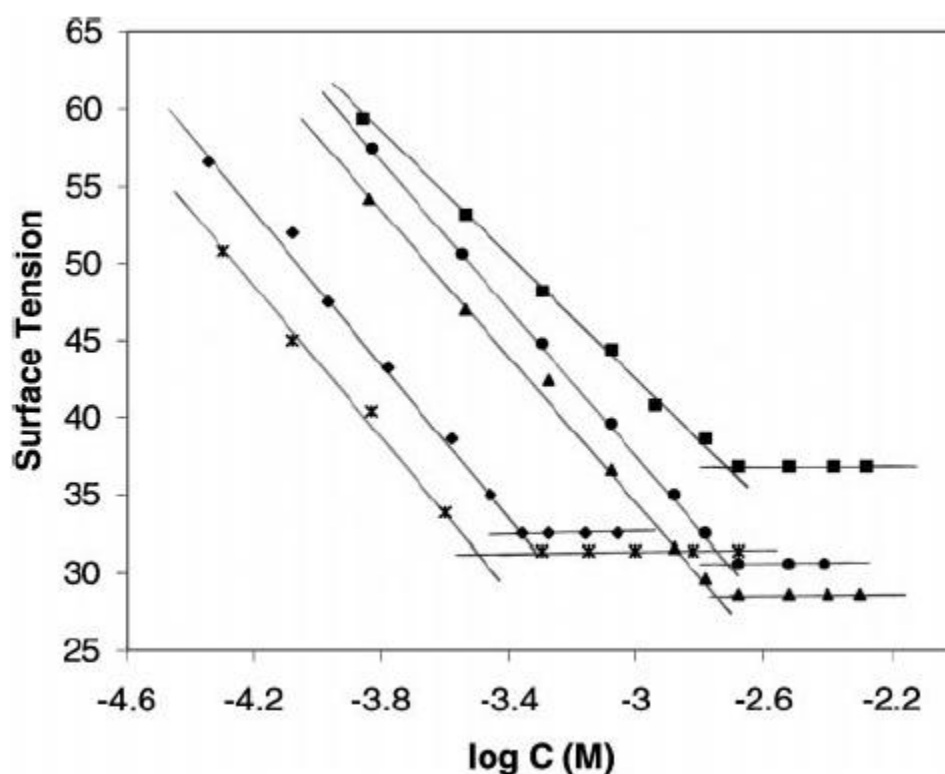


**Figure 6: Effect of absolute surfactant amount on physical state and viscosity of SLS and a mixture of alkylpolyglycosides in water (from (von Rybinski and Hill 1998))** L1 represents the simple micellar solution. At L1 Gel, the number of micelles is already high enough to increase viscosity.

At lower concentration nonionic surfactants usually dissolve as monomers and dimers. At the CMC associates to larger spherical structures, in which the part of the molecules is placed on the outside that shows a similar polarity as the solvent. In our case it is the carbohydrate part that is directed to the aqueous environment. By

increasing the concentration surfactants molecules form rod-like micelles, hexagonal or cubic phases and finally lamellar liquid-crystalline phases depending on the substance (see Figure 6). Theoretically some nonionic surfactants would also be able to form inverse micelles in an oily environment, but this is not of relevance for this work. For protein formulations only the concentration range in which spherical micelles are formed, is of interest as higher concentrations of surfactant would lead to cell rupture and hence adverse side effects during application (Soderlind et al. 2003).

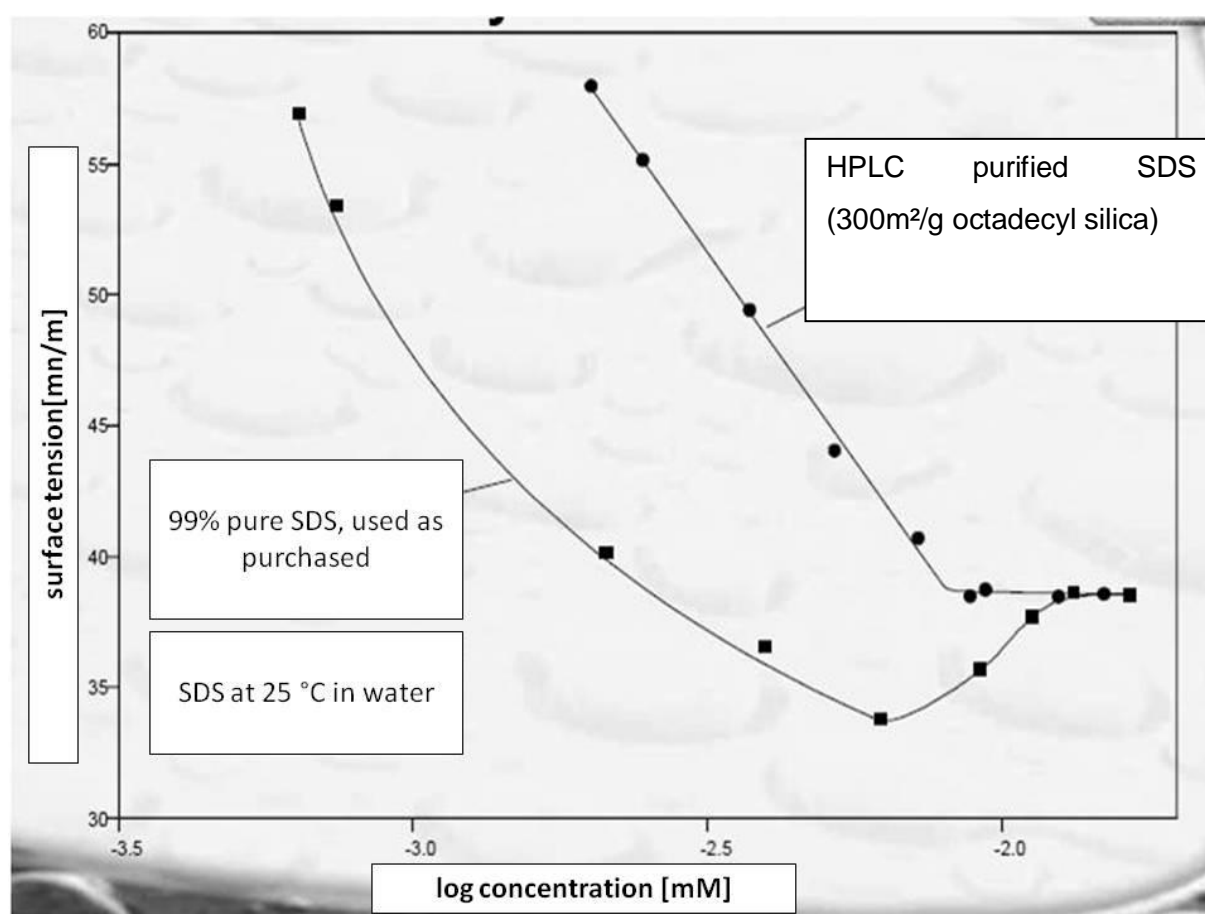
In contrast to the alterations of viscosity, the pronounced surface tension changes occur in the concentration range up to the CMC. Above the CMC the air liquid interface becomes fully occupied by surfactant molecules and the surface tension is not further decreased by the addition of more surfactant molecules (see Figure 7). By increasing the surfactant concentration the surface tension typically decreases in an inverse exponential fashion until the point of self association.



**Figure 7: Surface tension of different alkylpolyglycosides and mixtures with other surfactants (from (Rosen and Sulthana 2001))**

In reality this holds only true for highly pure systems. If technical mixtures of

surfactants are used surface tension decreases similarly, but the CMC is not a sharp break, instead one finds a minimum in between the two (Patist et al. 2000). This alteration can be explained by the different chain lengths of the various molecules in a technical product. In the mixture, there is a greater variability in the attractive forces between the molecules and the first molecules that form micelles are the ones with the highest self attraction. Successive inclusion of the better soluble molecules leads to mixed micelles and a higher surface tension. This ratio than describes the constant surface tension segment (Figure 8).



**Figure 8: Surface tension of different purities of SDS (from (Krüss-GmbH 2003))**

In this chapter different sugar based surfactants were studied and compared to polysorbates. These sugar based surfactants can contain a sugar moiety attached to a fatty alcohol (alkyl glycoside) or to a fatty acid (sugar ester). Different providers have these products in their portfolio. E.g., the Ryoto products for example can be obtained with different HLB values; adjusted values from 7 to 15 are on the market. These sucrose fatty acid esters are mainly added to food products. Cognis offers

alkyl glycosides as different brands. The Plantacare products are mainly used as surfactant in cosmetic cleansing preparations as they form clear solutions and the Emulgade products as emulsifiers. NV-10 is a product from Expedeeon. It is directly dedicated to stabilize proteins during the downstream process. In

Table 3 properties, applications, and the ingredients as declared by the supplier are listed.

Supplier	Brand name	Properties	Typical application	Declared components
Ryoto (Syntapharm)	S 1570	Water soluble HLB = 15	Food industry (beverages)	Sucrose fatty acid esters (mainly sucrose stearate)
	P 1570	Water soluble HLB = 15	Food industry (beverages)	Sucrose fatty acid esters (mainly sucrose palmitate)
	S 1170	Water soluble HLB = 11	Food industry (ice cream)	Sucrose fatty acid esters (mainly sucrose stearate)
	S 770	Water soluble HLB = 7	Food industry	Sucrose fatty acid esters (mainly sucrose stearate)
	Monoester P	Water soluble HLB 15-16	Pharmaceutical Industry	Sucrose fatty acid esters (> 70% sucrose palmitate)
Dai-Ichy Kogyo Seiyaku (Sisterna)	Sisterna Sucrose Esters	Water soluble HLB ~ 16	O/W emulsifier	Sucrose fatty acid esters (mainly mono-esters)
Cognis	Plantacare 2000 UP	Water soluble	Aqueous solutions in cosmetic industry	Oligosaccharides attached to fatty alcohols
	Plantacare 818 UP	Water soluble	Aqueous solutions in cosmetic industry	Oligosaccharides attached to fatty alcohols

	Plantacare 1200	Waxy flakes	Emulsifier pharmaceutical industry	in	Oligosaccharides attached to fatty alcohols (lauryl glucoside)
	Emulgade	Waxy flakes	Emulsifier pharmaceutical industry	in	Oligosaccharides attached to fatty alcohols
Expedeon	NV 10	Water soluble	Protein stabilizer during downstream		Oligosaccharides attached to hydrophobic residues

**Table 3: List of properties, applications, and ingredients as declared in the supplier's documents for different sugar based surfactants**

All these substances were checked for their application in aqueous pharmaceutical protein formulation. Hence, the applied amounts are low, but should be solubility up to 1% (m/V) has to be given. For the characterization of the different classes of substances the CMC was determined as well as their tendency to cause hemolysis in porcine erythrocytes. Some experiments were performed with more than one method as a proof of concept or to use an orthogonal method for CMC determination. In some instances additional information could be gained from these experiments (e.g. micelle size from PCS measurements). As some systems are not clearly chemically specified, we tried to elucidate the structure and composition of the materials and potential byproducts. Residual metal ions might catalyze chemical degradation (Stadtman 1993), other impurities might be hemolytic (Azaz et al. 1981), cause immunogenic reaction (Attwood 1983) and should be known before added to protein formulations for invasive application. Summarizing, the aim of this study was to determine, whether the new surfactants can be compared to polysorbates in terms of physical, chemical and biological properties, and whether the new surfactants will show advantages over the approved products in this regard.

## **2.2. Materials and Methods**

### **2.2.1 Materials**

Plantacare 818 UP (PC 818), Plantacare 2000 UP (PC 2000), Plantacare 1200 (PC 1200) and Emulgade PL and Emulgade S were kindly donated from Cognis (Düsseldorf, Germany). Nvoy 10 (NV 10) was donated by Expedeon Protein Solutions (Cambridge, United Kingdom). Sisterna Sucrose Ester (Sisterna) was donated by Dai-Ichyo Kogyo Seiyaku Co. (Kyoto, Japan). Furthermore Ryoto sugar esters were donated by Syntapharm (Mühlheim, Germany). The Ryoto products tested were: Monoester P (MP), Sugar Ester P1570 (P1570), Sugar Ester S1570 (S1570), Sugar Ester S1170 (S1170) and Sugar Ester S770 (S770). Polysorbate 20 (PS 20) and Polysorbate 80 (PS 80) were obtained from Merck chemicals (Darmstadt, Germany) and stored at 2-8 °C under nitrogen atmosphere until use.

The water used in all experiments was deionized water from the university supply purified in a PURELAB Plus system (ELGA labwater, Celle, Germany) with a conductivity of <0.055 µS/cm.

Acetonitrile was of HPLC grade (Merck chemicals, Darmstadt, Germany). The water/acetonitrile mixtures were degassed in an ultrasonic bath for 3 h at room temperature. The eluent was kept under stirring on a heating plate at 45 °C to prevent gas bubbles from entering into the system

Pyrene was of synthesis grade (Merck chemicals, Darmstadt, Germany) and ethanol for dilution of technical grade (Sigma-Aldrich, Munich, Germany). Buffer salts and other reagents were of analytical grade or higher.

### **2.2.2 Methods**

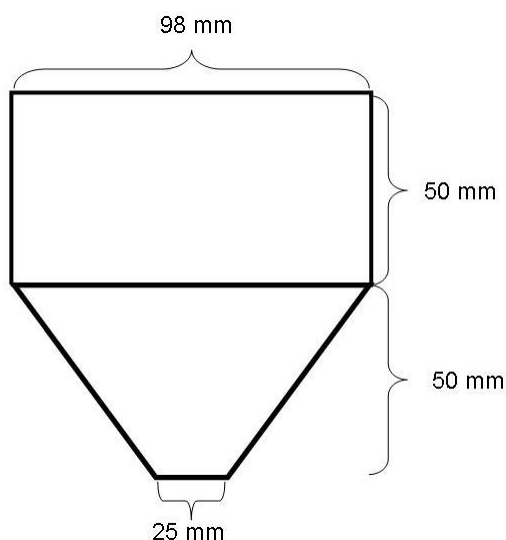
#### **2.2.2.1 Solubility testing**

Surfactant solutions of 0.1% to 20% (m/V) were prepared. If instant dissolution occurred, the products were equilibrated for 24 h. If turbid systems resulted, the

product was equilibrated either in a water bath at 50 °C or in an ultrasonic bath at ambient temperature for 24 h. When visual solubility could not be achieved the system was claimed insoluble. Optically clear solutions were centrifuged at 10000 g for 10 min and checked for undissolved material

### 2.2.2.2 Tensiometry

Surface tension was measured with a K100 MK2 tensiometer (Krüss, Hamburg, Germany) equipped with a F12 thermostat (Julabo, Ostfildern, Germany) and a 765 Dosimat (Metrohm, Leinfelden-Echterdingen, Germany) using LabDesk 3.1 software (Krüss, Hamburg, Germany). As a reference purified water was analyzed (requirement:  $72.0 \pm 0.1$  mN/m ;  $n=3$ ) before analysis of the surfactants. The Critical Micelle Concentration (CMC) was determined from sharp breaks in surface tension vs. logarithm of surfactant concentration plots. A custom-made glass vessel (dimensions are displayed in Figure 9) and a platinum iridium plate were used for all experiments. A total of 11 concentrations were tested for each surfactant. One measurement lasted at least 300 sec. Experiments ( $n = 3$ ) were performed at 25 °C. All samples were left for 30 min for temperature equilibration before measurements.

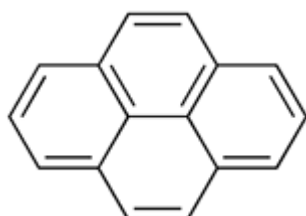


**Figure 9: Dimensions of the custom-made glass vessel for CMC determination via surface tension measurement**



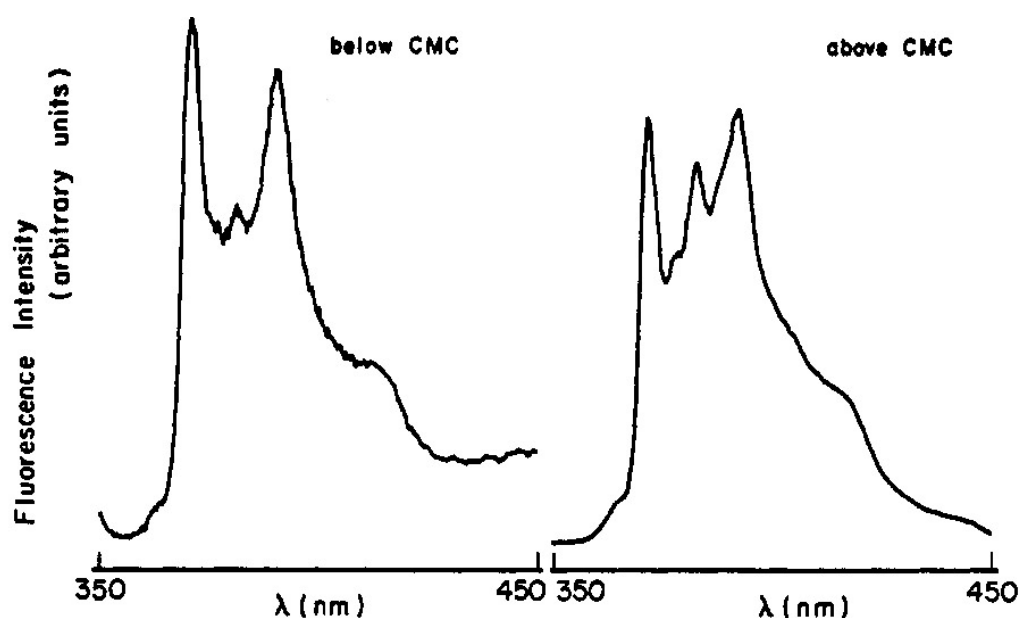
### 2.2.2.3 Pyrene Interaction Fluorescence

The interaction of the hydrophobic fluorescent dye pyrene (Figure 10) was used to determine the CMC of the surfactants alternatively to the surface tension measurements.



**Figure 10: Chemical structure of pyrene**

The fluorescence intensity of pyrene shows strong solvent dependency. In a polar environment such as the inner core of surfactant micelles the intensity of the 0-0 vibronic band is significantly enhanced at the expense of other bands. The strong perturbation of the vibronic band intensities has been used as a probe to accurately determine CMCs (Kalyanasundaram and Thomas 1977).



**Figure 11: Pyrene monomer fluorescence in aqueous sodium lauryl sulphate solutions, at concentrations below (left) and above the CMC (right).**

Fluorescence spectra were recorded and the ratio  $I_1/I_3$  was calculated, where  $I_1$  was the emission at 373 nm and  $I_3$  was the emission at 393 nm. Pyrene was excited at a

wavelength of 335 nm. Due to a change in polarity as the hydrophobic molecule pyrene is inserted into the hydrophobic alkyl residues in the inner core at the occurrence of micelles, the ratio becomes <1.

Surfactant concentrations comprised the same range as used in surface tension measurements. Experiments were performed with a Cary Eclipse spectrofluorimeter (Varian, Palo Alto, USA). Pyrene was added at 200 mM as an ethanolic solution. 10 µl of this solution was added to 1990 µl surfactant solution. 2 ml of the surfactant pyrene mixture were studied in quartz cuvettes with 1 cm pathway. Figure 11 shows the surfactant concentration dependent changes in the pyrene fluorescence.

#### 2.2.2.4 Hemolytic Activity

Hemolytic activity was measured with porcine erythrocytes. Coagulation was prevented by adding 200 ml 3.8 % (m/V) trisodium citrate buffer pH 7.4 to 800 ml fresh blood. Erythrocytes were washed six times with citrate buffer and centrifuged at 3.500 g and 5 °C for 45 min. Different surfactant concentrations (in the same citrate buffer as used for purification) were added to the porcine erythrocytes suspension at a 1:5 ratio. After incubation for 1 hour at 37 °C under gentle shaking samples were centrifuged (3.500 g for 15 min). The supernatant was tested for free hemoglobin at  $A = 415$  nm using an UV 8453 UV-spectrometer and UV-Visible ChemStation software (Agilent, Santa Clara, USA). Each experiment was performed with  $n=5$ . 100 % hemolysis was determined by diluting 10 µl erythrocytes in 990 µl Millipore water. By adding pure buffer to the erythrocyte suspension the basal hemolysis over the experimental time (0%) was tested. The degree of hemolysis produced by surfactants (%H) was calculated according to equation 2.

$$\%H = \frac{Hb - Hb_0}{Hb_{tot}} \times 100 \quad (2)$$

$Hb$  is the amount of hemoglobin found in the sample,  $Hb_0$  is the amount of basal hemoglobin found in the blank and  $Hb_{tot}$  is the amount of hemoglobin after 100 % hemolysis, resp. their absorbance at 415 nm at 37 °C. Hemolytic activity ( $HC_{5\%}$ ) was defined as the concentration at which at least 5 % hemoglobin was found in the supernatant (adapted from (Reinhart and Bauer 1995) and (Seyfert et al. 2002)).

#### **2.2.2.5 Photon Correlation Spectroscopy (PCS)**

Size determination of micelles in 1 % surfactant solutions was performed on a Zetasizer nano ZS (Malvern Instruments, Herrenberg, Germany) considering the sample viscosity (2.2.2.6) with a 633 nm laser. The hydrodynamic diameter was calculated with DTS NANO v5.10 software (Malvern Instruments, Herrenberg, Germany). The sample, in single-use UV-plastic cuvettes (Brand GmbH and Co KG, Wertheim, Germany), was at first equilibrated for 1 min at 25 °C and subsequently the time scale of the scattered light intensity fluctuations of the sample was measured. Each sample was recorded in triplicate, each with 20 runs. The size was obtained using the cumulants analysis by fitting a single exponential to the correlation function in order to obtain the volume-weighted mean size. For CMC determination PCS was also applied. For these experiments, the autocorrelation function was observed. In the presence of micelles a fit could be applied. Lacking micelles the calculated size drops by at least one order of magnitude or increases substantially due to few dust particles as the system tries to fit the few obtained signals into a size distribution. Thus, with increasing concentration at the CMC a clear correlation could be fit the first time and the micelle size as well as the CMC can be detected.

#### **2.2.2.6 Viscometry**

As viscosity is a crucial parameter for PCS measurements the viscosity of the surfactant solutions was determined on an AMVn falling sphere viscometer (Anton Paar, Graz, Austria). 800 µl of a 1 % surfactant solution was filled in a 1.6 mm diameter tube and viscosity was recorded at 25 °C ± 0.1 °C. A 60° angle was applied and every solution viscosity was determined 10 times.

#### **2.2.2.7 Elemental Analysis**

For elemental analysis for carbon, nitrogen, hydrogen and sulfur by the central analytics group at the Department of Chemistry at the Ludwig-Maximilians-Universität, Munich samples were cracked into an oxidized form (CO<sub>2</sub>, H<sub>2</sub>O, NO,

NO<sub>2</sub>, SO<sub>2</sub>, SO<sub>3</sub> and N<sub>2</sub>) of the respective atom. The resulting gases were analyzed using a gas chromatograph and a Thermal Conductivity Detector. The applied system was a Vario EL elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

Additionally Inductive Coupled Plasma analytics were performed for the Plantacare products as the material data sheet provided the information that traces of magnesium might be present in the products. Analysis for sodium (Na), magnesium (Mg) and calcium (Ca) was conducted.

#### **2.2.2.8 Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)**

For separation of the technical products and quantification of the ingredients, RP-HPLC was performed with a Refractive-Index (RI) detector ERC-7515A (Erma CR. Inc. Kawaguchi City Japan) and an Evaporative Light Scattering detector (ELSD). Both were coupled to an 1100 Agilent HPLC system using a HP 3590E digital/analog converter (Agilent Technologies, Waldbronn, Germany). RP 8 and RP 18 columns were applied (Klaffke et al. 1998; Kuehn and Neubert 2004). The eluent was composed of 60 % (w/w) acetonitrile and 40 % (w/w) water. The flow was set to 0.5 ml/min.

#### **2.2.2.9 Content of Peroxides**

H<sub>2</sub>O<sub>2</sub> equivalents were tested with Merckoquant® peroxide test strips (Merck, Darmstadt, Germany) with levels of detection between 1 mg/ml and 100 mg/ml according to the user's manual. If the test strip limit of 100 mg/l hydrogen peroxide equivalents was exceeded the samples were diluted by factor 10 until the result was in the specification range.

In additional experiments selected samples were placed in the Suntest CPS light stress testing machine (Heraeus, Hanau) at 54 W/m<sup>2</sup>, xenon lamp, for 72 hours to compare artificially aged surfactant solutions.

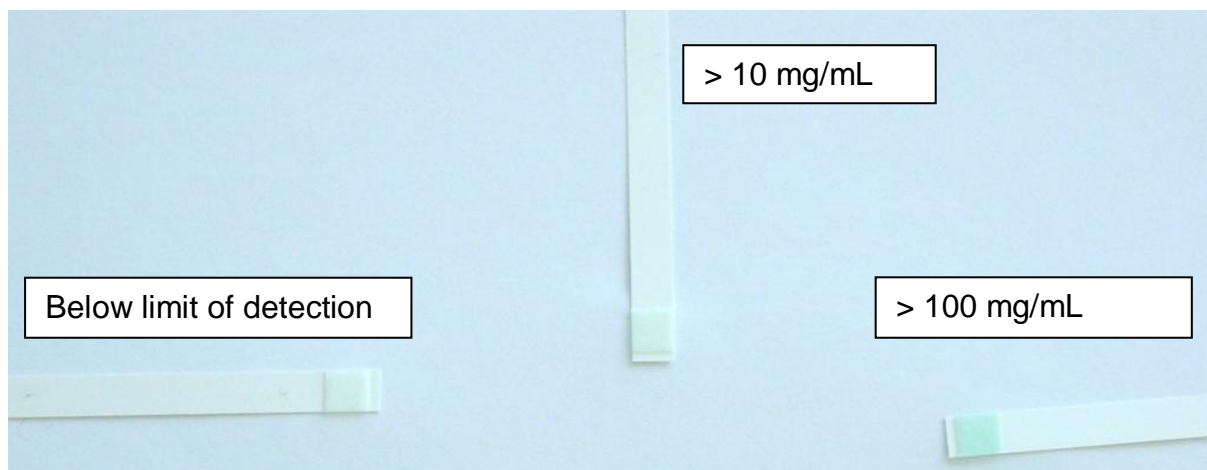


Figure 12: Example photograph of peroxide test

#### 2.2.2.10 Mass spectrometry

For mass spectrometry (MS) the central analytics group at the Department of Pharmacy at LMU ionization was achieved by electron spray ionization. Analysis was made in positive mode. The Q-TOF detector was coupled to an Agilent Liquid Chromatography (LC) system. Samples were prepared as 100 ppm solutions in methanol and not separated but injected via the LC system. As no internal standards were added to the samples all results are only of qualitative relevance. But as all nonionic surfactants are very similar in their ionization behavior (Kuehn and Neubert 2004) this method can also provide useful semiquantitative information on these products. For the NV10 product additionally different ionization mechanisms were applied in the central analytics group of the Department of Chemistry at LMU.

### 2.3. Results and Discussion

The tested surfactants can be classified into four subgroups:

Surfactants with solubility < 0.1%

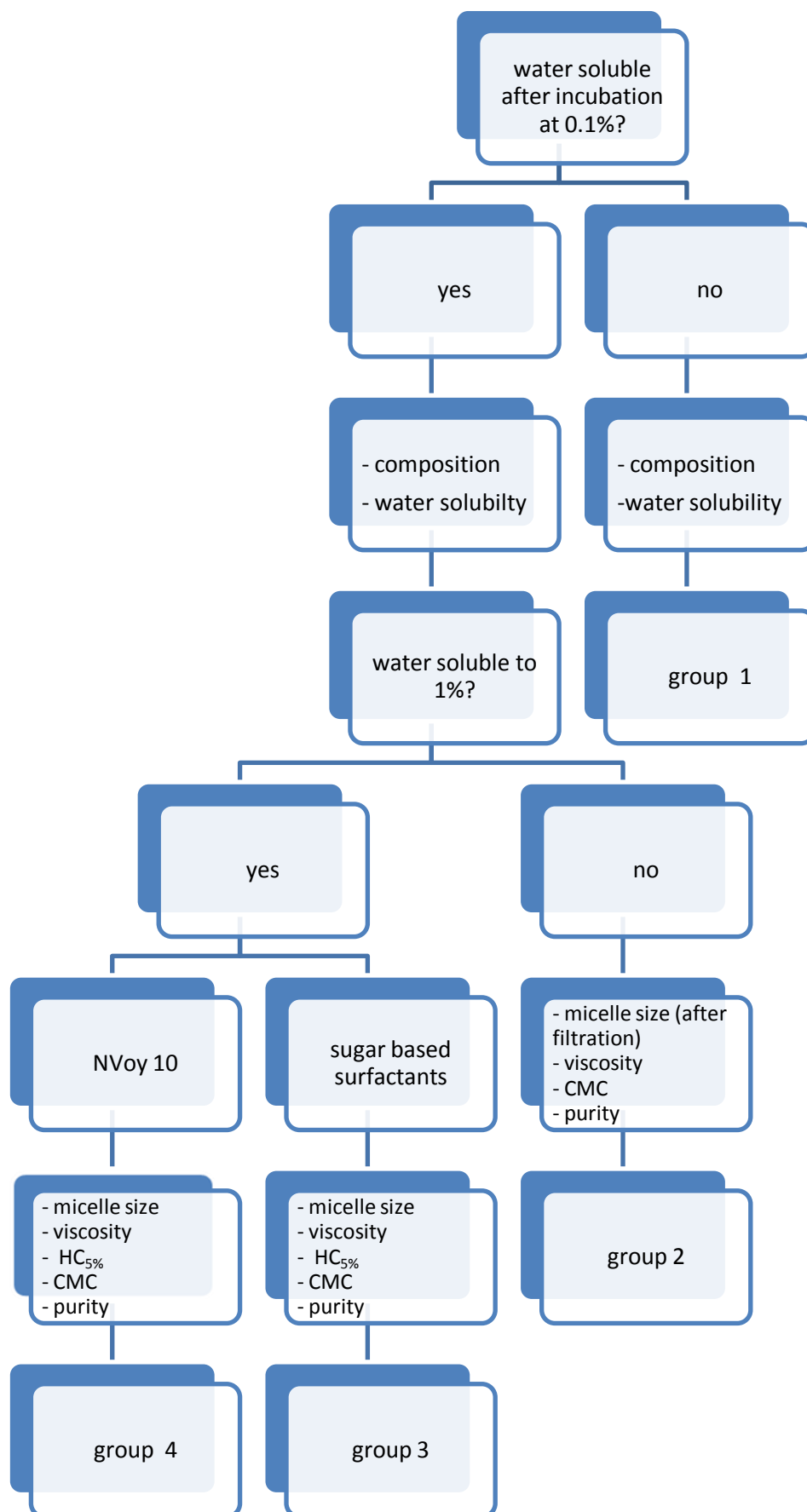
Surfactants with solubility < 1%

Surfactants with solubility > 1%

NVoy polymers (also with good water solubility)

The workflow is visualized in Figure 13. The first one is listing the poorly water soluble surfactants that were not further tested as they tend to form aqueous slurries or gels that could not be diluted or dissolved by heat or incubation in an ultrasonic bath.

To reduce unnecessary work for the analysis of these products, a workflow was used as described in Figure 13. Depending on the water solubility more and more experiments were performed to gain deeper knowledge of the physico-chemical behavior of these excipients. As stress testing of protein formulation is cost and work intensive only those excipients that show overall usefulness for potential application in parenteral formulation were included in the stress studies and the biophysical experiments.



**Figure 13: Work flow for surfactant analytics**

### 2.3.1 Poorly Soluble Surfactants

#### 2.3.1.1. Poorly Soluble Sugar Esters

Although the supplier claimed good water solubility for his products with a HLB of 15 (Ryoto 2007), most of the Ryoto sugar esters could not be dissolved adequately (see Table 4). This might be due to the higher amounts of long chain fatty acids (esters with the declared stearic acid), especially diesters in these products.

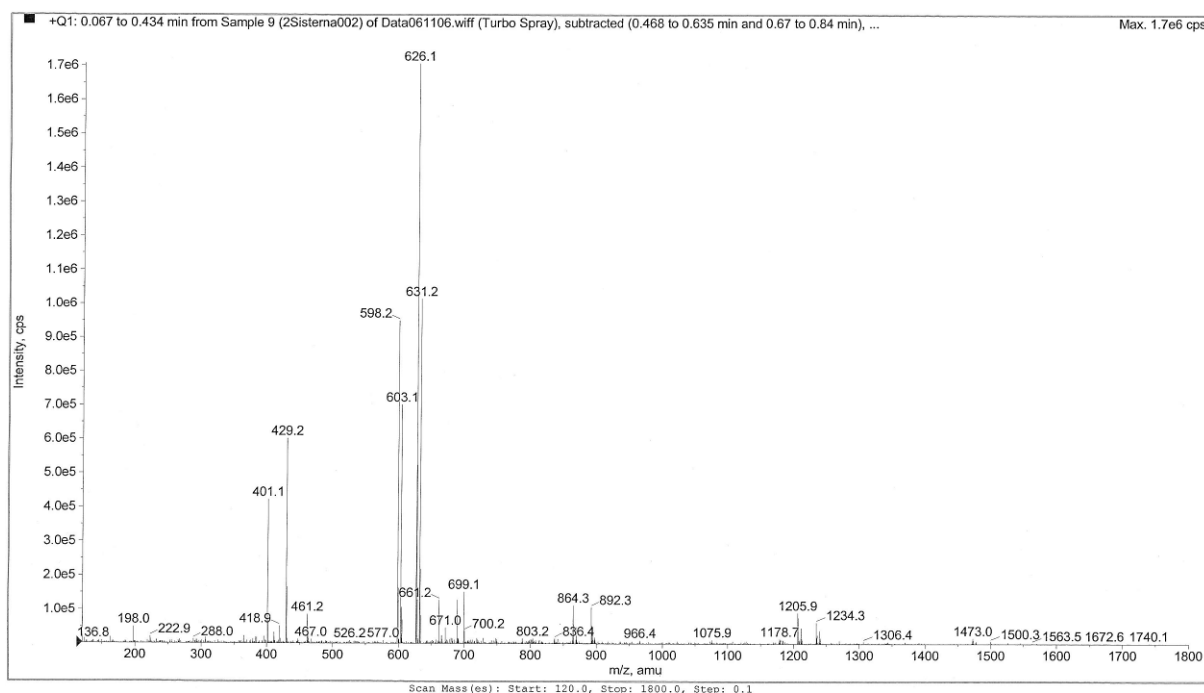
Substance	Solubility in water	Suppliers technical data sheet
S770	No	No solubility in water/HLB 7
S1170	No	No solubility in water/HLB 11
S1570	0.1% - 1%	Soluble at 25 °C/HLB 15
P1570	0.1% - 1%	Partially soluble at 25 °C in water/fully soluble at 75 °C/HLB 15

**Table 4: Summary of aqueous solubility of different Ryoto sucrose fatty acid esters.**

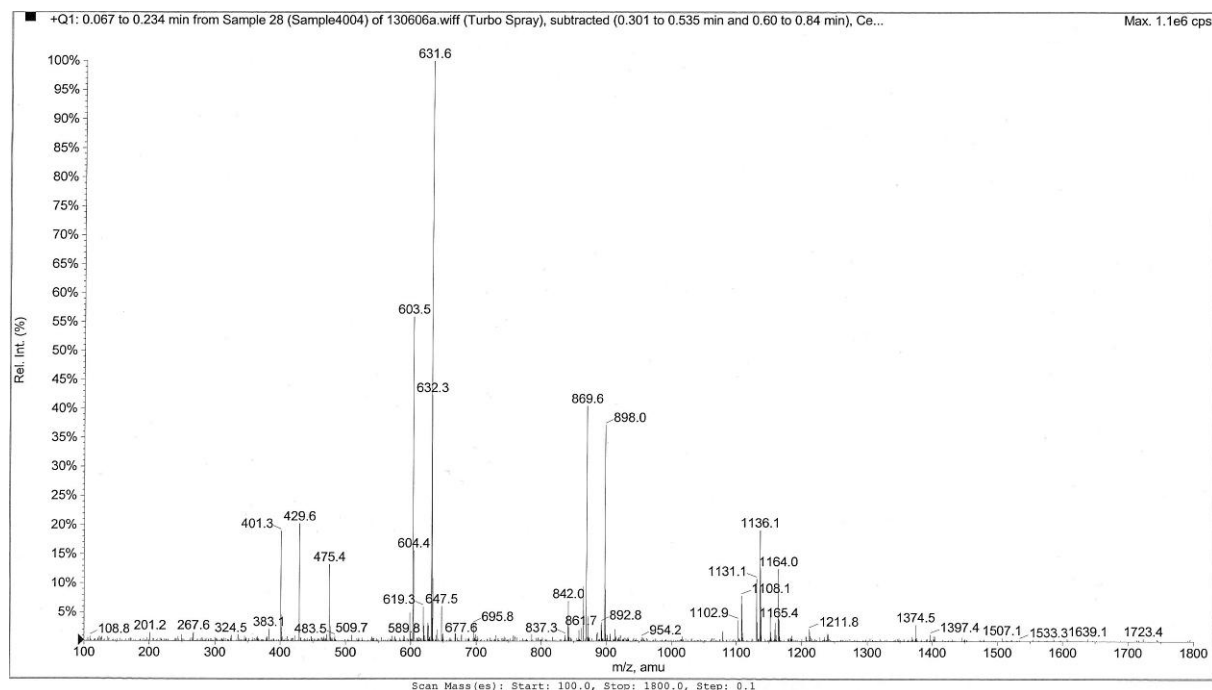
P1570 and S1570 showed partial solubility in water. The remaining solid fraction is most probably composed of long fatty acid esters, di- or triesters with poor solubility (described e.g. by (Aveyard et al. 1998)). Upon heating or sonification, the substances could be turned into highly viscous gel like systems that were still separated from the water phase. Similar results were obtained with the Sisterna product. The Sugar Ester® (HLB 16 according to manufacturer information) was only partially soluble in water. Since its solubility is below 0.1 mg/ml it was not further tested. Although the product data sheet refers to sucrose palmitate as the main substance, mass spectrometry gives a hint that sucrose stearate might be the main substance (see Figure 14). The main peak has  $m/z$  of 626.1 ( $m+H_2O^+$  or  $m+NH_4^+$ ) and the second largest peak is at  $m/z$  631.2 ( $m+Na^+$ ). The other large peaks represent sucrose palmitate with  $m/z$  598.2 ( $m+H_2O^+$ /  $m+NH_4^+$ ) and  $m/z$  603.1 ( $m+Na^+$ ). In literature the peaks  $m+Na^+$ ,  $m+H_2O^+$  and  $m+NH_4^+$  are described to form during ionization of glycosides (Schneider et al. 1997; Cuyckens et al. 2001).



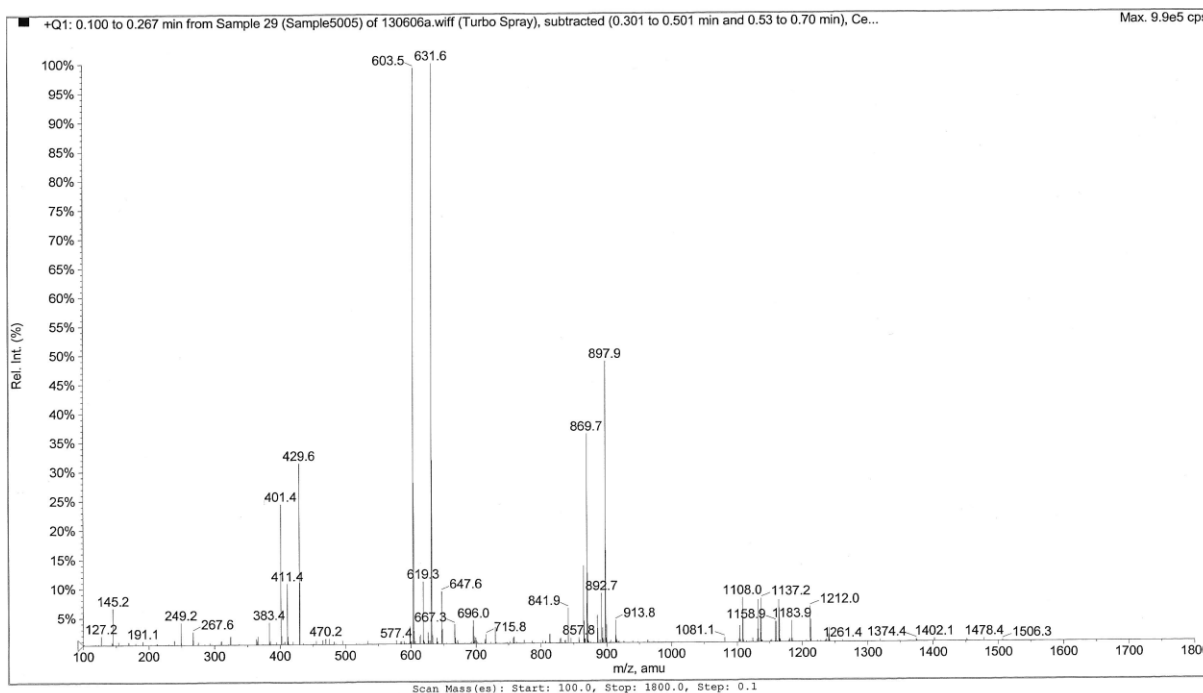
Furthermore, peaks of glucose stearate and palmitate ( $m/z$  429.2 and 401.1 =  $m+H^+$ ), as well as diesters can be detected ( $m/z$  864.3 mixed ester with palmitic and stearic acid and  $m/z$  892.3 sucrose distearate).



**Figure 14: Mass spectrum of Sisterna Sugar Esters**



**Figure 15: Mass spectrum of S770**



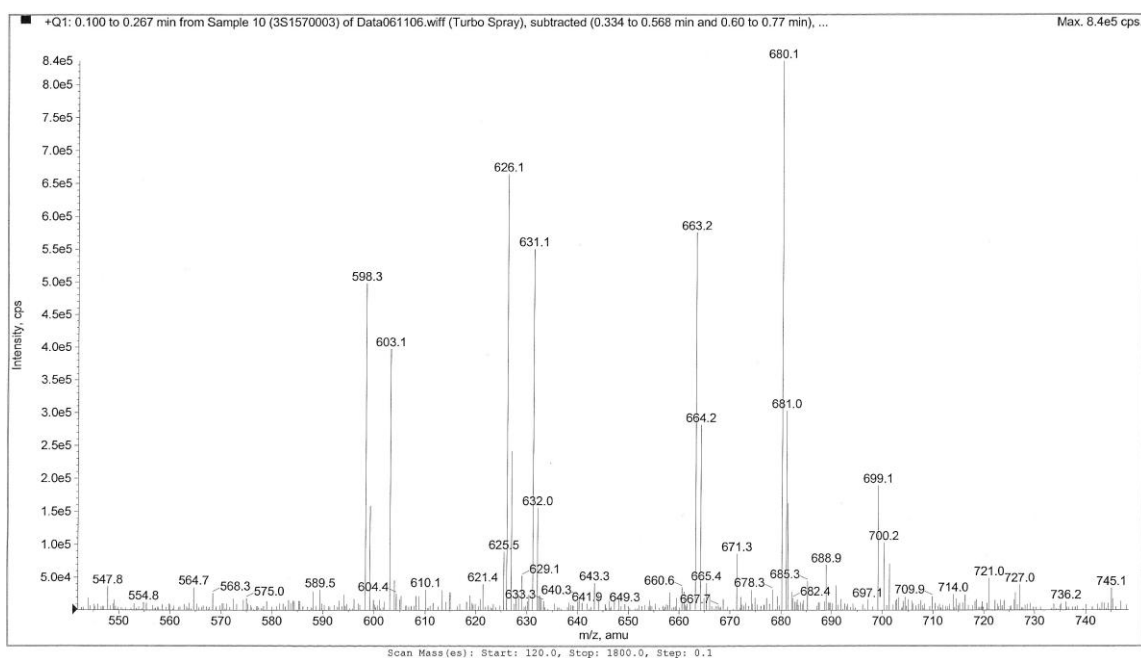
**Figure 16: Mass spectrum of S1170**

Figure 15 and 16 show the mass spectra of S770 and S1170. Both exhibit higher molecular weight species and in general a wide signal distribution indicating a very heterogeneous mixture. But the higher peak at a large molecular mass 1136 m/z occurs in the S770 sample, representing a possible sucrose triester. Whereas for S770 the larger peak is the sucrose stearate, for S1170 sucrose stearate is almost even to sucrose palmitate. Both spectra show similar relative intensities for the diester peaks (m/z 869 to 898). The higher amounts of triesters explain the lower HLB value as well as higher melting peaks (data not shown).

Furthermore, mass spectra of S1570 (Figure 17) and P1570 (Figure 18) were recorded. Again similar peaks were found in the samples for P1570. The product mainly contains mono- and diesters of palmitic and stearic acid with glucose.

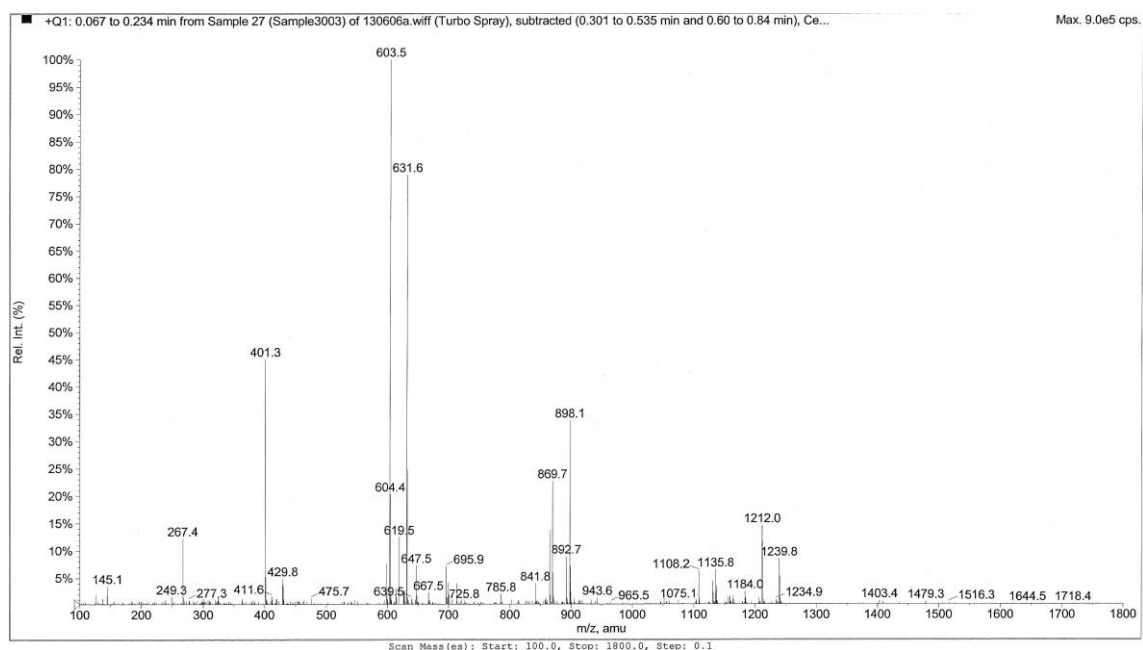
Although S1570 and P1570 should just differ in their main distribution of fatty acids, one could detect that the S1570 is a product of different monoesters of higher fatty acids and has a completely different mass spectrum compared to P1570. From this one spectrum, structure and composition of the product can not be evaluated as some artefacts occur in the spectrum that can not be explained by simple rules of multiple esterification, oligosaccharides or differences in fatty acid chain length. Probably due to free fatty acids in the mixture a  $m + H_2O + 55$  and a  $m + H_2O + 72$  (additional hydroxilation) peak are detected, representing a cyclic fragment of a fatty

acid attached to the sugar fatty acid ester .



**Figure 17: Mass spectrum of S1570**

The spectrum of P1570 shows a main peak at m/z 603.5 and an additional large peak at 631.6. These are the  $m + \text{Na}^+$  of sucrose palmitate and stearate, respectively. Furthermore the signal at 898.1 is sucrose palmitate +  $\text{Na}^+$ , whereas the signal at 869.7 is a mixed diester of sucrose with palmitic and stearic acid.



**Figure 18: Mass spectrum of P1570**

Some additional testing was performed with the supernatant of an 100 mg/ml dispersion after centrifugation. For S770 no adequate PCS correlation could be obtained. For the other products micelle sizes of 40-100 nm were detected (Table 5).

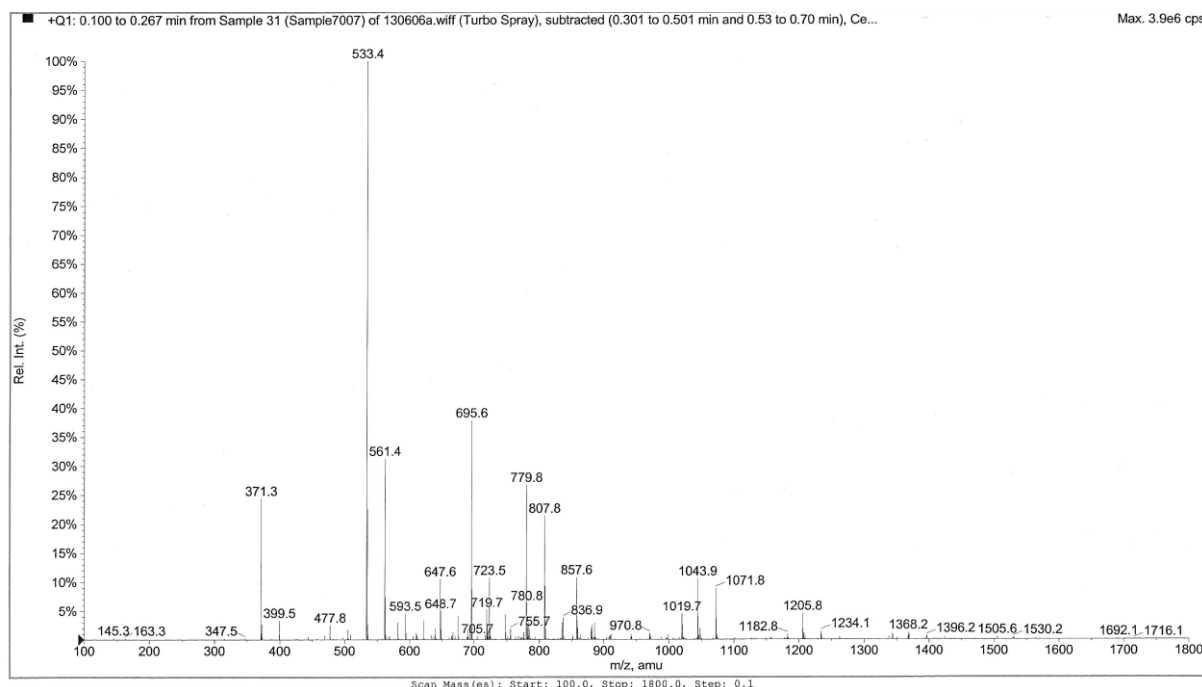
Compared to PS 20 and other nonionic surfactants (Al-Saden et al. 1982; Ericsson et al. 2004) the associates were substantially larger (see 3.2.2). This might be due to remainders of poorly water soluble components. As could be expected the viscosity of these supernatants was similar to the viscosity of water. The experimentally obtained viscosity values were applied for the PCS fitting.

Product name	Associate size / $Z_{ave}$ [nm]	Viscosity [mPas·s]
S1570	92	1.01
P1570	50	1.04
S1170	95	1.01
Sisterna Sugar Esters	43	1

**Table 5: Associate size (from PCS measurements) and viscosity at 20 °C of the soluble part of the insoluble sugar esters from centrifuged dispersions with 100 mg/l**

#### 2.3.1.2. Hardly Soluble Alkyl Glucosides

Similar to the poorly water soluble sugar esters some of the alkyl glycosides could not be completely dissolved in water. PC 1200 was supplied as a semisolid highly viscous bulk. Emulgade PL and Emulgade S were supplied as waxy flakes with melting points around 50 °C. These three substances could not be dissolved or processed into a gel even at higher temperatures or under sonification. Although PC 1200 was described as lauryl glucoside by the supplier and thus should show solubility at higher temperature. Again, this might be due to a higher content of longer fatty alcohol glycosides or poly alkyl glycosides. Mass spectrometry of this product was performed (see Figure 19).



**Figure 19: Mass spectrum of PC 1200**

The main peak at 533.4 m/z represents the  $m+Na^+$  peak of lauryl diglucoside. The second peak can be attributed to a dilauroyl diglucoside which might represent the insoluble part of the mixture. 371.3 m/z is lauroyl monoglucoside and 695.6 m/z is the lauroyl triglucoside. Aside of lauroyl glucosides myristyl glucosides are also present in the mixture with m/z 399.5, 561.4 and 723.5 as the mono- di- and trisaccharide. The signals at m/z 779.8 and 807.8 represent stearyl and eicosanyl triglucoside or dialkyl triglucosides. As the latter two show quite high intensities, these are probably responsible for the low aqueous solubility as well as the dodecyl and tetradecyl monoglucosides (m/z 371.3, 399.5) as these are the more hydrophobic molecules. Both Emulgade products are technical mixtures of not only alkyl glucosides but also PEG-alkyl ethers. As can be derived from the product name, they are typically utilized in emulsions in O/W or W/O systems and not as solubilizers in pure aqueous systems. No hydrogen peroxide equivalents could be detected in the diluted samples that were applied for viscosity and PCS measurements.

## 2.3.2. Soluble Surfactants

### 2.3.2.1 Water Soluble Sugar Esters

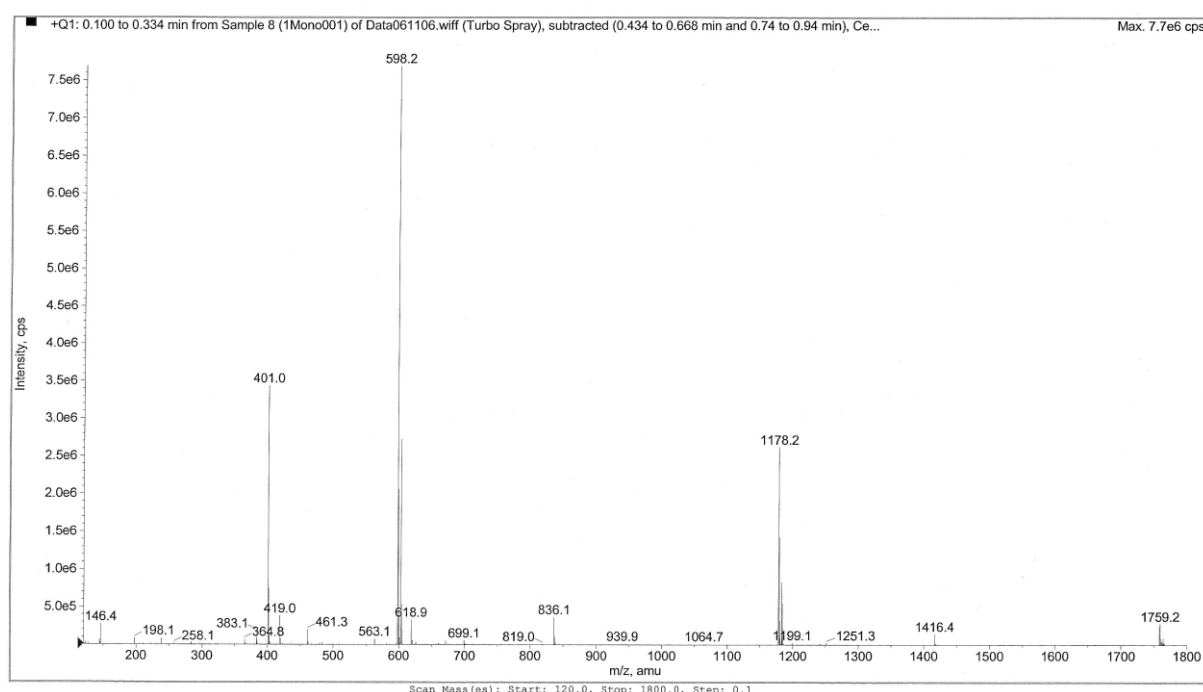
The product MP (Ryoto Monoester P) is declared to be a mixture of sucrose fatty acid esters with the main component sucrose monopalmitate with a minimum content of 70% in the powder. MP was soluble up to 0.1% (m/v) in water as well as in phosphate buffer at ambient temperature. Higher concentrations could be achieved by heating the samples. This thermodynamic solubility was sufficient to prepare 1% stock solutions for example for protein agitation tests. Solutions with 1% did not show visual precipitation or gelation within one week storage. As the product description by the supplier was similar to that of the Sisterna Sugar Ester this different behavior was unexpected. Subsequently, surface tension measurements were performed and a CMC of this excipient in water of ~50 mg/l and hence in the range of other nonionic surfactants (Wan and Lee 1974; Reinhart and Bauer 1995) was obtained. The same CMC was obtained in phosphate buffer. This is in accordance with work published by Zhang et al who could detect electrolyte effects on association behavior of surfactant molecules of n-dodecyl beta -D-maltoside solutions only at very high electrolyte concentrations. The effect can be described by structure forming or structure breaking effects, respectively (Zhang et al. 1996). The surface tension of an aqueous system above CMC is about 35.5 mN/m. Pyrene interaction revealed as well a CMC of ~ 50 mg/l.

PCS measurements at 10 mg/ml delivered a micelle size of 7 nm which is in the range of other nonionic surfactants (Al-Saden et al. 1982). In addition PCS measurements confirmed a CMC between 30 and 50 mg/l.

In an additional experiment the hemolytic activity of the surfactant was studied using porcine erythrocytes.  $HC_{5\%}$  was 100 mg/l and thus twice as high as the CMC. Thus, the application of the surfactant at the CMC is not expected to be a problem physiologically. For the ability to stabilize protein formulations, see chapter .

The purity of the product was described as a content of at least 70% sucrose monopalmitate ester. No further details were given on the composition of the products besides the HLB of 16. Inductive coupled plasma analytics were performed to gather knowledge on the purity of MP. There were significant amounts of Ca and

Mg present. Traces of ubiquitous Na could be detected. However, the concentration of Na is in the range of a few ppb. Figure 20 shows the mass spectrum of MP. The higher purity is also confirmed by a lower number of significant additional peaks. The  $m/z$  signal at 598.2 is showing the  $H_2O$  adduct of sucrose monopalmitate, the peak at  $m/z$  1178.2 is the a cluster of  $2M + H_2O^+$  of sucrose monopalmitate and the signal at  $m/z$  401 is either a fragment with one monosaccharide cracked or a side product and then most probably glucose or fructose palmitate, where the disaccharide was cleaved at the carbon and not at the oxygen atom. As you can see, MP is rather pure and is mostly free of stearic acid esters. Due to this, solubility in water is higher compared to the other Ryoto products.



**Figure 20: Mass spectrum of MP**

The samples were free of hydrogen peroxide equivalents and thus might show an advantage in storage tests compared to PS 20 and PS 80.

### **2.3.2.2. Water Soluble Alkyl Glucosides (plus analysis on PS 20 and PS 80)**

PC 818 and PC 2000 are ethers between sugars (mostly mono-, di-, and

trisaccharides) and fatty alcohols. PC 818 is composed of fatty alcohols with chain length between C8 and C18. For PC 2000 no such limitations on the fatty alcohol chain length are described by the manufacturer. Both products are completely water soluble at all ratios. As their structure and their composition is very close to polysorbates the analysis of polysorbates is described in this part (see also chapter 3).

Surface tension measurements show that micellization occurs at concentrations above 450 mg/l for PC 2000 and 210 mg/l for PC 818 respectively. Both substances decrease the surface tension to a similar level of  $29 \text{ mN/m} \pm 0.5 \text{ mN/m}$ . In phosphate buffer the CMC remains almost unaltered for both substances. Only pyrene interaction showed a difference for PC 2000 with a two times higher CMC. With the help of pyrene interaction fluorescence measurements CMC values of 400 (and 800 mg/l in phosphate buffer) and 200 mg/l for PC 2000 and PC 818 respectively were observed. All results regarding micellization and a comparison to PS 20 and PS 80 are shown in

Table 6

Product	CMC <sub>STM</sub> [mg/l]	CMC <sub>PF</sub> [mg/l]	Surface Tens. CMC [mN/m]	Micelle diameter [nm]
PC 818	210	200	29.4	29
PC 2000	450	400	29	15
PS 20	100	100	28	9
PS 80	20	20	42	11

**Table 6: Physico-chemical attributes of PC 818, PC 2000, PS 20, and PS 80. CMC<sub>STM</sub> is the CMC observed by tensiometry. CMC<sub>PF</sub> is the CMC observed via pyrene fluorescence interaction and hydrodynamic micelle diameter from PCS measurements.**

As expected, the hydrodynamic diameters of PC 818 and PC 2000 micelles are in a comparable range of a few nanometers, slightly bigger than PS 20 and PS 80 micelles. Results for polysorbates are in agreement with literature values (Wan and Lee 1974; Patist et al. 2000). The viscosity of 100 mg/l aqueous dilutions of PC 818 and PC 2000 is  $1 \text{ mPas}\cdot\text{s}$ .

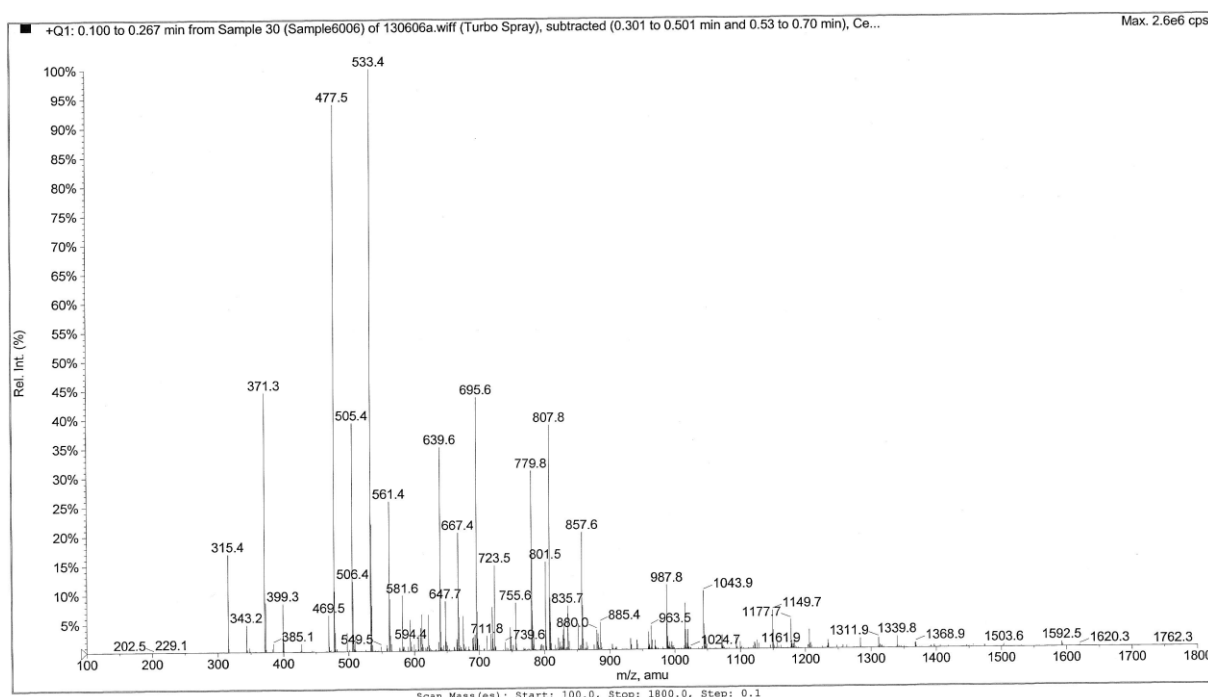
Also the hemolytic activity was tested for PC 818 and PC 2000. The HC<sub>5%</sub> is 200 mg/l



and 1000 mg/l, respectively. Thus, the ratio of HC<sub>5%</sub> to CMC is approximately two. For PS 20 the ratio is higher as the HC<sub>5%</sub> value is 1000 mg/l and the CMC of PS 20 is lower than that of the Plantacare products making the substance a bit safer with respect to hemolysis. Overall this safety margin is comparable with other sugar based surfactants in literature (Soderlind et al. 2003; Neimert-Andersson et al. 2006; Soederlind and Karlsson 2006). The difference to PS 20 might be a drawback when it comes to filing of such an excipient mixture for invasive application and hence further experiments with e.g. whole blood might become compulsory.

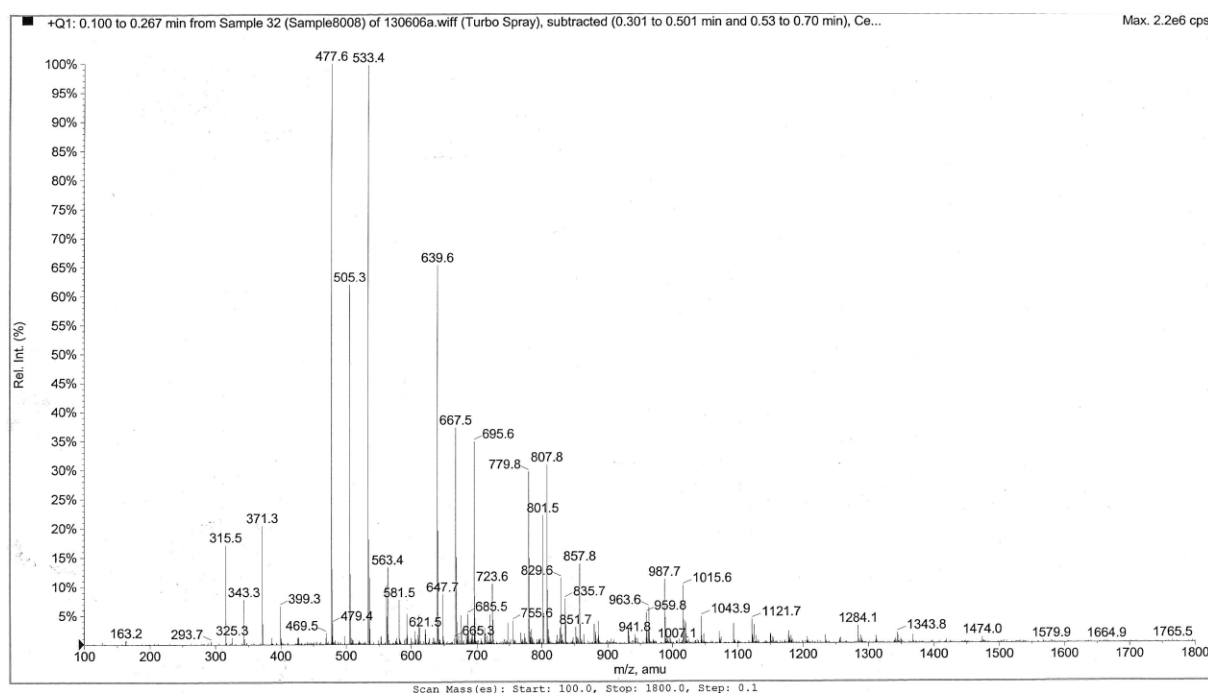
The purity of PC 818 and PC 2000 was tested with an RP-HPLC method according to the group of Neubert (Klaffke et al. 1998; Kuehn and Neubert 2004). As there was no UV-activity expected for these substances a RI-detector was applied for detection. With evaporative light scattering detection it was neither possible to gain linear results nor to achieve reproducibility. Our results were in accordance with literature values (Klaffke et al. 1998; Kuehn and Neubert 2004). The most prominent molecule in the mixtures is octyl diglucoside with area percentages of 30% and 40% for PC 818 and PC 2000, respectively. The dodecyl diglycoside made up 20% and 22% resp. (data not shown). The other substances showed peak areas of 5%. As no internal standards were available, only semiquantitative statements can be made based on the HPLC results. These data are also in general accordance with the results obtained from mass spectrometry (see Figure 21 and Figure 22) with main peaks at  $m/z$  477.6 ( $m + Na^+$ ), although direct comparisons can not be drawn as completely different physical properties have influence on the signal intensities. In the mass spectra the peak for dodecyl diglycoside appears bigger than in the HPLC ( $m/z$  533.4 =  $m + Na^+$ ). It is hard to find differences in the composition of both mixtures, but PC 2000 shows a slightly higher degree of shorter fatty alcohols and hence more hydrophilic species than PC 818 plus stronger signals at  $m/z$  639, representing octyl triglucoside, which is a more polar molecule. Also more monoglucosides (especially dodecyl monoglucoside 371.3 ( $M + Na^+$ )) are found for PC 818. This would explain the higher tendency for self aggregation of PC 818 as more hydrophobic surfactants have in general a lower CMC (Rulison 1995; Sierra and Svensson 1999; Chen et al. 2006; Neimert-Andersson et al. 2006). Diglucosides and are present with different chain lengths in a comparable frame with  $m/z$  signals of 477.6, 505.3, 533.4, 561.4 representing all even numbers chain lengths from C<sub>8</sub> to C<sub>14</sub>. These analogy is also

present in smaller extends for the triglucosides (639.6, 667.5, 695.6, 723.6), and monoglucosides ( $m/z$  315.5, 343.3, 371.3, 399.3) and even tetraglucosides can be detected for both substances ( $m/z$  801.5 and 857.1). And interestingly 779.8 and 807.8 ( $C_{18}$  and  $C_{20}$ ), which also can be dialkyl triglucosides. In the better water soluble mixtures these both peaks appear together with more substance of higher hydrophilicity. This might be the reason, why PC 818 and PC 2000 are water soluble.



**Figure 21: Mass spectrum of PC 818**

Both commercial products are mixtures of 50% water and 50% alkyl glucoside resulting in highly viscous fluids. When these are lyophilized fairly hygroscopic white powders can be obtained. The technical data sheet refers to traces of Mg that might be present in the products. These traces could not be observed via inductive coupled plasma analytics. Furthermore no Ca and only minimal amounts of Na could be detected. The pH of a pure 1% aqueous mixture is 3.0 providing autopreservation.



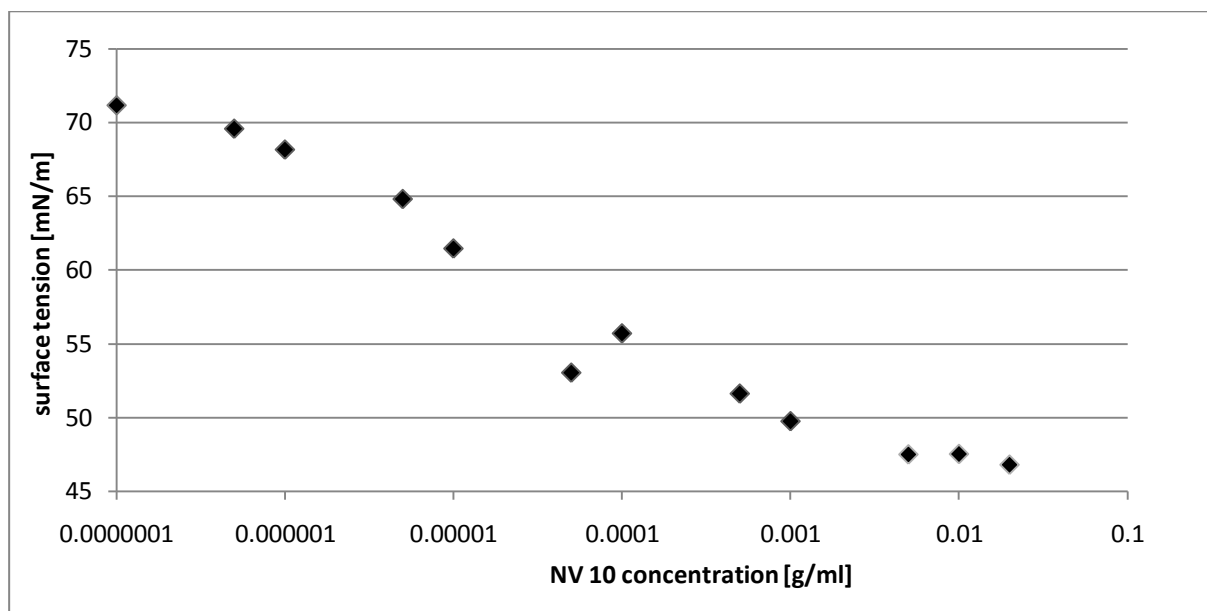
**Figure 22: Mass spectrum of PC 2000**

Furthermore the amount of oxidizing species (hydrogen peroxide equivalents) was determined in 10% solutions. Even after 72 h light stress in a SunTest system no peroxide degradation products could be detected using Merckoquant® test strips. On the other hand PS 20 and PS 80 showed remarkably high contents of oxidizing impurities when exposed to light. For both substances peroxide levels > 100 mg/l were measured. The pure substances freshly diluted from cooled and nitrogen stored PS 20 and PS 80 respectively showed peroxide levels of 5 mg/l.

### 2.3.2.3. NV 10

The Expedeon product NV 10 is declared by the supplier as an oligosaccharide with hydrophobic side chains, but with no statement of the linker between the hydrophilic and the hydrophobic part. From figures in the product manual one can assume that the hydrophobic parts are attached to various parts at a linear oligosaccharide. The oligosaccharide itself is a starch hydrolysate. NV 10 was tested under the assumption that this small polymer would behave similar to a classical surfactant. Indeed it was possible to lower surface tension to approx. 47-48 mN·m (see Figure 23). The molecule is claimed to have a molecular weight of 5 kDa. This is about four

times the size of hydroxypropyl  $\beta$  cyclodextrin (MW~1500), another starch hydrolysate derivative that decreases the surface tension to a similar level (Serno et al. 2010). The decrease of surface tension follows a similar shape as for regular surfactants. At 1% the decrease of surface tension seems to level off. A clear transition reflecting a CMC could not be identified.



**Figure 23: Surface tension of NV 10 in relation to its concentration**

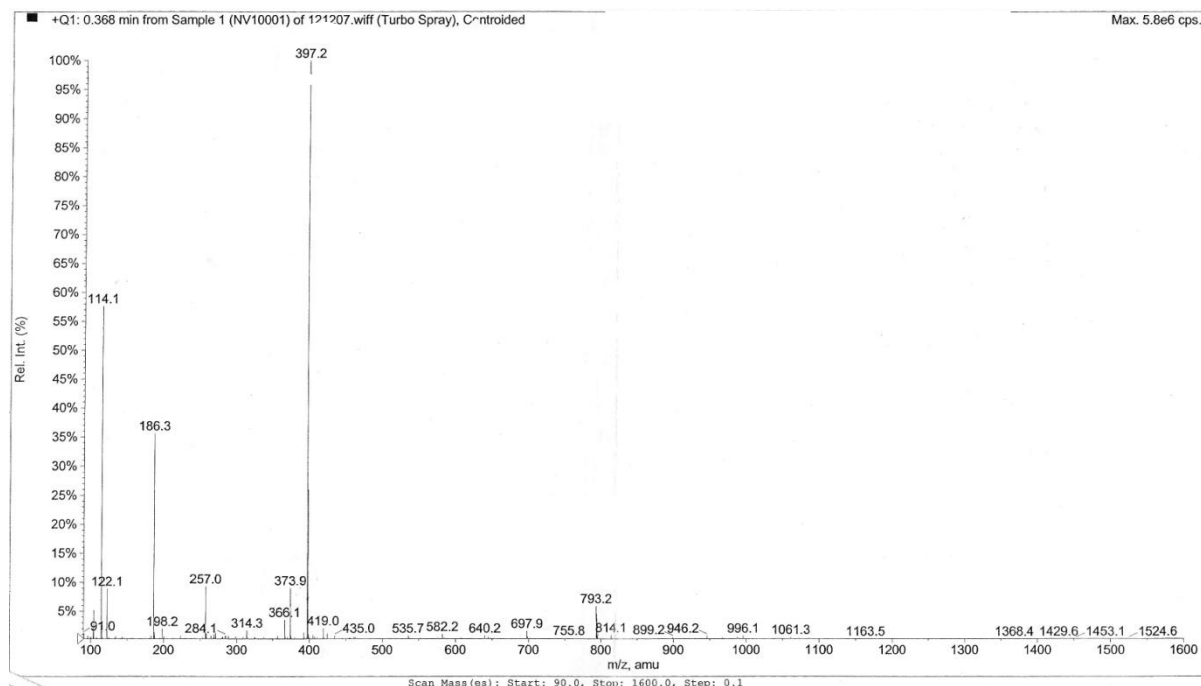
Pyrene interaction spectroscopy reveals a change in polarity of the whole system already at 0.001%. This leads to the assumption that the hydrophobic polymer molecules themselves or associates with a low number of participating molecules provide an unpolar environment.

PCS measurements were performed to determine the size of potential associates. But with the set-up applied it was not possible to define any structure or “micellar state” to the substance even at higher concentrations. This is another indicator that NV 10 is not behaving like a regular nonionic surfactant.

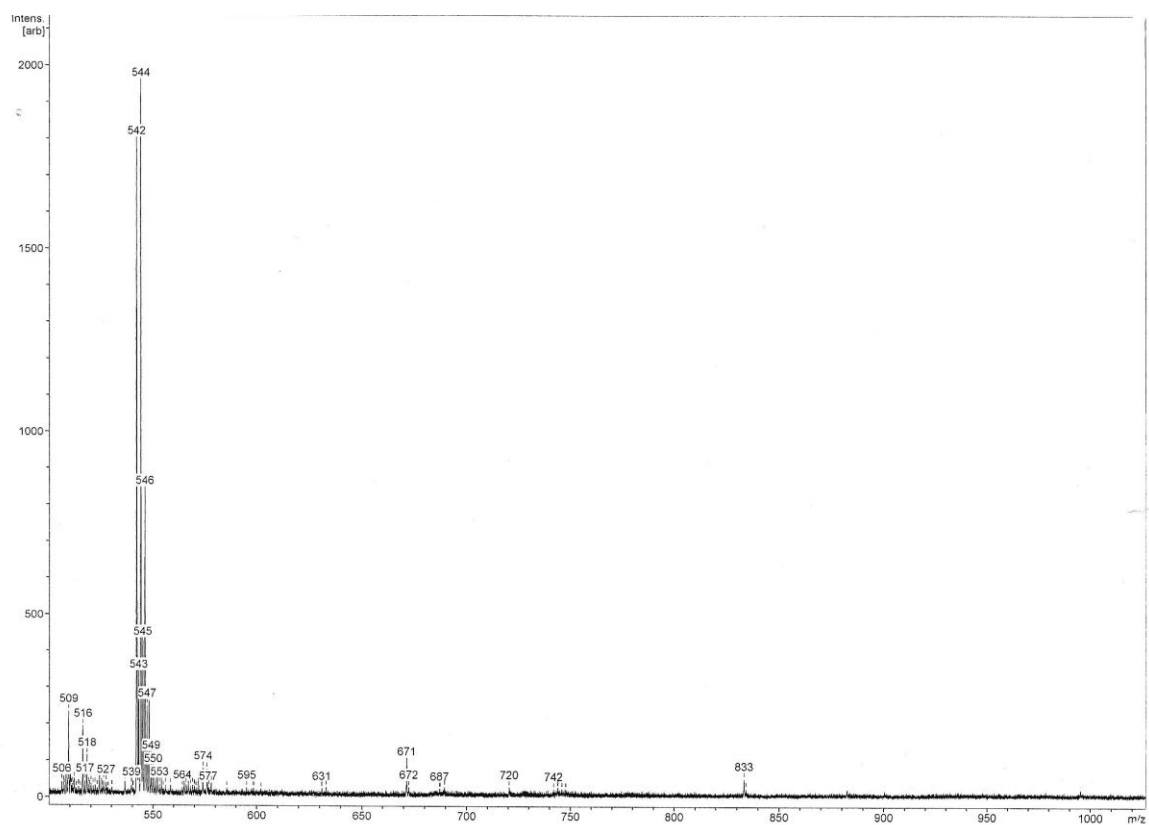
From patent literature (Daniel Brian Jones 2007) it was expected that the structure might be congruent with PC 818 or PC 2000 meaning that an oligosaccharide is attached to a fatty alcohol. It is stated from the manufacturer that the product is a small polymer in the size range of 5 kDa. One explanation for the attachment of the hydrophobic part is the bonding via glycosidic linkage. The elemental analysis indicated a nitrogen content of 0.8%. Thus, the linkage between lipophilic and

hydrophilic structural element by also by formed via the nitrogen atoms e.g. as amid bonds. Carbon and oxygen represent the main portions in the molecular structure with approximately 46% each. Hydrogen could be found with about 6.9%.

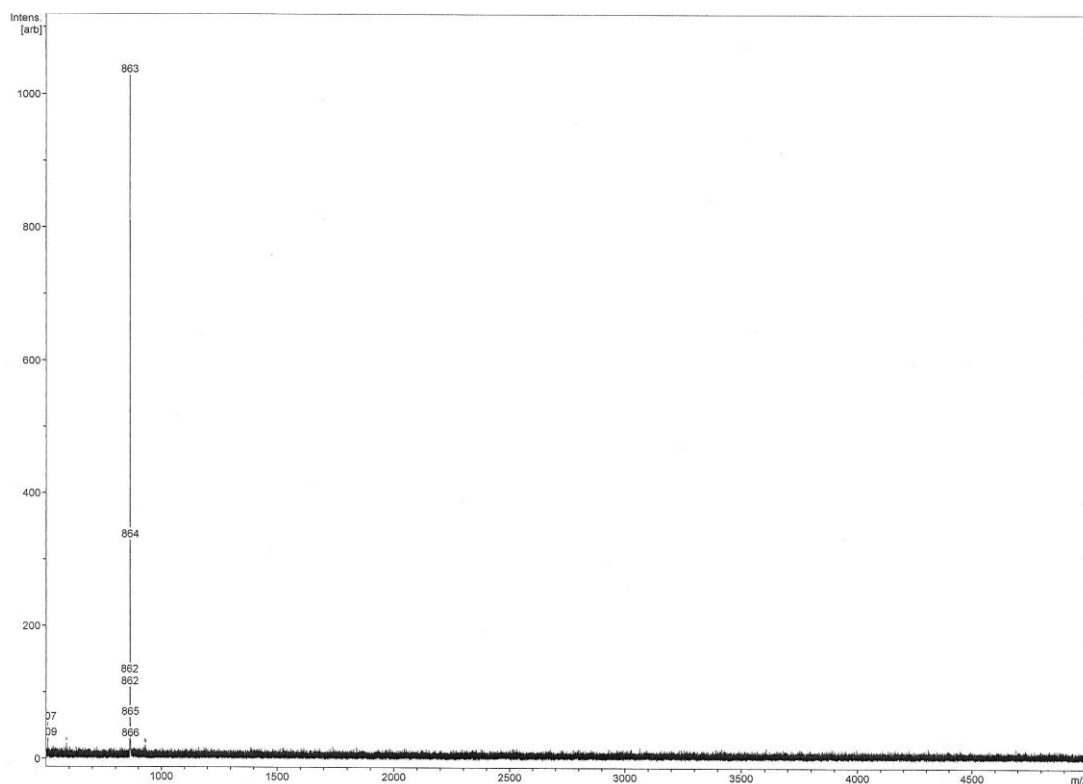
Mass spectrometry was performed with electrospray ionization, but could not provide further insight into the molecular structure (Figure 24). The main peak has a  $m/z$  of 397.2, which might be the Na adduct of the substance with a mass peak of 373.7 and a third peak is represent the  $m + H^+ / 2z$  signal ( $m/z$  186). These signals could of course also originate from a multiply charged molecule. The signal at 114  $m/z$  is most probably a fragment (octane, hexadecane) from the hydrophobic part of the polymer). When different detection and different ionization were performed the spectra were completely different (see Figure 25-22). On the one hand the mass peak at  $m/z$  397 disappeared and on the other hand peaks at  $m/z$  544 (MALDI, positive ionization) and  $m/z$  860 (MALDI, negative ionization) appeared. Using chemical ionization a broad mix of fragments and /or multiply charged molecules resulted. Thus, no conclusive results can be obtained from mass spectrometry on the structure of the NV 10 polymer.



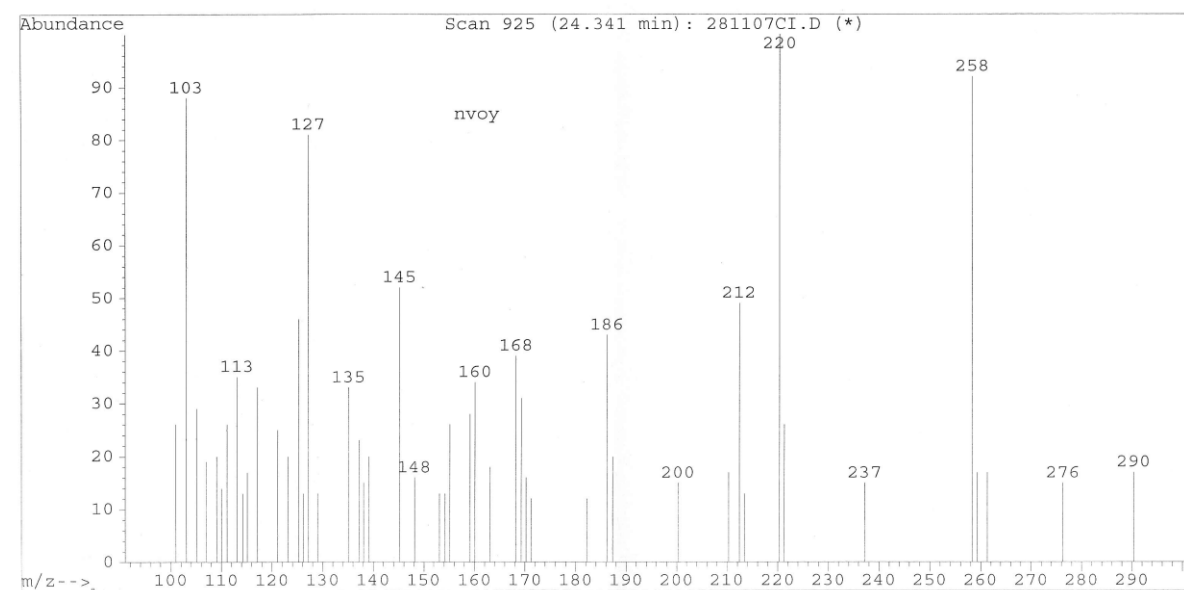
**Figure 24: Mass spectrum of NV10 (ES Ionization)**



**Figure 25: Mass spectrum of NV10 (Matrix assisted laser desorption ionization, positive mode)**



**Figure 26: Mass spectrum of NV10 (Matrix assisted laser desorption ionization, negative mode)**



**Figure 27: Mass spectrum of NV10 (chemical ionization)**

Furthermore for purity determination inductive coupled plasma experiments were performed to check for Mg, Ca and Na. No traces of these ions were present in the product. From overall analysis no decent statement can be given on the structure and composition of the product. However, NV10 is able to decrease the surface tension, but no CMC could be assigned. Nevertheless unpolar regions in the individual molecules or low number molecular associates form hydrophobic regions that are able to interact with pyrene. In addition, the product contains nitrogen which cannot be explained based on the provider's information. Hence, contrary to our prior hypothesis this product is not similar to the alkyl glucosides but is certainly derived from other classes and does not form higher molecular structures. When the hemolytic activity was tested for this substance the  $HC_{5\%}$  was in a high range of 1 mg/ml, which may be related to a rather polymeric nature.

## 2.4. Conclusions

Applicability of the sugar based surfactants is strictly dependent on their solubility in water and the formation of clear solutions in a broad temperature range and in concentrations up to at least 1%. Therefore insoluble and poorly water soluble surfactants needed to be identified and only characterized with limited efforts. To

better understand this class of surfactants it was useful to determine the composition of the products. Mass spectrometry gave insight into the distribution of hydrophobic residues and carbohydrate parts in technical mixtures. On the one hand most of them showed mostly alkyl residues ranging from C<sub>16</sub> to C<sub>18</sub> and from mono- to triesters. On the other hand the hydrophilic part in most of the substances was found to be a disaccharide, most probably either sucrose or maltose. So only the CMC and hence solubilization properties seems to be of higher importance for the manufacturers of the sugar esters. The solubility is mainly altered by the sugar moiety in sugar based surfactants (Boyd et al. 2000; Soederlind and Karlsson 2006), which in turn remained unaltered for all ester products. Sucrose itself is not sufficiently hydrophilic enough to overcome the low solubilities of palmitic or stearic acid. Hence most of the sucrose esters were not sufficiently water soluble (see also characterization of trehalose palmitate).

For the alkyl glucosides in turn from mono- to tetraglucosides different degrees of water solubility could be found and these might be attributed to the average amount of carbohydrate per molecule.

Only a minority of the products (MP, PC 818 and PC 2000) were sufficiently water soluble. The other products, being sucrose fatty acid esters (the other Ryoto products and the Sisterna sugar ester) or alkyl glucoside (PC 1200) were not, although the HLB value of these mixtures would lead to the conclusion that these products should show adequate solubility.

From the tested products the most promising candidates were PC 2000 and PC 818. These products mainly consist of octyl diglucoside and dodecyl diglycoside. Moreover some mono- tri and tetraglucosides are also present in these mixtures. The shorter chain lengths of the fatty alcohols and in turn the presence of longer carbohydrates leads to a better solubility. CMC, hemolytic activity, surface tension, as well as micelle size are similar to the properties of polysorbates. Of advantage for protein formulation might be, that the products show hardly any oxidizing potential, neither impurity nor structurally derived.

As a runner-up to the most promising candidates appears NV 10. The chemical analysis could not shed a clear light on the structure of the excipient and the underlying chemistry. A more detailed analysis of the excipient is necessary for evaluating its potential in pharmaceutical protein formulation, including



biodegradability, biocompatibility and chemical composition. As stated by the manufacturer some pharmaceutical companies already use the material in up- and downstream processing of pharmaceutical proteins. The low hemolytic activity is a major advantage.

Ryoto Monoester P, which mostly consists of sucrose monopalmitate, is another potentially useful candidate for protein formulation. It shows fairly high, but not complete aqueous solubility, exhibits reasonably low hemolytic activity and behaves physico-chemically similar to polysorbates.

Summarizing, from the screening of physical-chemical properties, there are possible alternatives to the polysorbate products. But it has to be kept in mind that all these products are not designed for parenteral use and other parameters such as endotoxin levels have not been considered for feasibility.

In further studies, these products, mainly the Plantacare mixtures, should be tested in protein formulations under stress conditions. In agitation stress studies it would be desired to show comparable protein stabilization. As no oxidizing species could be detected in these mixtures, it is expected that storage under elevated temperatures will lead to significantly lower amounts of chemically degraded protein with sugar based surfactants in contrast to the

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# 3. Synthesis, Characterization and Assessment of Suitability of Trehalose Fatty Acid Esters as Alternatives for Polysorbates in Protein Formulation

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## 3.1. Introduction

A large number of protein pharmaceuticals, both in liquid as well as lyophilized, is formulated with the addition of nonionic surfactants such as block copolymers and polysorbates, for example the insulin Lantus<sup>®</sup>, pegylated erythropoietin Mircera<sup>®</sup>, a human growth hormone formulation Nutropin<sup>®</sup> or the humanized monoclonal antibody Herceptin<sup>®</sup>. Different groups have shown that these surfactants pose dual effects on the protein. On the one hand they stabilize proteins physically against various kinds of surface correlated stress, e.g. adsorption at container surfaces (Charman et al. 1993; Wu et al. 2008), denaturation at the air-water interface (Bam et al. 1995; Bam et al. 1998) or the ice-water interface (Chang et al. 1996; Kreilgaard et al. 1998; Webb et al. 2002). On the other hand these polyethylene glycol based surfactants can put long-term chemical stability at risk (Knepp et al. 1996; Ha et al. 2002; Kiese et al. 2008; Wang et al. 2008). Mostly oxidizing reactions are described in the literature. These are explained by peroxide remnants in the technical products. An increase in hemolysis is referred to peroxide residues, too (Azaz et al. 1981). Adverse reactions in patients are known for some of these surfactants (Attwood 1983), which is another reason to search for other options for surface stabilization.

An alternative for these substances should show a good safety profile, the same surface related stabilizing effects, but no impact on long-term chemical stability of pharmaceutical proteins. Sugar based surfactants feature surface behavior similar to

polysorbates. They are already in pharmaceutical and cosmetical use for topical and oral formulations (Savic et al. 2005; El-Laithy Hanan 2009; Kirchherr et al. 2009). In addition, they are known as reagents for the stabilization of membrane proteins (Prive 2007; Lopez et al. 2009). Alkyl glycosides and sugar fatty acid esters are environmentally friendly (Garcia et al. 1997; Baker et al. 2000) as only renewable components, sugars (mono- to oligosaccharides) for the hydrophilic and fatty alcohols or fatty acids for the hydrophobic part are required. Synthesis can be performed both chemically (von Rybinski and Hill 1998; Chen et al. 2006; Hill 2007) and biochemically (von Rybinski and Hill 1998; Chen et al. 2005; Hill 2007). Technical mixtures are already on the market and well characterized (Rades and Mueller-Goymann 1997; von Rybinski and Hill 1998; Kuehn and Neubert 2004). Of course, the lack of PEG-residues and hence peroxides should pose the advantage regarding oxidizing behaviour of this class of surfactants against the already approved substances.

In this chapter we describe the synthesis and scrutiny of trehalose fatty acid monoesters. Trehalose is a well known stabilizer in protein formulations (Uritani et al. 1995; Sun and Davidson 1998; Cleland et al. 2001). Having this in mind the authors were looking for a good water soluble surfactant. We designed a synthetic strategy in four steps resulting in highly pure products. These surfactants are unlike polysorbates, which are technical mixtures of all kinds of molecules with a broad heterogeneity. The authors were furthermore interested in a correlation of chain length and protein stability. Thus we sought for substances that are comparable to Polysorbate 20 (PS 20) and Polysorbate 80 (PS 80) in regard of formulation feasibility. We characterized the physico-chemical properties of trehalose esters, such as CMC, micelle size, viscosity and calorimetric behavior. Furthermore they were tested for hemolytic activity as surfactants tend to rupture cell membranes (Reinhart and Bauer 1995; Soderlind et al. 2003; Soederlind and Karlsson 2006). Finally, the protein stabilizing capacity of the trehalose esters was tested in agitation stress studies of human growth hormone .

## 3.2. Materials and Methods

### 3.2.1 Materials

Palmitic acid, lauric acid, capric acid, anhydrous pyridine, trimethylsilyl chloride, hexamethyldisilazane, 4-(dimethylamino)pyridine (4-DMAP) and *N,N'*-dicyclohexylcarbodiimide (1,3-DCC) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Solvents for NMR were purchased from Euriso-Top SA (Saint-Aubin Cedex, France).  $\alpha,\alpha$ -Trehalose was purchased from Georg Breuer GmbH (Königstein, Germany). Flash column chromatography was performed using silica gel 60 (40 – 63  $\mu$ m) (VWR International GmbH, Bruchsal, Germany). All other chemicals were reagent grade, commercially available products. If indicated, solvents were dried using standard procedures. For the physicochemical analysis of the sugar based surfactants the water used in all experiments was purified in a PURELAB Plus system (ELGA labwater, Celle, Germany) and had a conductivity  $<0.055 \mu\text{S/cm}$ . Pyrene for synthesis and all buffer salts were obtained from Merck KGA (Darmstadt, Germany). Buffer salts were of analytical grade or higher. Human growth hormone (hGH) was gratefully donated by Bernina Plus & HDS. Porcine blood was collected at the municipal slaughterhouse. Further purification and processing to an erythrocyte suspension is explained in the methods part.

#### 3.2.1.1 Preparation of 2,3,4,6,2',3',4',6'-octa-O-(trimethylsilyl) trehalose (1)

$\alpha,\alpha$ -Trehalose dihydrate (7.00 g, 18.5 mmol) was placed in a flask containing 100 ml of anhydrous pyridine. Trimethylsilyl chloride (20.0 ml, 158.0 mmol) was added over a period of 30 minutes, hexamethyldisilazane (40.0 ml, 188.0 mmol) was added the same way and the mixture was mechanically stirred under nitrogen atmosphere for 18 hours at room temperature. The solvent was removed by flash evaporation, the resulting residue was treated with 100 ml ice water and extracted with hexane (3  $\times$  50 ml). The combined organic extracts were dried over anhydrous  $\text{MgSO}_4$  and then the solvent was removed by flash evaporation. The residue was crystallized in

methanol to give 2,3,4,6,2',3',4',6'-octa-O-(trimethylsilyl)- $\alpha,\alpha$ -trehalose (**1**) (15.7 g, 17.0 mmol, 92 %) as a white solid. MP: 82 °C (lit.: 80 – 82 °C). IR (KBr):  $\nu$  (cm<sup>-1</sup>) = 2959, 1251, 1155, 1117, 1071, 1023, 901, 878, 840, 748, 682, 536, 457. <sup>1</sup>H-NMR (CDCl<sub>3</sub>):  $\delta$  (ppm) = 4.91 (d, 2 H,  $J$  = 3.1 Hz, 1-H, 1'-H), 3.88 (t, 2 H,  $J$  = 9.0 Hz, 2-H, 2'-H), 3.78 (m, 2 H, 5-H, 5'-H), 3.66 (m, 4 H, 6-H, 6'-H), 3.42 (t, 2 H,  $J$  = 9.1 Hz, 4-H, 4'-H), 3.37 (dd, 2 H,  $J_1$  = 3.2 Hz,  $J_2$  = 9.3 Hz, 3-H, 3'-H), 0.14 – 0.09 (m, 72 H, 8 SiMe<sub>3</sub>). <sup>13</sup>C-NMR (CDCl<sub>3</sub>):  $\delta$  (ppm) = 96.8 (C-1, C-1'), 76.1 (C-2, C-2'), 75.7 (C-5, C-5'), 75.3 (C-3, C-3'), 74.3 (C-4, C-4'), 64.7 (C-6, C-6'), 3.6 (2 SiMe<sub>3</sub>), 3.5 (2 SiMe<sub>3</sub>), 2.7 (2 SiMe<sub>3</sub>), 2.2 (2 SiMe<sub>3</sub>). MS (ESI):  $m/z$  (rel. int. in %) = 941 [M+Na]<sup>+</sup> (87), 936 (100), 977 (47), 361 (40).

### 3.2.1.2. Preparation of 2,3,4,2',3',4'-hexa-O-(trimethylsilyl)- $\alpha,\alpha$ -trehalose (**2**)

2,3,4,6,2',3',4',6'-Octa-O-(trimethylsilyl)- $\alpha,\alpha$ -trehalose (**1**) (0.920 g, 1.00 mmol) was placed in a 250 ml flask containing 25 ml of methanol and cooled to 0 °C. A solution of potassium carbonate (0.140 mg, 1.0 mmol) in methanol (30 ml) was added dropwise over a period of 20 minutes. The reaction mixture was stirred for 5 hours at 0 °C, complete conversion was checked by TLC. The reaction was stopped by adding glacial acetic acid (115  $\mu$ L, 2.0 mmol). The solvent was removed by flash evaporation, the crude product was treated with brine and extracted with diethyl ether (3 x 30 ml). The combined organic extracts were dried over magnesium sulfate, removing the solvent by flash evaporation yielded 2,3,4,2',3',4'-hexa-O-(trimethylsilyl)- $\alpha,\alpha$ -trehalose (**2**) as a white solid (0.640 g, 0.82 mmol, 82 %). MP: 114 °C (lit. 115 – 118 °C). IR (KBr):  $\nu$  (cm<sup>-1</sup>) = 3503, 2959, 1405, 1251, 1171, 1126, 1110, 1076, 1011, 948, 897, 873, 843, 748, 683, 623, 589, 519, 456. <sup>1</sup>H-NMR (CDCl<sub>3</sub>):  $\delta$  (ppm) = 4.90 (d, 2 H,  $J$  = 3.1 Hz, 1-H, 1'-H), 3.86 (t, 2 H,  $J$  = 9.0 Hz, 2-H, 2'-H), 3.84 (m, 2 H, 5-H, 5'-H), 3.71 (m, 4 H, 6-H, 6'-H), 3.48 (t, 2 H,  $J$  = 9.2 Hz, 4-H, 4'-H), 3.41 (dd, 2 H,  $J_1$  = 3.2 Hz,  $J_2$  = 9.3 Hz, 3-H, 3'-H), 0.16 – 0.12 (m, 54 H, 6 SiMe<sub>3</sub>). <sup>13</sup>C-NMR (CDCl<sub>3</sub>):  $\delta$  (ppm) = 94.8 (C-1, C-1'), 73.5 (C-2, C-2'), 73.0 (C-5, C-5'), 72.9 (C-3, C-3'), 71.5 (C-4, C-4'), 61.8 (C-6, C-6'), 1.1 (2 SiMe<sub>3</sub>), 1.0 (2 SiMe<sub>3</sub>),



0.2 (2 SiMe<sub>3</sub>). MS (ESI):  $m/z$  (rel. int. in %) = 797 [M + Na]<sup>+</sup> (21), 792 (100), 379 (28), 361 (10), 289 (18).

### 3.2.1.3 Preparation of 6-O-monopalmitoyl-, 6-O-monolauroyl- and 6-O-monocaprinoyl-2,3,4,2',3',4'-hexa-O-(trimethylsilyl)- $\alpha,\alpha$ -trehalose (**3a**, **3b**, **3c**)

2,3,4,2',3',4'-Hexa-O-(trimethylsilyl)-trehalose (**2**) (0.770 g, 1.00 mmol), together with 4-DMAP (0.160 g, 1.30 mmol) and the corresponding carboxylic acid (for **3a**: palmitic acid C<sub>16</sub>H<sub>32</sub>O<sub>2</sub>; for **3b**: lauric acid C<sub>12</sub>H<sub>24</sub>O<sub>2</sub>; for **3c**: capric acid C<sub>10</sub>H<sub>20</sub>O<sub>2</sub>) (1.30 mmol) were placed in a flame dried 25 mL Schlenk flask, containing anhydrous dichloromethane (5 mL). A solution of 1,3-DCC (0.250 g, 1.20 mmol) in anhydrous dichloromethane (3 mL) was added dropwise. The mixture was mechanically stirred for 18 hours at room temperature under a nitrogen atmosphere. The resulting precipitate was removed by filtration under reduced pressure and washed with dichloromethane (5 mL). The crude product was obtained by removing the solvent from the combined, dried dichloromethane solutions by flash evaporation and purified by flash column chromatography (hexane, diethyl ether 7:3).

**3a**: 6-O-Monopalmitoyl-2,3,4,2',3',4'-hexa-O-(trimethylsilyl)- $\alpha,\alpha$ -trehalose (0.330 g, 0.33 mmol, 33 %, colorless oil). IR (NaCl):  $\nu$  (cm<sup>-1</sup>) = 3447, 2925, 2855, 2360, 2342, 1743, 1457, 1251, 1166, 1111, 1077, 1009, 965, 898, 874, 844, 748, 668, 518. <sup>1</sup>H-NMR (CDCl<sub>3</sub>):  $\delta$  (ppm) = 4.93 (t, 2 H,  $J$  = 3.1 Hz, 1-H, 1'-H), 4.30 (dd, 1 H,  $J_1$  = 11.8 Hz,  $J_2$  = 2.3 Hz, 6-H), 4.07 (dd, 1 H,  $J_1$  = 11.8 Hz,  $J_2$  = 4.5 Hz, 6-H), 4.02 (m, 1 H, 5-H), 3.92 (m, 2 H, 2-H, 3-H), 3.85 (m, 1 H, 5'-H), 3.71 (m, 2 H, 6'-H), 3.47 (m, 4 H, 4-H, 2'-H, 3'-H, 4'-H), 2.35 (m, 2 H, 2''-H), 1.63 (m, 2 H, 3''H), 1.27 (m, 24 H, 4''-H – 15''-H), 0.88 (m, 3 H, 16''-H), 0.15-0.12 (m, 54 H, 6 SiMe<sub>3</sub>). <sup>13</sup>C-NMR (CDCl<sub>3</sub>):  $\delta$  (ppm) = 173.7 (C-1''), 94.3 (C-1'), 94.2 (C-1), 73.3 (C-5), 73.1 (C-2), 72.8 (C-5'), 72.6 (C-4'), 72.5 (C-3'), 71.7 (C-2'), 71.2 (C-4), 70.6 (C-3), 63.1 (C-6), 61.5 (C-6'), 34.0 (C-2''), 31.8 (C-14''), 29.5 (C-4'' – C-10''), 29.3 (C-11''), 29.2 (C-12'', C-13''), 24.7 (C-3''), 22.6 (C-15''), 14.0 (C-16''), 1.3 – 0.6 (6 SiMe<sub>3</sub>).

**3b**: 6-O-Monolauroyl-2,3,4,2',3',4'-hexa-O-(trimethylsilyl)- $\alpha,\alpha$ -trehalose (0.402 g, 0.42 mmol, 42 %, colorless oil). IR (NaCl):  $\nu$  (cm<sup>-1</sup>) = 3522, 2957, 2926, 2856, 2361, 1744, 1455, 1385, 1327, 1251, 1165, 1110, 1076, 1009, 965, 898, 873, 842, 749,

681.

$^1\text{H-NMR}$  ( $\text{CDCl}_3$ ):  $\delta$  (ppm) = 4.92 (t, 2 H,  $J = 3.2$  Hz, 1-H, 1'-H), 4.29 (dd, 1 H,  $J_1 = 11.9$  Hz,  $J_2 = 2.5$  Hz, 6-H), 4.06 (dd, 1 H,  $J_1 = 11.9$  Hz,  $J_2 = 4.7$  Hz, 6-H), 4.01 (m, 1 H, 5-H), 3.91 (m, 2 H, 2-H, 3-H), 3.85 (m, 1 H, 5'-H), 3.70 (m, 2 H, 6'-H), 3.45 (m, 4 H, 4-H, 2'-H, 3'-H, 4'-H), 2.35 (m, 2 H, 2''-H), 1.63 (m, 2 H, 3''H), 1.26 (m, 16 H, 4''-H – 11''-H), 0.88 (m, 3 H, 12''-H), 0.14 – 0.10 (m, 54 H, 6 SiMe<sub>3</sub>).  $^{13}\text{C-NMR}$  ( $\text{CDCl}_3$ ):  $\delta$  (ppm) = 173.7 (C-1''), 94.4 (C-1'), 94.3 (C-1), 73.4 (C-5), 73.2 (C-2), 72.8 (C-5'), 72.6 (C-4'), 72.5 (C-3'), 71.8 (C-2'), 71.3 (C-4), 70.7 (C-3), 63.2 (C-6), 61.6 (C-6'), 34.1 (C-2''), 31.8 (C-10''), 29.5 – 29.1 (C-4'' – C9''), 24.7 (C-2''), 22.6 (C-11''), 14.0 (C-12''), 1.3 – 0.3 (6 SiMe<sub>3</sub>).

MS (ESI):  $m/z$  (rel. int. in %) = 979 [ $\text{M} + \text{Na}$ ]<sup>+</sup> (34), 975 (100), 471 (43), 271 (90).

**3c**: 6-O-Monocaprinoyl-2,3,4,2',3',4'-hexa-O-(trimethylsilyl)- $\alpha,\alpha$ -trehalose (0.420 g, 0.45 mmol, 45 %, colorless oil). IR (NaCl):  $\nu$  ( $\text{cm}^{-1}$ ) = 3521, 2957, 2926, 2856, 1743, 1457, 1404, 1385, 1326, 1251, 1165, 1110, 1077, 1008, 964, 898, 874, 843, 749, 683.

$^1\text{H-NMR}$  ( $\text{CDCl}_3$ ):  $\delta$  (ppm) = 4.92 (t, 2 H,  $J = 2.55$  Hz, 1-H, 1'-H), 4.29 (dd, 1 H,  $J_1 = 11.9$  Hz,  $J_2 = 2.2$  Hz, 6-H), 4.06 (dd, 1 H,  $J_1 = 11.7$  Hz,  $J_2 = 4.6$  Hz, 6-H), 4.01 (m, 1 H, 5-H), 3.89 (m, 2 H, 2-H, 3-H), 3.83 (m, 1 H, 5'-H), 3.70 (m, 2 H, 6'-H), 3.45 (m, 4 H, 4-H, 2'-H, 3'-H, 4'-H), 2.34 (m, 2 H, 2''-H), 1.63 (m, 2 H, 3''H), 1.26 (m, 12 H, 4''-H – 9''-H), 0.88 (t, 3 H,  $J = 7.1$  Hz, 10''-H), 0.13 – 0.10 (m, 54 H, 6 SiMe<sub>3</sub>).  $^{13}\text{C-NMR}$  ( $\text{CDCl}_3$ ):

$\delta$  (ppm) = 173.9 (C-1''), 94.6 (C-1'), 94.5 (C-1), 73.6 (C-5), 73.4 (C-2), 73.0 (C-5'), 72.8

(C-4'), 72.7 (C-3'), 72.0 (C-2'), 71.5 (C-4), 70.9 (C-3), 63.4 (C-6), 61.7 (C-6'), 34.3 (C-2''), 32.0 (C-8''), 29.5 – 29.2 (C-3'' – C-6''), 24.9 (C-3''), 22.8 (C-9''), 14.2 (C-10''), 1.3 – 0.3 (6 SiMe<sub>3</sub>). MS (ESI):  $m/z$  (rel. int. in %) = 953 [ $\text{M} + \text{Na}$ ]<sup>+</sup> (23), 946 (91), 361 (100), 279 (63).

#### 3.2.1.4 Preparation of 6-O-monopalmitoyl-, 6-O-monolauroyl- and 6-O-monocaprinoyl- $\alpha,\alpha$ -trehalose (4a, 4b, 4c)

The corresponding esters **3a**, **3b**, **3c** of 2,3,4,2',3',4'-hexa-O-(trimethylsilyl)- $\alpha,\alpha$ -trehalose (0.74 mmol) were placed in a 25 ml flask, containing 8 ml of a mixture of

trifluoroacetic acid, tetrahydrofuran and water in a ratio of 8:17:3. The resulting solution was mechanically stirred for 1 hour at room temperature. **4a** was obtained as a white precipitate which was collected by filtration under reduced pressure and crystallized in acetone. **4b** and **4c** did not precipitate and thus were purified by removing the solvent using flash evaporation, followed by flash column chromatography (ethyl acetate, methanol 4:1).

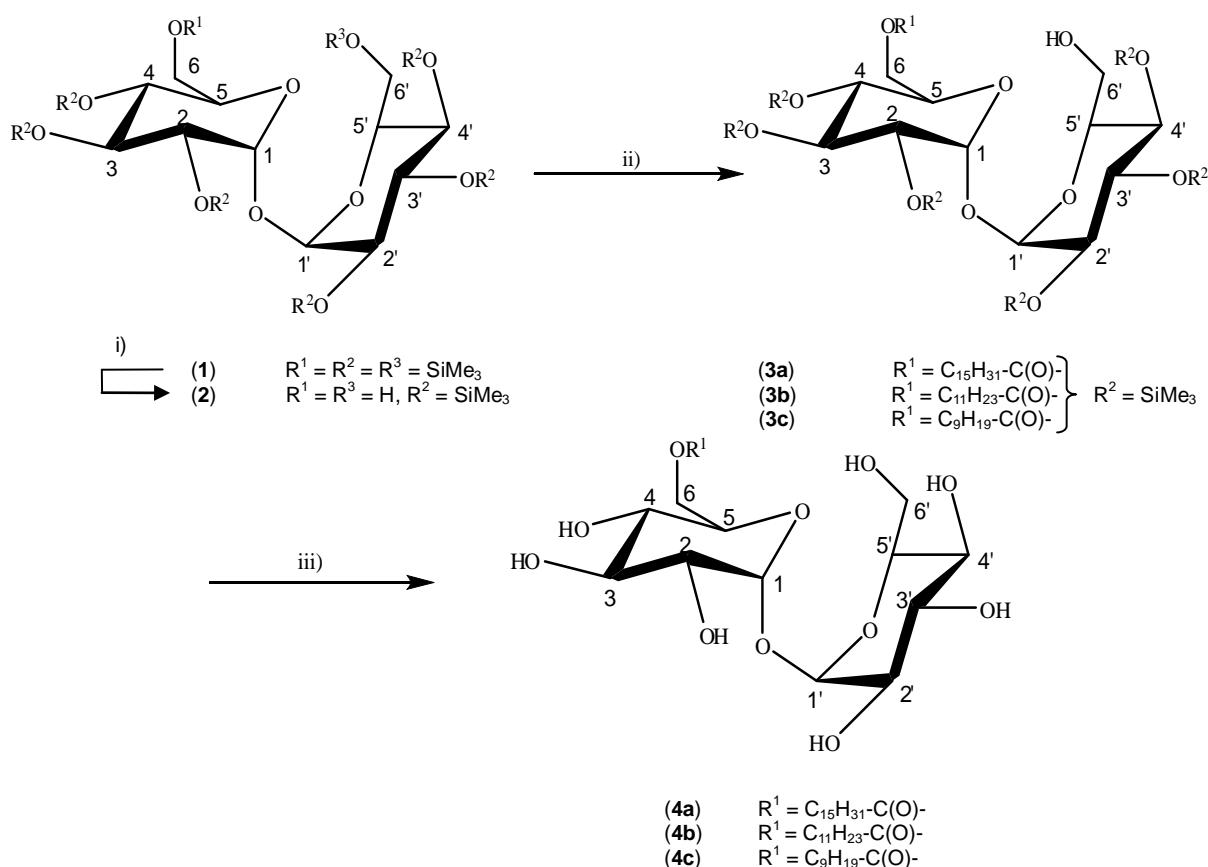
**4a:** 6-O-Monopalmitoyl- $\alpha,\alpha$ -trehalose (Mono-PT) (0.310 g, 0.53 mmol, 72 %, white solid). MP: softening at 130 – 135 °C, melting at 203 – 207 °C; (lit. 198 – 200 °C). IR (KBr):  $\nu$  (cm<sup>-1</sup>) = 3421, 2926, 2854, 2361, 1740, 1636, 1467, 1378, 1150, 1109, 1078, 1046, 989, 939, 805, 721, 576, 527. <sup>1</sup>H-NMR (CDCl<sub>3</sub>):  $\delta$  (ppm) = 5.08 (dd, 2 H,  $J_1$  = 11.2 Hz,  $J_2$  = 3.6 Hz, 1-H, 1'-H), 4.36 (dd, 1 H,  $J_1$  = 12.0 Hz,  $J_2$  = 1.6 Hz, 6-H), 4.19 (dd, 1 H,  $J_1$  = 12.0 Hz,  $J_2$  = 5.1 Hz, 6-H), 4.02 (m, 1 H, 5-H), 3.79 (m, 4 H, 2-H, 2'-H, 5'-H, 6'-H), 3.66 (m, 1 H, 6'-H), 3.47 (m, 2 H, 3-H, 3'-H), 3.34 (m, 2 H, 4-H, 4'-H), 2.34 (t, 2 H,  $J$  = 7.4 Hz, 2''-H), 1.61 (m, 2 H, 3''-H), 1.29 (m, 24 H, 4''-H - 15''-H), 0.90 (t, 3 H,  $J$  = 7.2 Hz, 16''-H). <sup>13</sup>C-NMR (CDCl<sub>3</sub>):  $\delta$  (ppm) = 175.5 (C-1''), 95.2 (C-1'), 95.1 (C-1), 74.6 (C-2'), 74.4 (C-2), 73.9 (C-5'), 73.2 (C-3, C-3'), 71.9 (C-4, C-4'), 71.4 (C-5), 64.4 (C-6), 62.6 (C-6'), 35.0 (C-2''), 33.1 (C-14''), 30.9 – 30.3 (C-4'' – C-13''), 26.1 (C-3''), 23.8 (C-15''), 14.5 (C-16''). MS (ESI):  $m/z$  (rel. int. in %) = 1183 [2·M + Na]<sup>+</sup> (20), 603 [M + Na]<sup>+</sup> (100).

**4b:** 6-O-Monolauroyl- $\alpha,\alpha$ -trehalose (Mono-LT) (0.294 g, 0.56 mmol, 76 %, white solid). MP: softening at 130 – 134 °C, melting at 159 – 163 °C; (lit.: 156 – 158 °C). IR (KBr):  $\nu$  (cm<sup>-1</sup>) = 3421, 2926, 2854, 2361, 1740, 1636, 1467, 1378, 1150, 1109, 1078, 1046, 989, 939, 805, 721, 576, 527. <sup>1</sup>H-NMR (CDCl<sub>3</sub>):  $\delta$  (ppm) = 5.08 (dd, 2 H,  $J_1$  = 11.1 Hz,  $J_2$  = 3.6 Hz, 1-H, 1'-H), 4.36 (d, 1 H,  $J$  = 11.7 Hz, 6-H), 4.19 (dd, 1 H,  $J_1$  = 11.9 Hz,  $J_2$  = 4.9 Hz, 6-H), 4.02 (m, 1 H, 5-H), 3.79 (m, 4 H, 2-H, 2'-H, 5'-H, 6'-H), 3.66 (m, 1 H, 6'-H), 3.47 (m, 2 H, 3-H, 3'-H), 3.33 (m, 2 H, 4-H, 4'-H), 2.34 (t, 2 H,  $J$  = 7.2 Hz, 2''-H), 1.61 (m, 2 H, 3''-H), 1.29 (m, 16 H, 4''-H - 11''-H), 0.90 (t, 3 H,  $J$  = 6.6 Hz, 12''-H). <sup>13</sup>C-NMR (CDCl<sub>3</sub>):  $\delta$  (ppm) = 175.5 (C-1''), 95.2 (C-1'), 95.1 (C-1), 74.6 (C-2'), 74.4 (C-2), 73.9 (C-5'), 73.2 (C-3, C-3'), 71.9 (C-4, C-4'), 71.4 (C-5), 64.4 (C-6), 62.7 (C-6'), 35.0 (C-2''), 33.1 (C-10''), 30.8-30.2 (C-4'' – C-9''), 26.1 (C-3''), 23.8 (C-11''), 14.5 (C-12''). MS (ESI):  $m/z$  (rel. int. in %) = 547 [M + Na]<sup>+</sup> (41), 563 [M + K]<sup>+</sup> (100), 1071 [2·M + Na]<sup>+</sup> (43), 1087 [2·M + K]<sup>+</sup> (45).

**4c:** 6-O-Monocaprinoyl- $\alpha,\alpha$ -trehalose (Mono-CT) (0.286 g (0.57 mmol, 78 %, white

solid). MP: 163 - 164 °C. IR (KBr):  $\nu$  (cm<sup>-1</sup>) = 3500, 3355, 2933, 2908, 2361, 2343, 1685, 1457, 1149, 1100, 1081, 1030, 998, 956, 803, 612. <sup>1</sup>H-NMR (CDCl<sub>3</sub>):  $\delta$  (ppm) = 5.08 (dd, 2 H,  $J_1 = 11.1$  Hz,  $J_2 = 3.7$  Hz, 1-H, 1'-H), 4.36 (dd, 1 H,  $J_1 = 11.9$  Hz,  $J_2 = 2.1$  Hz, 6-H), 4.19 (dd, 1 H,  $J_1 = 12.1$  Hz,  $J_2 = 5.2$  Hz, 6-H), 4.01 (m, 1 H, 5-H), 3.79 (m, 4 H, 2-H, 2'-H, 5'-H, 6'-H), 3.66 (m, 1 H, 6'-H), 3.47 (m, 2 H, 3-H, 3'-H), 3.32 (m, 2 H, 4-H, 4'-H), 2.34 (t, 2 H,  $J = 7.6$  Hz, 2''-H), 1.61 (m, 2 H, 3''H), 1.30 (m, 12 H, 4''-H - 9''-H), 0.90 (t, 3 H,  $J = 6.9$  Hz, 10''-H). <sup>13</sup>C-NMR (CDCl<sub>3</sub>):  $\delta$  (ppm) = 175.5 (C-1''), 95.2 (C-1'), 95.1 (C-1), 74.6 (C-2'), 74.4 (C-2), 73.9 (C-5'), 73.2 (C-3, C-3'), 71.9 (C-4, C-4'), 71.4 (C-5), 64.4 (C-6), 62.6 (C-6'), 35.0 (C-2''), 33.1 (C-8''), 30.6 - 30.3 (C-4'' - C-7''), 26.1 (C-3''), 23.8 (C-9''), 14.5 (C-10''). MS (ESI):  $m/z$  (rel. int. in %) = 519 [M + Na]<sup>+</sup> (100), 516 (56), 509 (7).

All three steps are summarized in Figure 28.



**Figure 28:** Reaction scheme of the preparation of Mono-PT (4a), Mono-LT (4b) and Mono-CT (4c): i) K<sub>2</sub>CO<sub>3</sub>, MeOH, 0 °C, 5 h; ii) 1,3-DCC, 4-DMAP, carboxylic acid, dry dichloromethane, 18 h, rt; iii) TFA, THF, H<sub>2</sub>O, 1 h, rt.

### **3.2.1.5 Instruments used for identification of the synthesized products**

IR spectra were recorded on a Perkin Elmer FT-Infrared Spectrometer Paragon 1000 (Perkin Elmer, Waltham, MA, USA).  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were taken on a Jeol JNMR-GX400 (400 MHz/100 MHz), and a Jeol JNMR-GX500 (500 MHz/125 MHz) (Jeol, Tokyo, Japan), and chemical shifts are reported in ppm, downfield from tetramethylsilane. ESI-mass spectroscopy was performed on an API 2000 (Applied Biosystems, Foster City, CA, USA). Melting points were taken on a Büchi Melting Point B-540 (BÜCHI Labortechnik GmbH, Essen, Germany). TLC was performed on POLYGRAM SIL G/UV<sub>254</sub> plates (Macherey-Nagel, Düren, Germany).

## **3.2.2. Methods**

### **3.2.2.1 Tensiometry**

Surface tension was measured with a K100 MK2 tensiometer (Krüss, Hamburg, Germany) equipped with a F12 thermostat (Julabo, Ostfildern, Germany) and a 765 Dosimat (Metrohm, Leinfelden-Echterdingen, Germany) using LabDesk 3.1 software (Krüss, Hamburg, Germany). Before the surface tension of surfactants was determined, the system was tested with purified water. When surface tensions of  $72.0 \pm 0.1$  mN/m were found for water in three consecutive measurements, surfactant solutions were tested. The Critical Micelle Concentration (CMC) was determined from sharp breaks in surface tension vs. logarithm of surfactant concentration plots. A custom-made glass vessel and a platinum iridium plate were used for all experiments. A total of 11 concentrations were tested for each surfactant. One measurement lasted at least 300 sec. Experiments ( $n = 3$ ) were performed at 25 °C for Mono-LT and Mono-CT. Because of its low solubility at room temperature Mono-PT was tested at 45 °C.

### **3.2.2.2 Pyrene Interaction Fluorescence**

Pyrene fluorescence was applied to alternatively determine the CMC according to (Kalyanasundaram and Thomas 1977). Fluorescence spectra were recorded and the ratio  $I_1/I_3$  was calculated, where  $I_1$  was the emission at 373 nm and  $I_3$  was the emission at 393 nm. Excitation wavelength was 335 nm. Due to a change in polarity when micelles occur, the ratio becomes  $<1$  at the CMC. Surfactant concentrations comprised the same range as applied to surface tension measurements. Experiments were performed in quartz cuvettes with 10 mm pathlength using a Cary Eclipse spectrofluorimeter (Varian, Palo Alto, USA). 10  $\mu$ l of a 200 mM pyrene solution in ethanol was added to 1990  $\mu$ l surfactant solution (N=3).

### **3.2.2.3 Hemolytic Activity**

Hemolytic activity was measured with porcine erythrocytes. Coagulation was prevented by adding 200 ml 3.8 % (m/V) trisodium citrate buffer pH 7.4 to 800 ml fresh blood. Samples were washed six times with citrate buffer and centrifuged at 3.500 g and 5 °C for 45 min. Different surfactant concentrations (in the same citrate buffer used for purification) were added to the porcine erythrocytes suspension at a 1:5 ratio. After incubating for 1 hour at 37 °C under gentle shaking samples were centrifuged (3.500 g for 15 min). The supernatant was tested for free hemoglobin at  $A = 415$  nm using an UV 8453 UV-spectrometer and UV-Visible ChemStation software (Agilent, Santa Clara, USA). Each experiment was performed with N=5. 100 % hemolysis was determined by diluting 10  $\mu$ l erythrocytes in 990  $\mu$ l Millipore water. 0 % hemolysis was tested by adding pure buffer to the erythrocyte suspension. The degree of hemolysis produced by surfactants (%H) was calculated according to equation (adapted from (Reinhart and Bauer 1995) and (Seyfert et al. 2002)).

### **3.2.2.4 Photon Correlation Spectroscopy (PCS)**

Size determination of micelles in 1 % (m/V) surfactant solutions was performed on a

Zetasizer nano ZS (Malvern Instruments, Herrenberg, Germany) with a 633 nm laser. The hydrodynamic diameter was calculated with DTS NANO v5.10 software (Malvern Instruments, Herrenberg, Germany). The sample, in single-use UV-plastic cuvettes (Brand GmbH and Co KG, Wertheim, Germany), was at first equilibrated for 1 min at 25 °C (and 5 min for 45 °C, respectively) and subsequently the time scale of the scattered light intensity fluctuations of the sample was measured. Each sample was recorded in triplicate, each with 20 runs. The size was obtained using the cumulants analysis by fitting a single exponential to the correlation function in order to obtain the volume-weighted mean size. The viscosity at 25 °C was used as input value for the fitting.

### **3.2.2.5 Viscometry**

As viscosity is a crucial parameter for PCS measurements the viscosity of the surfactant solutions was determined on an AMVn falling sphere viscometer (Anton Paar, Graz, Austria). 800 µl of a 1 % (m/V) surfactant solution was filled in a 1.6 mm diameter tube and viscosity was recorded from 20 °C to 45 °C  $\pm$  0.1 °C for every 5 °C. A 60° angle was applied and every solution viscosity was determined 10 times.

### **3.2.2.6 Differential Scanning Calorimetry (DSC)**

1 % (m/V) aqueous surfactant solutions (N=1) were analyzed using a DSC821 calorimeter (Mettler Toledo, Columbus, USA) and heated from 10 °C to 75 °C at 10 °C/min. Transitions were analyzed by STAR software supplied with the instrument.

### **3.2.2.7 Agitation stressing of human growth hormone formulations**

0.95 ml of a 0.2 mg/ml human growth hormone formulation was sterile filtrated into 2R vials. The protein was formulated in 10 mM phosphate buffer pH 7.2 containing different surfactant concentrations. Every concentration was tested in triplicate. A MM200 swinging mill (Retsch, Haan, Germany) was applied for a quick agitation stress for 10 min at 8 Hz. Consequently protein recovery was studied using size

exclusion chromatography (SEC), and protein aggregation was studied via turbidity ( $A_{350\text{nm}}$ ), light obscuration and visual inspection for particulate matter.

#### **3.2.2.8 Size Exclusion Chromatography (SEC)**

The monomer content of the protein formulation as well as the dimer formation was investigated via SEC. A HP 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector and a 3000 SWXL gel filtration column (Tosoh Bioscience, Tokyo, Japan) was used for this experiment. 50  $\mu\text{l}$  of a centrifuged protein sample was injected into the HPLC. Within the HPLC a 0.2  $\mu\text{m}$  online filter was used. The running buffer was composed of 50 mM  $\text{NaH}_2\text{PO}_4$  and 150 mM NaCl at a pH of 7.2. The analytics were performed at a flow-rate of 0.5 ml/min with UV-detection at 216 nm.

#### **3.2.2.9 Turbidity Measurement**

Turbidity of the shaken samples was quantified by means of UV-absorption at 350 nm. 200  $\mu\text{l}$  samples were placed in a 96 well plate and tested in a Fluostar Omega reader with analysis software (BMG Labtech, Offenburg, Germany). Pure buffer was used as a blank sample.

#### **3.2.2.10 Light Obscuration**

Sub-visible particle counting was carried out via a light obscuration method. The particles are deduced by the size from the amount of light blocked as the particles pass in a single file fashion. According to Ph. Eur. method 2.9.19 and USP method <788> the experiment was adapted using a reduced sample volume. The analysis was performed using a SVSS-c particle counting system (Pamas, Stuttgart, Germany). At fixed fill and ejection rates of 10 ml/min, each sample was measured at a volume of 0.3 ml per injection. A first injection of 0.5 ml was discarded to assure that the sample will end up directly in the Hamilton syringe and the mean value was

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obtained from three injections. Between each sample analysis, the system was rinsed with water to the point at which the 1  $\mu\text{m}$  particle count of the apparatus was less than 20. Sub-visible particles bigger than or equal to 10 and 25  $\mu\text{m}$  are presented as cumulative counts per ml.

#### **3.2.2.11 Visual Inspection**

A visual inspection for particulate matter was performed in front of a black and a white screen manually adapted to Ph. Eur. method 2.9.20.

### **3.3. Results and Discussion**

#### **3.3.1 Synthesis of Sugar Based Surfactants Mono-PT, Mono-LT und Mono-CT**

Few papers have previously been published on the preparation of fatty acid monoesters of  $\alpha,\alpha$ -trehalose, in any cases the primary hydroxyl group at C-6 was esterified (Suzuki et al. 1999; Raku et al. 2003; Chen et al. 2005). Due to the symmetrical character of the disaccharide  $\alpha,\alpha$ -trehalose, both primary hydroxyl groups at C-6 and at C-6' show equal reactivity. Consequently, the two monoesters at C-6-OH or C-6'-OH can be considered equivalent, but double acylation to give a 6,6'-diester has to be avoided. Chen et al. (Chen et al. 2005) obtained monolinoleoyl  $\alpha,\alpha$ -trehalose by a lipase-catalyzed condensation of trehalose with linoleic acid under sophisticated reaction conditions, Raku et al. (Raku et al. 2003) performed transesterifications of  $\alpha,\alpha$ -trehalose with a couple of fatty acid vinyl esters catalyzed by *Bacillus subtilis* protease to obtain various  $\alpha,\alpha$ -trehalose monoesters. In contrast, a lipase-catalyzed esterification of  $\alpha,\alpha$ -trehalose with a fatty acid, accelerated by microwave irradiation, gave mainly the 6,6'-diester.

For the preparation of the target surfactants an improved protocol based on Datta's work was developed (Figure 28). Fully silylated 2,3,4,6,2',3',4',6'-octa-O-(trimethylsilyl)- $\alpha,\alpha$ -trehalose (**1**) was prepared from  $\alpha,\alpha$ -trehalose dihydrate using the silylating agents trimethylsilyl chloride and hexamethyldisilazane in anhydrous

pyridine following with improved stoichiometry. Product **1** was easily purified by crystallization from methanol. Selective deprotection of both primary hydroxyl groups to give 2,3,4,2',3',4'-hexa-O-(trimethylsilyl)- $\alpha,\alpha$ -trehalose (**2**) was performed using a methanolic potassium carbonate solution. After complete conversion, the reaction was stopped by addition of a defined amount of glacial acetic acid to neutralize the reaction mixture. Using an excess of glacial acetic acid results in complete deprotection of the silylated  $\alpha,\alpha$ -trehalose. The crude product **2** could be used for the subsequent acylation step without further purification. Predominant monoacylation of the symmetrical diol **2** to give the monoesters **3a**, **3b**, **3c** of 2,3,4,2',3',4'-hexa-O-(trimethylsilyl)- $\alpha,\alpha$ -trehalose was achieved using Steglich conditions. In this reaction, the carboxylic acids add 1,3-DCC formatting O-acylisoureas. Esterification of the hydroxyl group is facilitated by an acyl group carrier like 4-DMAP. As a side reaction, the O-acylisourea can undergo a 1,3-rearrangement to give a non-reactive *N*-acylurea. Typically, this side reaction is very slow, but if sterically ambitious molecules like the sixfold silylated  $\alpha,\alpha$ -trehalose and long chained fatty acids are used, this side reaction can cause a significant loss in yield. We found, that using stoichiometric instead of catalytic amounts of 4-DMAP leads to a significant increase in yields of the monoacyl products. But still, this step gives only moderate yields (33 – 45 %). The products were separated from unreacted starting material **2** and from 6,6'-diesters by flash column chromatography and were obtained as colorless oils. The aspired monoesters of  $\alpha,\alpha$ -trehalose **4a**, **4b**, **4c** were prepared by complete removal of the remaining six silyl protecting groups with trifluoroacetic acid in aqueous tetrahydrofuran. Mono-PT precipitated from the reaction mixture and could be separated by filtration. This compound could not be purified by silica gel flash column chromatography, since it is able to dissolve significant amounts of silica gel. Finally, crystallization from acetone gave pure Mono-PT (**4a**) as a white solid. Mono-LT (**4b**) and Mono-CT (**4c**) did not precipitate from the solution after deprotection. Removing the solvent using flash evaporation resulted in oily crude products which could be purified by flash column chromatography, giving Mono-LT and Mono-CT as white solids. The structures of all compounds were confirmed by the data from IR,  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and mass spectroscopy.

Potential impurities within the trehalose-based excipient could be the agents used during synthesis of the excipient. Furthermore the excipient could contain 6,6'-diester

which is obtained due to double acylation of the 2,3,4,2',3',4'-hexa-O-(trimethylsilyl)- $\alpha,\alpha$ -trehalose. After purification of the product, none of these substances could be detected by NMR spectroscopy or mass spectrometry.

### 3.3.2 Physical Characterization

#### 3.3.2.1 Surface Tension and Critical Micelle Concentration

Both surface tension measurements as well pyrene interaction spectroscopy yielded comparable CMC results in water (Table 7).

	CMC <sub>STM</sub> (mg/ml)	CMC <sub>PF</sub> (mg/ml)	CMC <sub>STM</sub> (mM)	CMC <sub>PF</sub> (mM)
Mono-CT	2.14	1.92	4.32	3.87
Mono-LT	0.21	0.33	0.40	0.62
Mono-PT	0.006	n.d.*	0.01	n.d.

**Table 7: CMC of Mono-CT (4c), Mono-LT (4b) and Mono-PT (4a) in water as derived from surface tension measurements (STM) and pyrene fluorescence (PF). \*not determined.**

An increase in hydrophobic chain length resulted in a decrease of the CMC. This is in accordance with other publications on sugar based surfactants (Aveyard et al. 1998; Chen et al. 2006). The general rule that a two carbon atoms longer fatty acid results in an approximately 10-fold lower CMC could be applied to the trehalose monoesters (Aveyard et al. 1998). The CMC at 25 °C was not significantly affected by the buffer salts used for hemolytic activity and protein stability tests (data not shown). This was expected as the surfactants are nonionic and should show only minor interaction with ions in the solution (Thorsteinsson et al. 2005), when salts are used in concentrations to control pH and regulate osmolarity of the final drug product. Mono-CT showed a CMC of 1.92 mg/ml and a decrease of surface tension to 29 mN/m. Mono-LT reached

a decrease of surface tension of ~39 mN/m at a CMC of 0.33 mg/ml. For Polysorbates a similar values were observed as PS 20 has its CMC at 0.15 mg/ml with a decrease in surface tension ~38 mN/m and PS 80 has a CMC of 0.014 mg/ml with surface tension values of ~45 mN/m (Wan and Lee 1974).

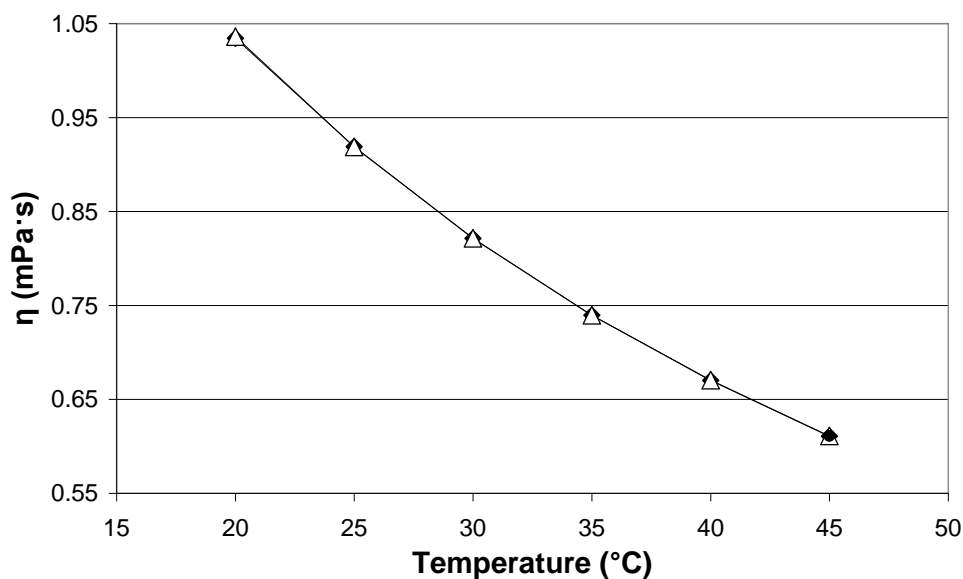
### 3.3.2.2 Micelle Size Characterization and Rheometry

PCS experiments were performed at 25 °C and 45 °C. Due to the relatively poor solubility at 25 °C Mono-PT could not be analyzed at the lower temperature. As reported by Soderman for other sugar based surfactants, all hydrodynamic diameters were in the range of 5-15 nm (Ericsson Caroline et al. 2005).

	Volume Mean Diameter (nm) at 25 °C	Volume Mean Diameter (nm) at 45 °C
Mono-CT	5.0	5.5
Mono-LT	6.3	7.3
Mono-PT	n.d.*	10.12
PS 20	6.9	7.9
PS 80	7.4	9.1

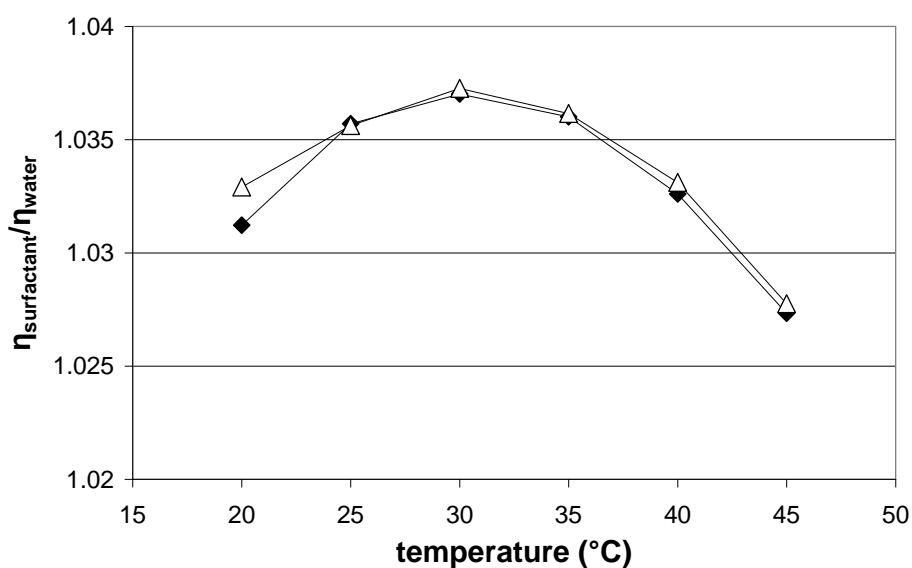
**Table 8: Volume mean diameter of different sugar surfactants and PS 20 and PS 80 at 25 and 45 °C \*not determined**

Mono-CT had a size of 5.0 nm at 25 °C and 5.5 nm at 45 °C, Mono-LT had a size of 6.3 nm at 25 °C and 7.3 nm at 45 °C, Mono-PT had a size of 10.1 nm at 45 °C (Table 8). The size increased with fatty acid chain length. The size range was comparable to other surfactants such as PS 20 (6.9 nm at 25 °C and 7.9 at 45 °C) or PS 80 (7.4 nm / 9.1 nm) and is in good agreement with the data reported for other non-ionic Macrogol-based surfactants (Becher and Arai 1968). Results were strongly dependent from viscosity and consequently from temperature. Temperature-dependent viscosities are displayed in Figures 29a and 29b.



**Figure 29a: Temperature dependence of the kinematic viscosity of 1 % solutions of Mono-CT (♦) and Mono-LT (Δ) (Mono-PT (■) at 45 °C).**

For both Mono-CT and Mono-LT, viscosities of 1 % (m/V) solutions were almost identical, with marginally lower values for Mono-CT. Mono-PT was only measured at 45 °C and in this area, the increase in viscosity was comparable for all three trehalose monoesters, and in the range of the viscosity of water. The ratio of viscosity of a 1 % (m/V) surfactant solution to pure water at any temperature between 20 and 45 °C was between 1.025 and 1.038.



**Figure 29b: Relative viscosity  $\eta_{\text{Surfactant}}/\eta_{\text{water}}$  (b) of 1 % solutions of Mono-CT (♦) and Mono-LT (Δ) (Mono-PT (■) at 45 °C).**

The maximum increase could be detected at 30 °C. In contrast, Sodermann et al., who worked with the same concentrations of tetradecylmaltoside found a far more pronounced maximum of >1.2 at 60 °C (with 1.1 at 30 °C) indicating a shape transition of the micelles. Thus, the surfactants described here are expected to form spherical micelles and to preserve the structure in the temperature range evaluated.

### **3.3.2.3 DSC Characterization**

Calorimetric measurements of 1 % (w/w) solutions showed no transitions for Mono-CT and Mono-LT in the range between 10 °C and 75 °C. In contrast, Mono-PT exhibited an endothermic transition at 43 °C. In the temperature range the solubility also increases substantially. At this point the flexibility of the fatty acid increases and melting occurs. Therefore, the applicability of Mono-CT and Mono-LT in a temperature range which is relevant for biopharmaceuticals is given. In an additional test no cloud point could be detected for 1 % (m/m) aqueous solutions between 20 and 90 °C for Mono-CT and Mono-LT (data not shown).

### **3.3.2.4 Hemolytic Activity**

An important safety concern of surface active substances is possible rupture of cell membranes and formation of mixed micelles with membrane lipids. All trehalose-based surfactants tended to destabilize blood cell membranes below their CMC. The HC<sub>5%</sub> values were approximately 10 and 5 times below the CMC for Mono-CT and Mono-LT, respectively. Mono-PT shows a HC<sub>5%</sub> at a concentration 4 times above CMC. For comparison, PS 20 shows the same hemolytic activity at a concentration of 1 mg/ml, which is factor 10 higher than the CMC (

Table 9). Concentrations usually applied in protein stabilization are approximately at or slightly below CMC. Hence, Mono-CT and Mono-LT appear to be suitable candidates for further testing concerning protein stabilization, but hemolytic side effects should be carefully evaluated. For Mono-PT the calculated HLB was 12.7, for Mono-LT 14.0 and for Mono-CT 14.8. In comparison PS 20 has a HLB-value of 16.7

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and PS 80 has a value of 15.0. Hence, both polysorbates are slightly more hydrophilic than the trehalose monoesters.

	HC <sub>5</sub> % (mg/ml)	HC <sub>5</sub> % (mM)
Mono-CT	0.1984	0.400
Mono-LT	0.0524	0.140
Mono-PT	0.025	0.05

**Table 9: Hemolytic activity of Mono-CT, Mono-LT and Mono-PT.**

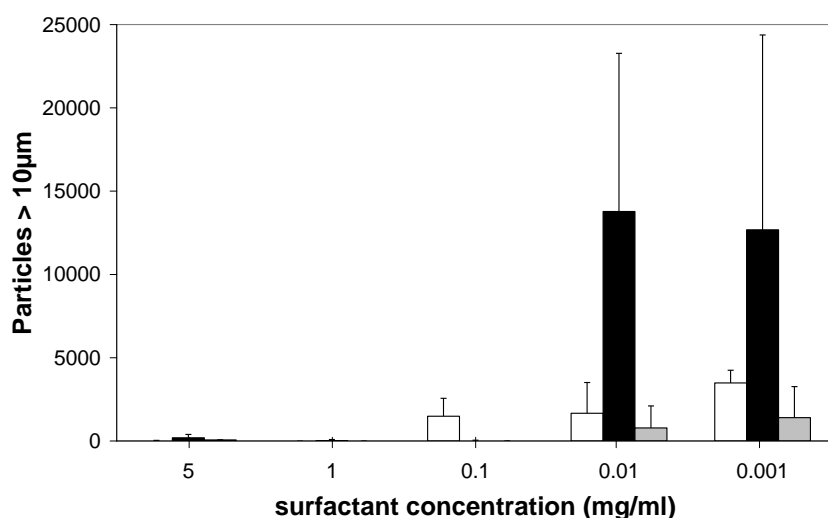
In literature HLB-values are described as a less significant factor for hemolytic activity, however the membrane interaction appears to be more dependent on the structural alignment of the surfactants (Zaslavsky et al. 1978; Ohnishi and Sagitani 1993). Somfai et al. studied other sugar based surfactants and found similar results for both highly pure substances as well as technical products, but the cell compatibility could be improved by redesigning the hydrophobic chain, e.g. branching at the hydroxyl group of 12-hydroxystearic acid (Soderlind et al. 2003; Neimert-Andersson et al. 2006; Soederlind and Karlsson 2006). In general, determining hemolytic activity of substances using washed red cells might result in lower HC<sub>5</sub>% values than in whole blood as other substances present in blood could compete with cell membranes for binding to surfactants.

### 3.3.3 Agitation stress of human growth hormone

Due to its poor solubility at 2 – 8 °C and 25 °C Mono-PT was not included in the stability testing of hGH. The expected required concentration of surfactant for protein stabilization is in the range of 0.1 to 1 mg/ml (0.01 - 0.1 % (m/V)) as PS 20 and 80 tend to show their best stabilizing behavior at these concentrations, just below and approximately at CMC. Consequently a range of 0.001 to 5 mg/ml was included in the studies.

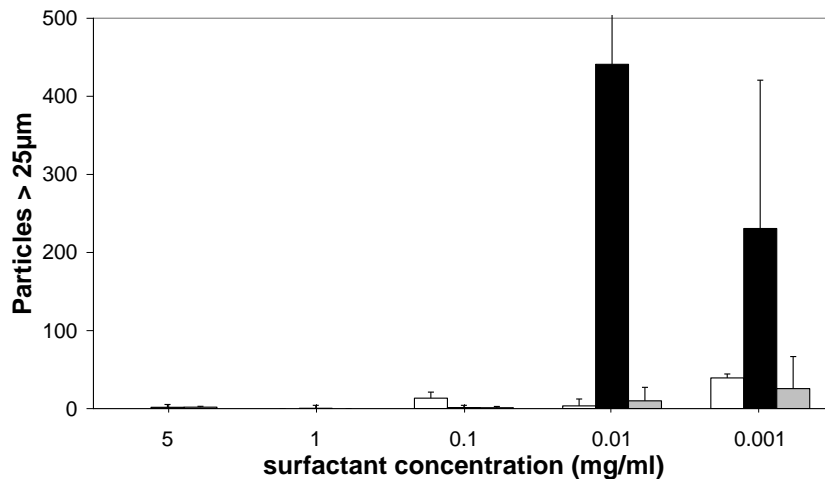
### 3.3.3.1 Particle formation

Visible particles were found in all formulations with surfactant concentrations  $\leq 0.1$  mg/ml. Analysis of subvisible particles via light obscuration also indicated strong formation of particles  $\geq 10$   $\mu\text{m}$  and  $\geq 25$   $\mu\text{m}$  upon shaking (Fig. 30). During agitation for a short period, concentrations of 0.1 mg/ml PS 80 and Mono-CT suppress the formation of visible and subvisible aggregates. Samples with lower Mono-CT concentrations showed higher particle numbers than all other formulations but also a high standard deviation. For Mono-LT 1 mg/ml was necessary for the stabilization. The turbidity measurements, detecting particles in the sub- $\mu\text{m}$  range with high sensitivity indicated the same trend.



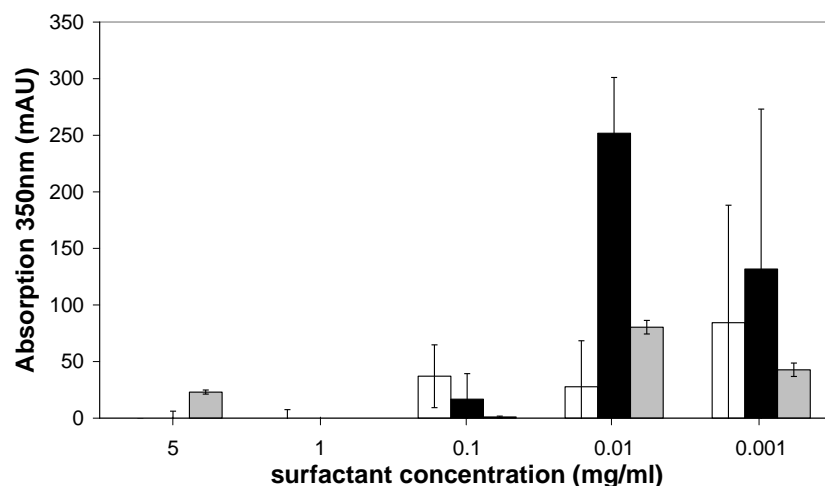
**Figure 30a: Subvisible particles  $\geq 10\mu\text{m}$ ) of hGH solutions after agitation in presence of Mono-LT (white), Mono-CT (black) und PS 80 (grey).**





**Figure 30b: Subvisible particles >25µm of hGH solutions after agitation in presence of Mono-LT (white), Mono-CT (black) und PS 80 (grey).**

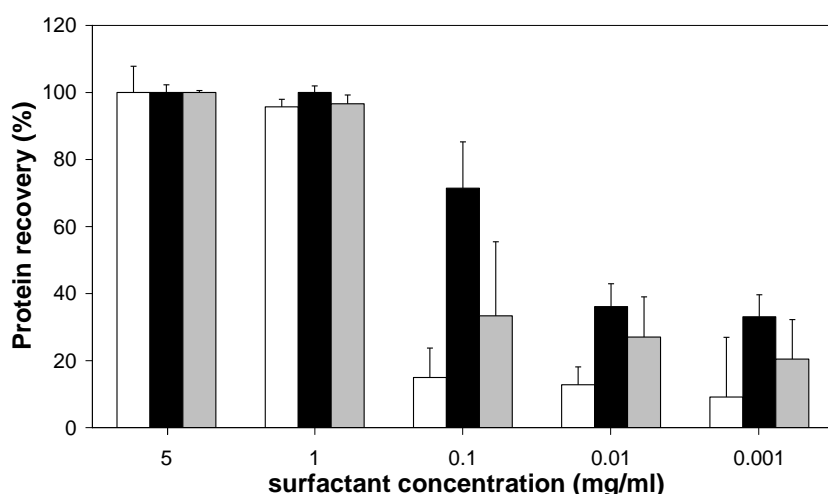
As for visible and subvisible particles extensive protein aggregation occurs at concentrations below 1 mg/ml for Mono-CT and Mono-LT and below 0.1 mg/ml for PS 80 (Fig. 4). Thus, particle analysis demonstrates the stabilizing effect of Mono-CT and Mono-LT. Similar or slightly higher concentrations as compared to PS 80 appear to be necessary for sufficient stabilization of hGH. HGH is known for its high aggregation tendency during agitation stress and the stabilizing effects of surfactants have been described for example by Katakam et al. (Katakam et al. 1995).



**Figure 31: Turbidity of hGH solutions after agitation in presence of Mono-LT (white), Mono-CT (black) und PS 80 (grey)..**

### 3.3.3.2 Size Exclusion Chromatography

Results on SEC show a clear dependency of monomer recovery from surfactant concentration for all surfactants (Fig. 5). Very low recovery of less than 40 % resulted in the formulations containing 0.01 or 0.001 mg/ml surfactant as well as in the 0.1 mg/ml mono-LT and PS80 containing formations.



**Figure 32: SEC monomer recovery of hGH solutions after agitation in presence of Mono-LT (white), Mono-CT (black) und PS 80 (grey).**

This compared to a recovery of only  $24.6 \pm 8.6$  % in surfactant free formulations. The amount of dimers and trimers recovered in SEC after agitation was negligible. This loss in recovery corresponded to substantial formation of large aggregates which were excluded from SEC but detected by particle analysis and turbidity measurements. This could also be prevented by the new trehalose-based surfactants. For hGH adsorption plays a major role and has been shown to occur at filter materials, silica or methylated silica surfaces (Maa and Hsu 1996; Buijs and Hlady 1997; Buijs et al. 1998; Maa and Hsu 1998). In order to study this effect, hGH was also stored statically without shaking in surfactant free formulation. The recovery of hGH decreased over time and leveled off after 6 hours. After 24 h 80 % hGH were lost due to adsorption. Addition of 0.1 % (m/V) PS 80, Mono-CT and Mono-LT could suppress this effect providing up to 95 % recovery. Thus, the loss in monomer recovery in the shaken samples was due to both aggregation and adsorption.

Recovery higher than 95 % could only be assured at concentrations of 1 mg/ml and 5 mg/ml for all three surfactants. These concentrations were slightly above the minimum concentration necessary to suppress particle formation. Overall, Mono-CT trends to provide the highest recovery values.

Mono-CT as well as Mono-LT are comparable to PS 80 with regard to their protein stabilizing ability in this case study with hGH. A concentration of 1 mg/ml seems to be sufficient to stabilize this hydrophobic protein against stress from harsh agitating with a high headspace volume. Polysorbate is usually applied in concentrations between 0.017 mg/ml and 1.6 mg/ml (Hawe et al. ; Wang 1999; Treuheit et al. 2002; Hawe and Friess 2008) for this purpose. This corresponds to the results of this study with a stabilizing concentration for both trehalose esters and PS 80 of 1 mg/ml. SEC revealed that hGH did not form dimers nor trimers upon agitation. Instead, marked formation of larger particles as demonstrated by turbidity measurement, subvisible and visible particles occurs. This corresponds to results seen by other groups (Katakam and Banga 1997). Depending on the type of stress different types of aggregates can be produced (Ha et al. 2002; Mahler et al. 2009). In this case shaking induced the formation of very large vesicles.

### **3.4. Conclusions**

Three trehalose-based surfactants were synthesized in high purity. Mono-PT with the lowest HLB value in this group shows insufficient water solubility at 2 – 8 °C and 25 °C and therefore appears to be less suitable for protein formulation. The two substances with shorter fatty acid chains, Mono-CT and Mono-LT seem to have the potential to be applied in biopharmaceutical formulations. The formation of micelles and the reduction in surface tension are comparable to PS 80 and PS 20. Both these sugar based surfactants show slightly higher hemolytic activity as compared to polysorbates. A change from a linear to a branched hydrophobic part of the surfactant might lead to an improvement in regards of the ratio of CMC to haemolytic activity as described by Somfai et al. for other sugar based surfactants (Soederlind and Karlsson 2006). Mono-CT and Mono-LT form transparent and colorless aqueous solutions and do not show phase transitions or separation at pharmaceutically

applied temperatures. Due to their high purity and their molecular structure, induction of chemical instability of biopharmaceuticals is less likely compared to polysorbates. Similar as polysorbates, Mono-CT and Mono-LT were able to stabilize hGH against aggregation upon shaking as well as adsorption. As trehalose is known to be a good bulking agent and lyoprotector (Wang 2000) these substances might show an even better performance in freeze-dried formulations. Consequently, trehalose-based surfactants offer great potential for application in biopharmaceutical formulations. Potential use requires large toxicological studies and enormous regulatory hurdles would have to be met, but some manufacturers of sugar based surfactants got approval for clinical use after only a few animal studies (Maggio 2008) and increased IP space by application of new excipients could trigger this.

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# 4. Stress test of protein formulations with different nonionic surfactants

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## 4.1. Introduction

For the development of pharmaceutical formulations stress tests are an important tool to evaluate the shelf life of different recipes. As already described in the introduction chapter proteins can be exposed to different kinds of stresses. The addition of nonionic surfactants in general reduces interface induced stress. These interfaces typically exist between the aqueous protein solution and the neighboring phases. The most important interface is formed towards the gas phase, typically air or nitrogen (Maa and Hsu 1997; Baszkin et al. 2001). This interface can be expanded and becomes more dynamic via shaking or stirring, which increases the applied stress and speed up the development process (Kiese et al. 2008; Eppler et al. 2010; Serno et al. 2010). The other important interface is between the protein formulation and its container. Proteins are also stressed at the water-ice interface during freezing and thawing (Tang and Pikal 2005). In all these cases nonionic surfactants were able to stabilize the protein against the interfacial stress. In this chapter the comparison of different sugar-based surfactants against polysorbates as the gold standard in pharmaceutical formulations (Wang 2005) is made. Some drawbacks can come along with the use of polyethyleneglycol-based surfactants, such as oxidation of the protein (Wang et al. 2008). Also direct side effects (Donbrow et al. 1978; Azaz et al. 1981) have already been described already in the literature, see also chapter 2. The most promising sugar based surfactants candidates selected based on their physical chemical properties plus self-synthesized trehalose esters were tested for their protein stabilizing capacity during agitation and storage at elevated temperatures. For the agitation stress tests it would be desirable for the new surfactants to show a behavior comparable to polysorbates as the stabilizing abilities of the latter are already satisfying. With respect to storage at elevated temperatures it would be hoped that the sugar based surfactants would score better as peroxide equivalents

were not detectable in these mixtures even after longer storage under UV radiation (see chapter 2).

## **4.2. Materials and Methods**

### **4.2.1. Material**

For synthesis of the trehalose esters, please refer to chapter 3. All sugar based surfactant mixtures were kindly donated by their suppliers. Plantacare products were donated by Cognis (Düsseldorf, Germany), Ryoto Products were donated by , NV10 was donated by Novexin. Polysorbate 20 (PS 20) and Polysorbate 80 (PS 80) were obtained from Sigma Aldrich (Munich, Germany). Analytical grade buffer salts and HPLC grade Acetonitrile and Trifluoroacetic acid were obtained from Merck Chemicals (Darmstadt, Germany). The deionized water used for the experiments was taken from a PURELAB Plus system (ELGA labwater, Celle, Germany). The water had a conductivity  $<0.055 \mu\text{S/cm}$ .

### **4.2.2. Methods**

#### **4.2.2.1. Stress Tests**

##### **4.2.2.1.1. Agitation stress test of hGH containing formulations**

hGH is a very sensitive protein when it comes to surface related stress. But the problem is to find the amount of stress that is sufficient to lead to aggregation and stop at a time point where you can differentiate between a stabilizing and a non stabilizing formulation (Eppler et al. 2010). Shaking (St. John et al. 2001), vortexing in centrifugation tubes (Katakam et al. 1995), or stirring with magnet bars (Maa and Hsu 1997) are typically used to stress hGH. None of these approaches worked as it was either too harsh or not sufficiently stressful. Ultimately, a Retsch MM 200

swinging mill (Retsch GmbH, Haan, Germany) was employed. For hgH containing formulations, agitation of 1.9 ml of 0.1 mg/ml hGH in 10 mM phosphate buffer pH 7.4 together with different concentrations of nonionic surfactants was performed in 2R-Vial at 8 Hz for 10 min.

#### **4.2.2.1.2. Stress Tests with IL-11 Containing Formulations**

For IL-11, agitation of 2 ml of 0.5 mg/ml IL-11 10 mM phosphate buffer and the respective amount of nonionic surfactant was performed on a horizontal shaker at 20 Hz for 172 h at ambient temperature.

Storage stability of 0.5 mg/ml protein formulations containing nonionic surfactants was performed at 2-8, 25, 40 and 50°C on the basis of the ICH guidelines ICH Q1. Storage was performed in 2R-vials with a fill volume of 1 ml. Samples contained 10 mM glycine and 10 mM phosphate buffer pH 7.0 plus one type of surfactant. At 0.01% were stored PS 20, PC 2000, PC 818 and NV-10 and trehalose caprylate (n=2 / plus a surfactant free control sample). Furthermore PS 80 and trehalose laurate were applied at 0.1% and 0.001% as well. Additional samples of pure PC 2000 and PS 80 have been exposed to UV-radiation (366 nm) for a period of one week. Samples of stressed surfactants were prepared by spreading of a 10 ml sample of 10% surfactant solutions. Samples were filled and crimp sealed into cleaned and heat sterilized vials. Storage stress experiments in incubators were performed in paper container boxes to exclude light radiation induced oxidation effects.

Furthermore, additional experiments were performed with freeze-dried formulations. All freeze-dried formulations contained additionally 1% glycine to the buffer salts and the surfactant. The amino acid is used as bulking agent. This excipient is also used in the market formulation of Neumega® in a concentration of 2.3%. Freeze-drying was performed with an Epsilon 2-6D freeze dryer (Martin Christ GmbH, Osterode, Germany). 2 ml of the above mentioned formulations were lyophilized as follows:

Freezing: 3 h from ambient temperature to -50 °C, no vacuum

Primary Drying: 21 h at -20 °C, 0.05 mbar

Secondary Drying: 48 h at -10 °C, 0.01 mbar

Samples were closed at 800 mbar vacuum. All vials revealed cosmetically elegant cakes. Formulations were stored under 40 °C and 50 °C and samples were taken

both after one and two weeks.

#### **4.2.2.2. Size Exclusion Chromatography**

hGH: A HP 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector and a 3000 SWXL gel filtration column (Tosoh Bioscience, Tokyo, Japan) was used for this experiment (50 µl injection volume). The running buffer was composed of 50 mM NaH<sub>2</sub>PO<sub>4</sub> and 150 mM NaCl at a pH of 7.2. The analysis were performed at a flow-rate of 0.5 ml/min with UV-detection at 216 nm.

Il-11: Size exclusion chromatography was performed using a TSK G2000 SWXL column (Tosoh Bioscience, Tokyo, Japan) at 4±2 °C (50 µl injection volume at 0.1 mg/ml) on the same HPLC system. A running buffer containing 0.1mM glycine, 500mM sodium chloride and 50mM morpholinoethanesulfonic acid (MES) at pH6.0 was used at a flow rate of 0.75 ml/min. Detection wavelength was set at 225 nm.

Concentrations of the samples of both proteins were calculated based on AUC in comparison with freshly prepared protein standards.

#### **4.2.2.3. Turbidity measurments**

Turbidity of the shaken samples was quantified via UV-absorption at 350 nm. 200 µl samples were placed in a 96 well plate and tested in a Fluostar Omega reader with analysis software (BMG Labtech, Offenburg, Germany). Pure buffer was used as a blank sample. Samples were corrected against an unshaken control, hence negative values occur for the more stable formulations.

#### **4.2.2.4. Light Obscuration measurements**

Sub-visible particle counting was carried out via a light obscuration method. The particle size is deduced from the amount of light blocked as the particles pass in a single file fashion. According to Ph. Eur. method 2.9.19 and USP method <788> the experiment was adapted using a reduced sample volume. The analysis was

performed using a SVSS-c particle counting system (Pamas, Stuttgart, Germany). At fixed fill and ejection rates of 10 ml/min, each sample was measured at a volume of 0.3 ml per injection. A first injection of 0.5 ml was discarded to assure that the sample will end up directly in the Hamilton syringe and the mean value was obtained from three injections. Between each sample analysis, the system was rinsed with water to the point at which the 1 µm particle count of the apparatus was less than 20. Sub-visible particles bigger than or equal to 10 and 25 µm are presented as cumulative counts per ml. To prevent artifact particles from the pure water, the system was rinsed 15 min prior to sampling.

#### **4.2.2.5. Visual Inspection**

A visual inspection for particulate matter was performed in front of a black and a white screen manually adapted to Ph. Eur. method 2.9.20.

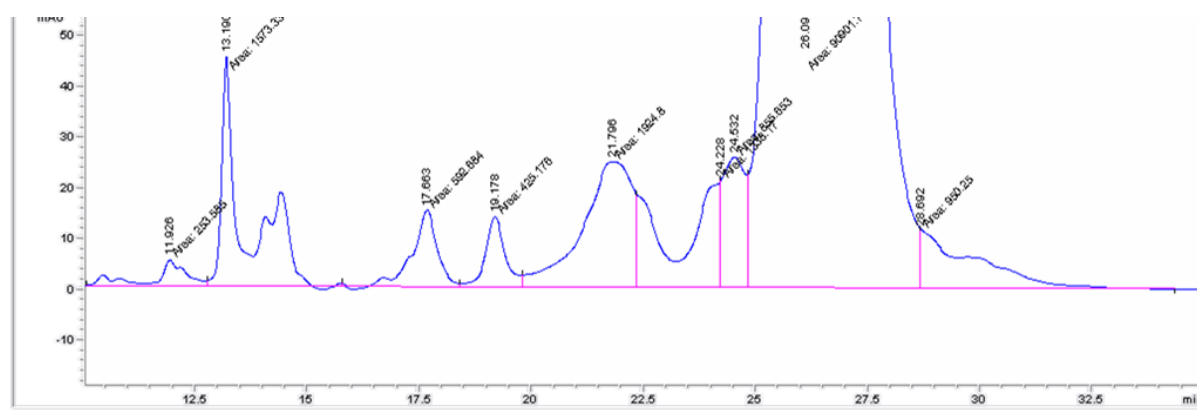
#### **4.2.2.6. SDS-Page**

Covalent aggregation of Interleukin 11 was analyzed via non-reducing denaturing SDS polyacrylamid gel electrophoresis. NuPage 10% and NuPage running buffer (Invitrogen, Karlsruhe, Germany) were used for these experiments. The elecetrophoresis was performed at a constant current of 0.03 A per gel. NuPage LDS sample buffer was added to the samples, which were denaturated for 10 minutes at 95 °C. 20µl of the solution was transferred into the gel. The amount of protein was set to 5 µg protein. The gels were stained with Colloidal Blue staining kit (Invitrogen, Karlsruhe, Germany).

#### **4.2.2.7. Reversed Phase HPLC**

Related species of IL-11 were detected at a wavelength of 214nm at the variable wavelength detector and the diode array detector (adapted from (Yokota et al. 2000)). Two eluents, water plus 0.1% triflouric acetic acid [A] and water/acetonitril (20/80(m/m)) plus 0.1% triflouric acetic acid [B] were used in the gradient. The

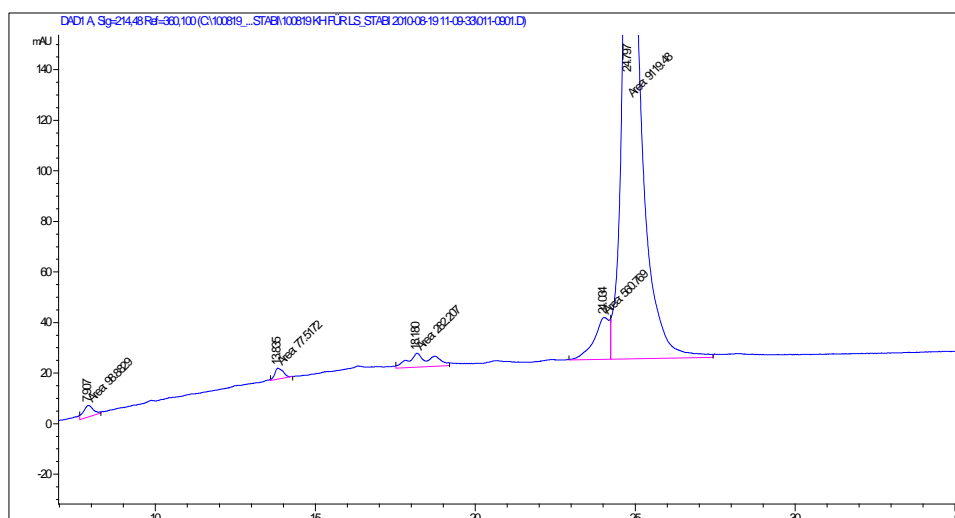
gradient ranged from 30% B to 100% B and flow rate from 0.5-2 ml/min. One experiment lasted 35min. A Poros R1/10 4.6x100 (Applied Biosystems, Darmstadt, Germany) RP column was used at ambient temperature for separation. A blank buffer chromatogram was subtracted from every sample to flatten and smooth the base line. The percentage of the related species was calculated from this subtracted system by determining the AUC of the observed peaks as a percentage of the total peak area. In Figure 33 a typical chromatogram is shown. Nature of the single peaks is explained in Kenley and Warne (Kenley and Warne 1994).



**Figure 33: Chromatogram of related species detection of IL-11 after RP-HPLC**

For RP-HPLC of oxidized IL-11 the eluent for the detection was the same as for the detection of related species. For detection of oxidized methionine the column temperature was set to 40 °C and the samples were diluted to a final concentration of 0.08 mg/ml. The amount of oxidized methionine was detected as area under the curve from the shoulder peak eluting shortly before the main protein peak (Yokota et al. 2000). The amount of oxidized methionine was detected as a peak shoulder of the protein peak as visualized in Figure 34.

Peak recovery, % oxidized species, and % oxidized methionine were calculated from the AUC of the chromatograms. For further details on the single degradation products separated with these methods, please refer to Fitzner (Fitzner 2003).



**Figure 34: Typical chromatogram of an RP-HPLC analysis for oxidized methionine species. The shoulder eluting before the main protein peak at 24 min represents the IL-11 with oxidized methionine. All other peaks are summed up to other oxidized species.**

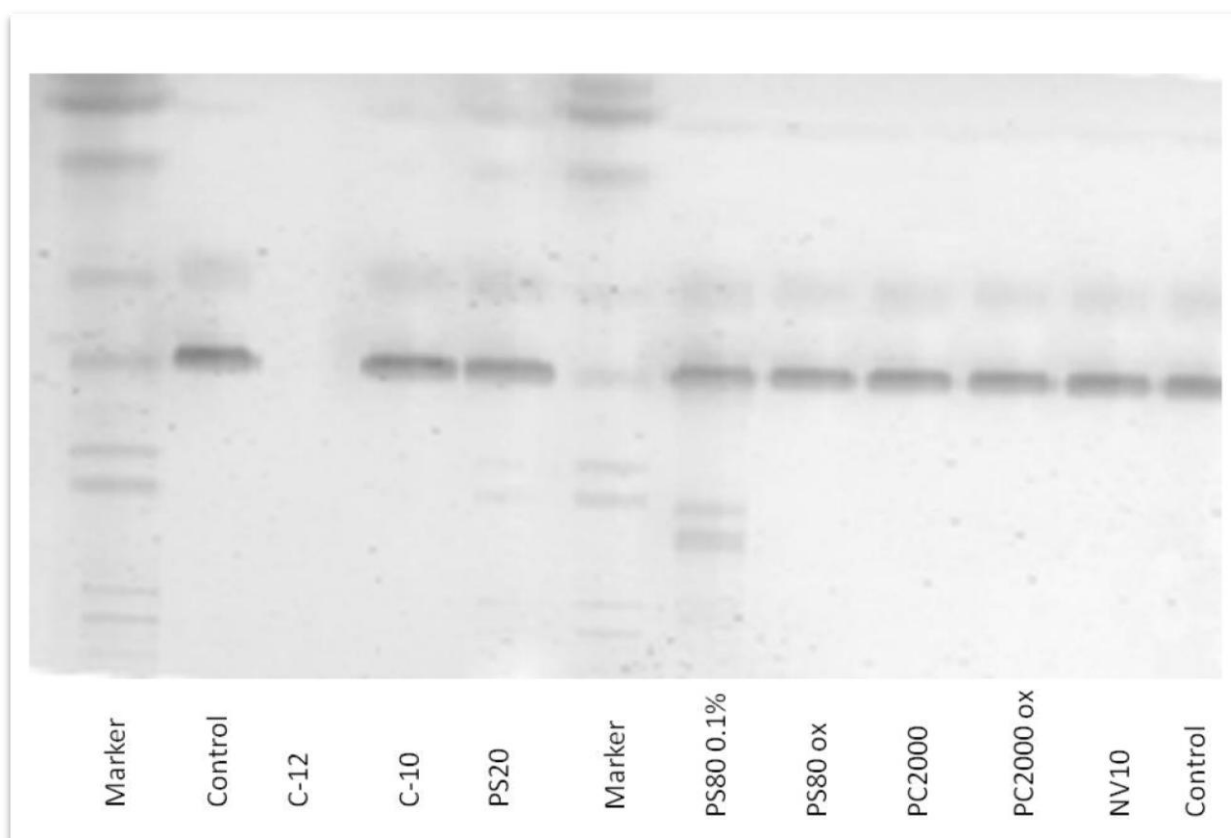
## 4.3. Results and Discussion

### 4.3.1. Stress Testing of IL-11

#### 4.3.1.1. Thermal Stress

All liquid IL-11 formulations revealed a significant loss in monomer content and high amounts of insoluble aggregates already after short periods of time at 50 °C. HPLC analysis revealed lower drug content in the soluble phase and most samples were visibly turbid as detected by the naked eye. UV measurements at 350 nm confirmed the increased turbidity.

SDS page was performed with the highest temperature samples. Analysis revealed the absence of covalent aggregate formation with Coomassie blue staining (Figure 35).

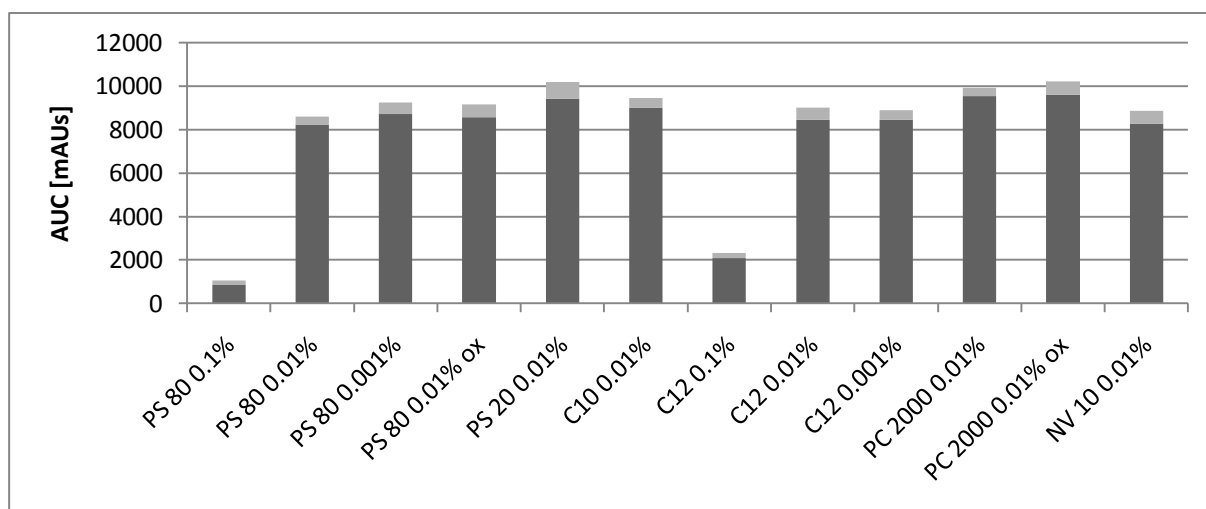


**Figure 35: SDS page of IL-11 formulations with different nonionic surfactants after storage 50 °C for 6 weeks (Coomassie Blue staining). Control corresponds to unstressed IL-11, C-12 and C-10 to trehalose laurate and capriate, respectively, PS80 0.1% to 0.1% polysorbate 80 (higher surfactant concentration), PS80 ox and PC 2000 ox to the preoxidized surfactant formulations.**

The only sample that showed differences to the control was the samples with the highest amount of PS 80 (PS80 0.1%) and to a slightly lesser extend the sample containing 0.01% PS 20. But contrary to the expected covalent dimers it could be found that the protein seems to form fragments. Extra bands for dimers are present in all samples, but to a very low extend. One can only speculate that the formulations with PC2000 perform better on this measure.

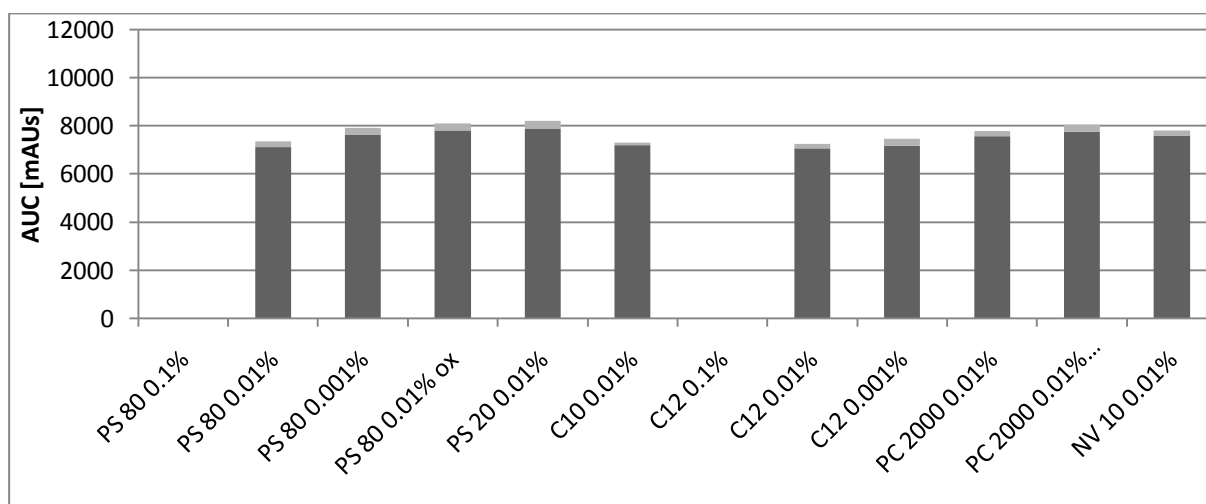
RP-HPLC data for related species detection are shown in Figure 36-7. For all surfactants the amount of total protein was decreased and the oxidized content of all samples increased. Differentiation between the effect of the different surfactant types is not feasible.





**Figure 36: IL-11 recovery in [mAU\*s] from samples stored at 40 °C for 2 weeks with different nonionic surfactants. Dark grey corresponds to native IL-11, light grey to related species. Start values 12100 mAU\*s and 600 mAU\*s.**

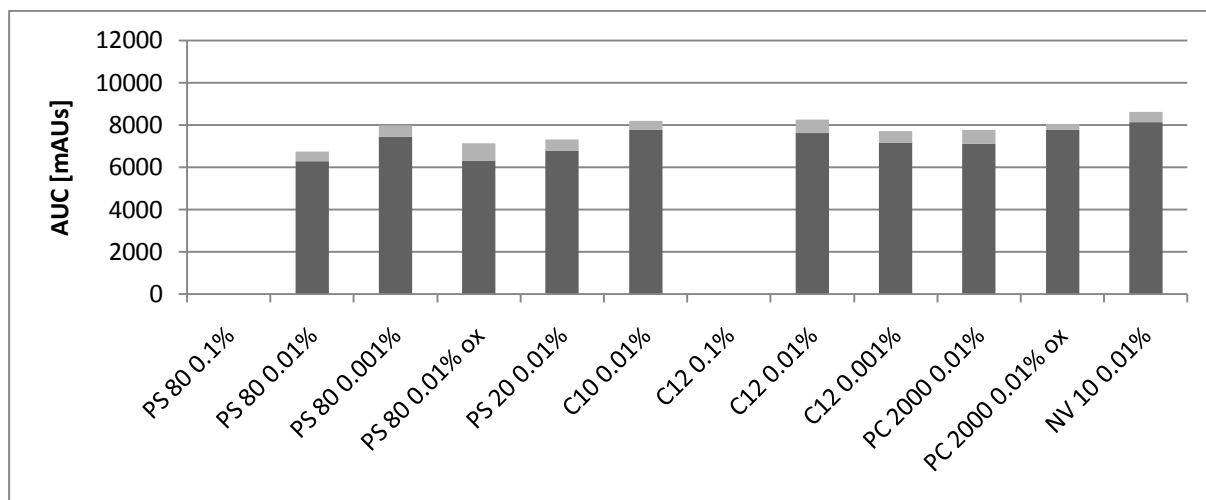
After a storage time of two weeks at 40 °C in the samples with high surfactant content (0.1% PS80 or C10, respectively) the protein was almost vanished. Highest protein recovery is gained from PC 2000 containing samples. Compared to the samples before storage, protein loss is about 15% for all samples at 0.01% or 0.001% surfactant concentration.



**Figure 37: IL-11 recovery in [mAU\*s] from samples stored at 40 °C for 6 weeks with different nonionic surfactants. Dark grey corresponds to native IL-11, light grey to related species. Start values 12100 mAU\*s and 600 mAU\*s.**

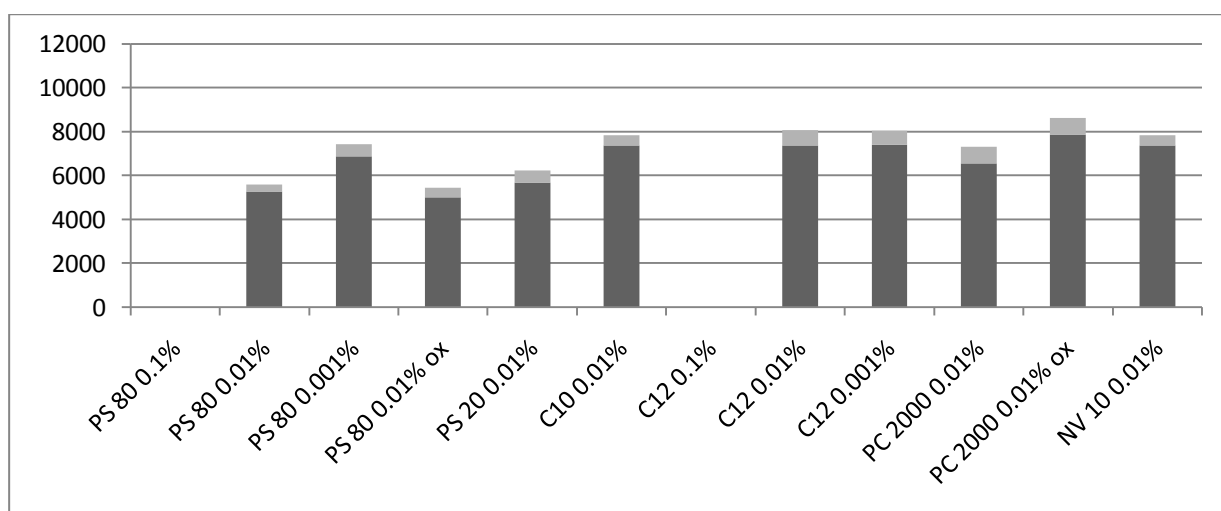
After six weeks at 40 °C the protein content decreased to a concentration of 75-80% of the original value, except for the high surfactant concentration formulations, which

were completely precipitated. At this sampling point the 0.01% and 0.001% polysorbates containing samples together with the PC 2000 containing sample showed the highest recoveries.



**Figure 38: IL-11 recovery in [mAU\*s] from samples stored at 50 °C for 2 weeks with different nonionic surfactants. Dark grey corresponds to native IL-11, light grey to related species. Start values 12100 mAU and 600 mAU.**

Samples stored at 50 °C showed stronger aggregation than the 40 °C samples. Only approximately 60-75% of the starting protein content could be recovered. Also at 40 °C the high surfactant concentrations caused complete precipitation of the protein.

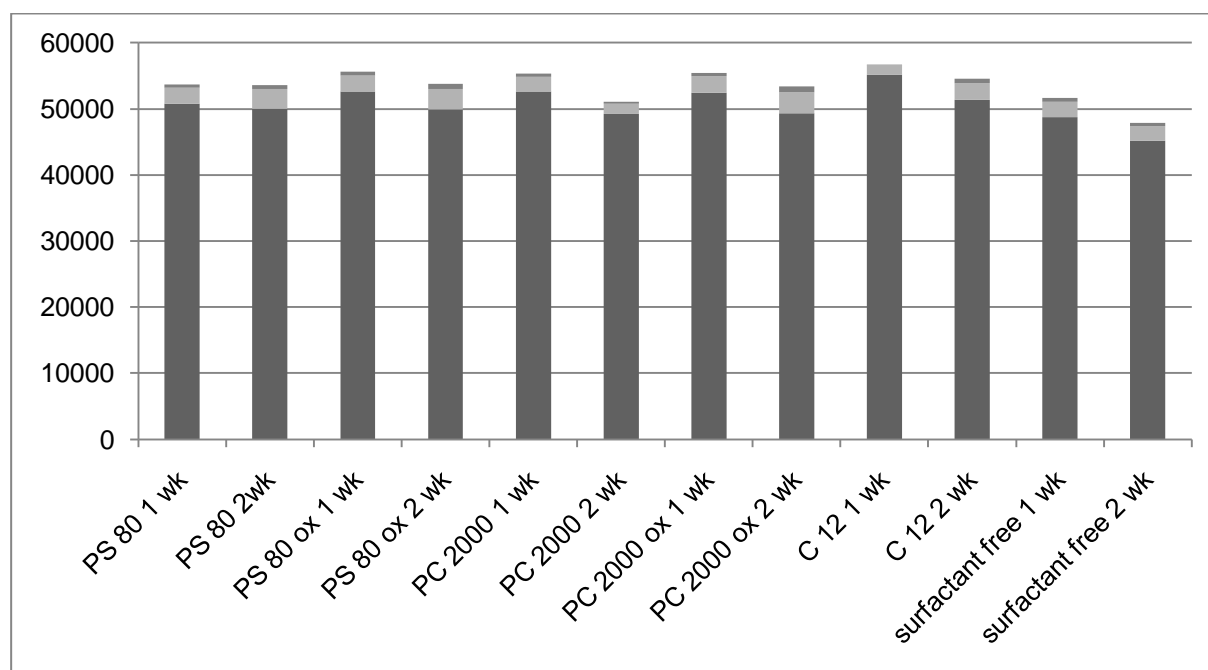


**Figure 39: IL-11 recovery in [mAU\*s] from samples stored at 50 °C for 6 weeks with different nonionic surfactants. Dark grey corresponds to native IL-11, light grey to related species.**

Here a clear trend can be seen that the PEG based surfactants score lower. The

same holds true for the samples stored for six weeks. After six weeks, recoveries for the sugar based surfactants stay at the two weeks level, but the polysorbate samples exhibit further decrease. Another observation is that the absolute amount of related species remains similar at 50 °C.

Thus one can say that a differentiation between surfactants is difficult. It seems at higher temperatures that the sugar based surfactants, especially PC 2000, are superior to the standard polysorbates. But recoveries were quite low and the analytics have only been performed with the soluble part of the protein. PS 80 at 0.1% showed the highest amounts of fragments in SDS-page. The experiments at 40 °C showed no clear trend. An explanation for this might be that polysorbates tend to destabilize the native state more and favor unfolding and hence precipitation of the protein at elevated temperatures. This would also explain, the almost complete precipitation of the high surfactant concentration formulations. Phenomena like this are already discussed in literature from the groups of Randolph for the intermediate molten globule state of hGH or Westh for the binding and unfolding behavior of SDS with cutinase from *Humicola insolens* (Bam et al. 1996; Nielsen et al. 2005).



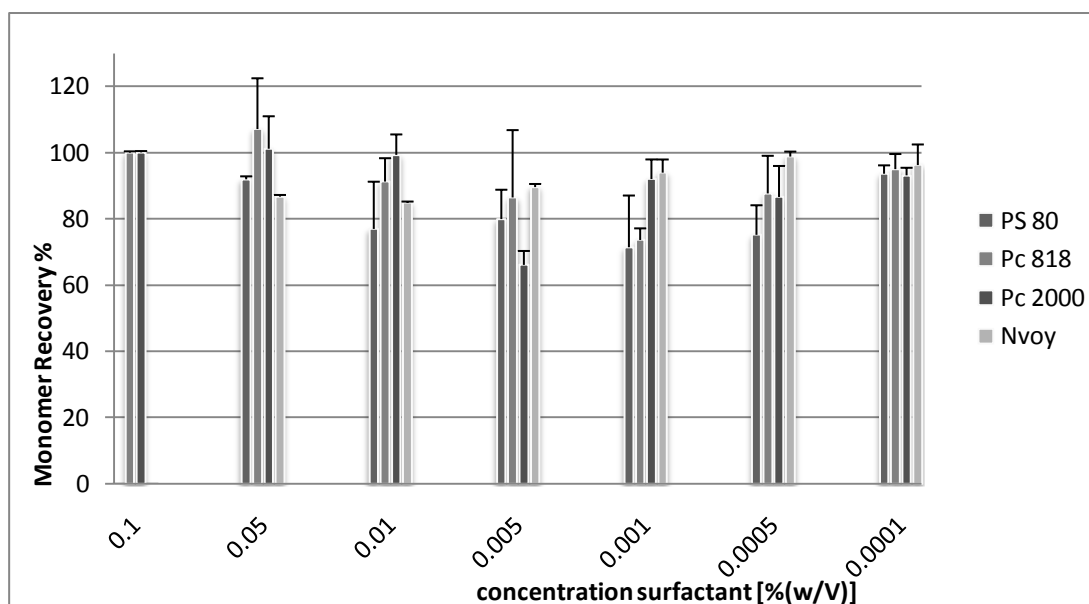
**Figure 40: RP-HPLC results in [mAU\*s] of lyophilized IL-11 with different nonionic surfactants at 0.01 % (w/V) after 1 week storage at 50 °C (Control values: 500 for oxidized species, 1200 for oxidized Methionine, and 60000 for the native protein peak). Dark grey represents unaltered IL-11, light grey oxidized methionine, and grey other oxidized species.**

After lyophilization, the protein formulations behaved all very similar. Figure 40 shows

exemplarily the RP-HPLC analytics for oxidized methionine. The same picture was found for 40 °C storage and for the analysis of related species. All surfactant formulations showed higher protein recovery than surfactant free II-11 formulation after two weeks, but further differentiation is not possible.

#### **4.3.1.2. Agitation Stress**

After shaking for 24 h no visible changes in protein content, particle counts or turbidity could be found in comparison to the surfactant free formulations. SEC recovery for all formulations was between  $97\pm3\%$  and  $99\pm2\%$ . After 172 h the highest surfactant concentrations showed the highest recovery of protein (Figure 41). PS 80 showed a trend to slightly lower recovery than the sugar based surfactants and NV 10 a trend to the best protein protection. Generally, these differences lie within the standard deviations and are hence insignificant. Interestingly, the lowest surfactant concentration of 0.0001% showed almost as good results as 0.1 and 0.05% surfactant. The fact that intermediate surfactant concentrations reveal detrimental results is already described in literature for IgG monoclonal antibodies (Mahler et al. 2005; Matheus 2006). The reason for this behavior remains unclear. Another observation is, similar to hGH, that the amount of dimers and trimers remains constant (data not shown). This is confirmed by the light obscuration and turbidity results. Unfolded protein seems to aggregate to larger particles in the subvisible and visible size range. For all surfactants the highest particle counts after 172 h are found at 0.005-0.0005%. The lowest concentration showed lower amounts of visible and subvisible particles. Yet, all concentrations were lower in particle counts and turbidity than the surfactant free formulation. As this experiment was performed in a very early stage of the thesis, trehalose fatty acid esters were not yet available, and were therefore not tested. Turbidity and light obscuration measurements were also performed with a corresponding outcome (Figure 43 and 44). The higher the surfactant concentration is the lower the turbidity of the formulations. The turning point of protein stability lies between 0.01% and 0.1% surfactant. Although the protein content is still decreased at 0.01%, no visible particles are formed. This concentration is comparable to the concentration needed for II-11 stability.

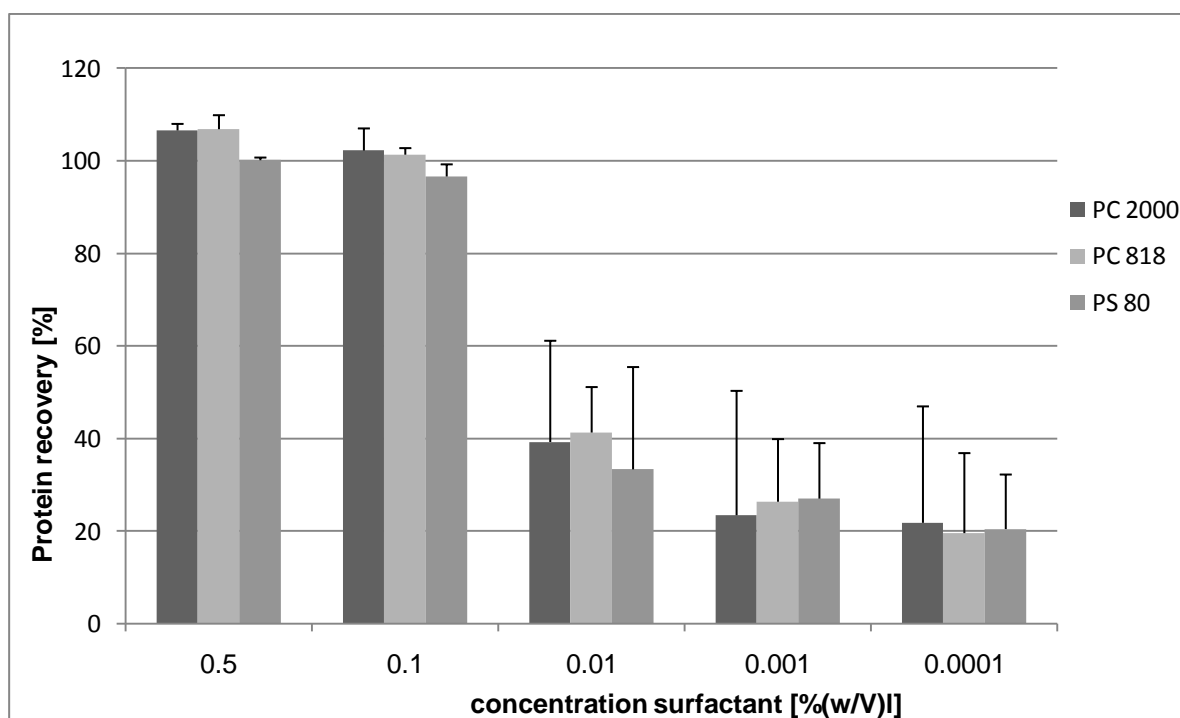


**Figure 41: II-11 recovery from SEC after shaking at various concentrations of nonionic surfactants (control 100%, surfactant free formulations 25±5%).**

#### 4.3.2. Stress Testing of hGH

##### 4.3.2.1. Agitation Stress

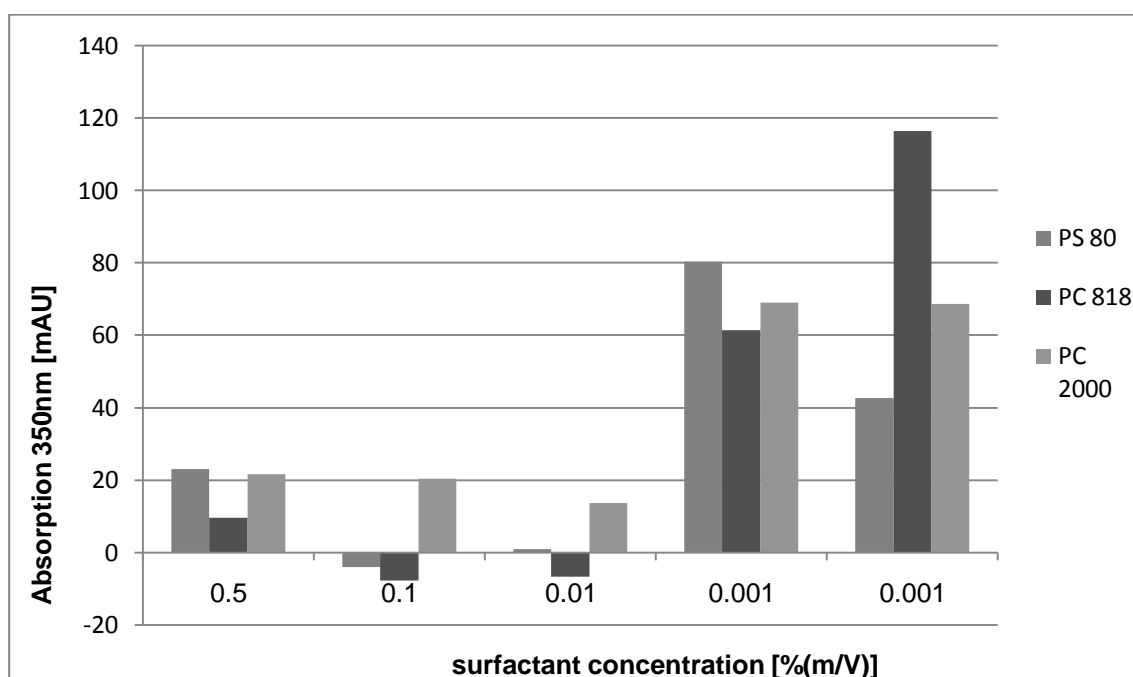
The results from shaking hGH in the presence of trehalose esters are also displayed in chapter 3.



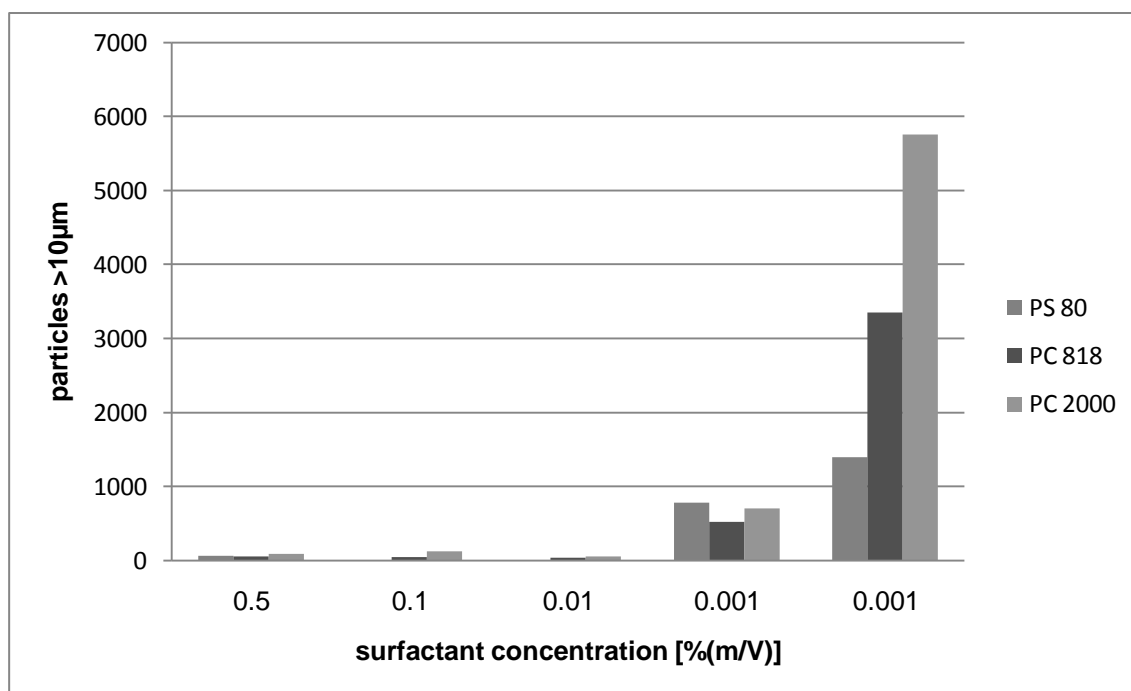
**Figure 42: Protein recovery from SEC results of hGH after shaking**

Similar results were obtained from shaking hGH together with technical mixtures of sugar based surfactants. Also the Plantacare products show comparable behavior to PS 80 in terms of protein protection. A concentration of 0.1% provided good protein protective effect. As a negative control, surfactant free hGH had a residual protein content of only 16±4%.

As the surfaces generated with the stress methods are more dynamic, higher surfactant content may be needed to cover all surfaces generated by shaking in the swinging mill. As proved by Friess et al. and also protected as patent, the head space is crucial for shaking stability of the protein (Friess et al. 2008; Kiese et al. 2008) We used a large headspace to let the fluid spread widely and build new and large surfaces to substantially stress the protein formulations.



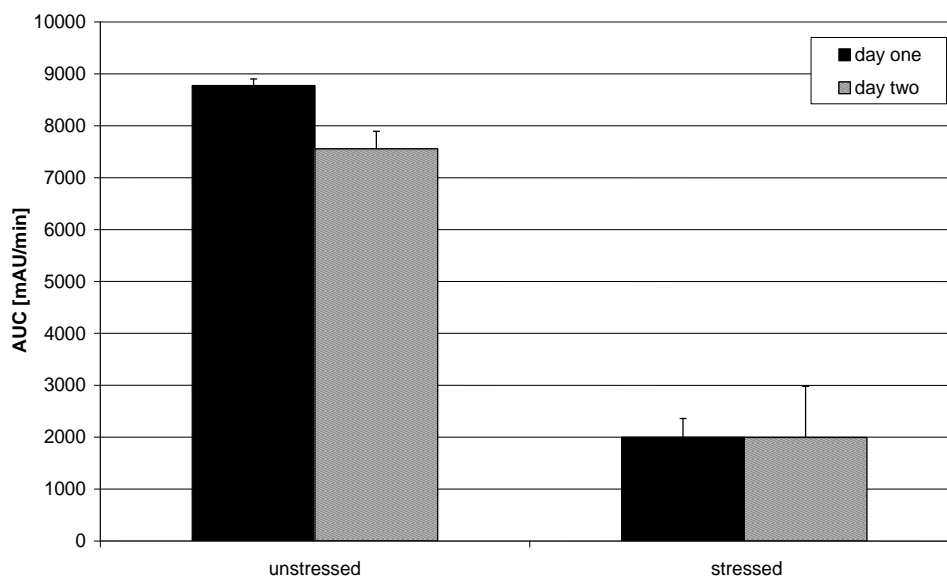
**Figure 43: Turbidity of hGH after shaking.**



**Figure 44: Particles >10 μm of hGH containing formulations after shaking.**

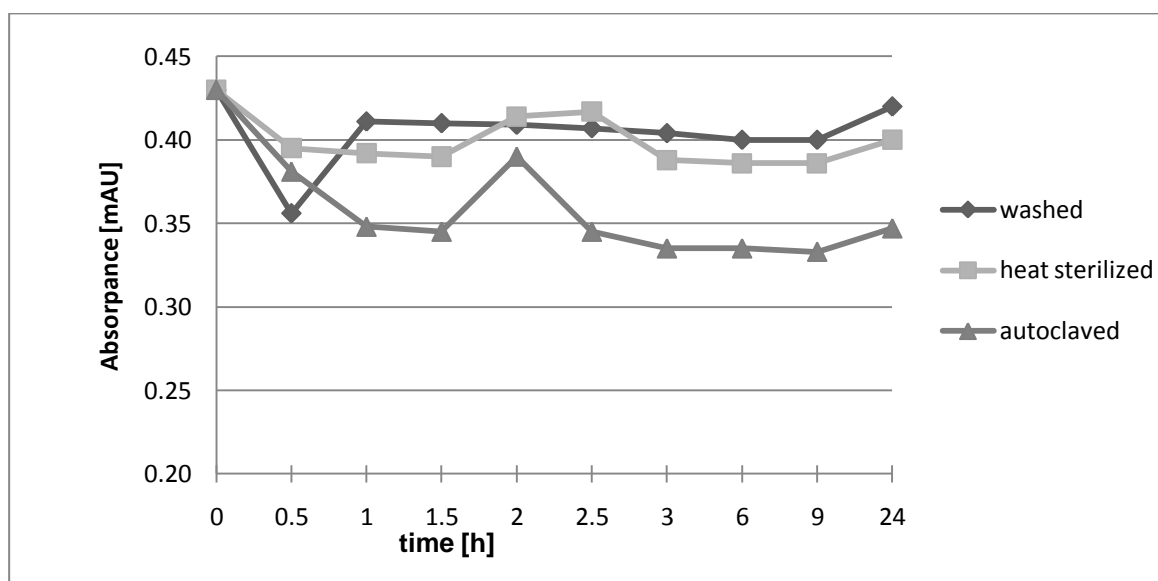
#### **4.3.2.2. Adsorption at Container Surfaces During Storage**

Proteins are eager to adsorb to different kinds of surfaces (Mathes 2010). Especially hydrophobic proteins tend to have reduced contents after simple storage due to adsorption to different container materials (Johnston 1996). During the shaking stress studies some problems occurred with the content of unstressed hGH as control standard. The protein content within the HPLC vial decreased up to 10% over 24 h when no surfactant was added (Figure 45). hGH is known for its tendency to adsorb on different kinds of liquid-solid interfaces, such as filter material (Maa and Hsu 1998), polystyrene surfaces (Katakam and Banga 1997), and different silicas (Buijs and Hlady 1997). Hence, we tested also for the ability of the surfactants to prevent adsorption to container walls.



**Figure 45: hGH adsorbs to HPLC vial wall at 4 °C rack temperature.**

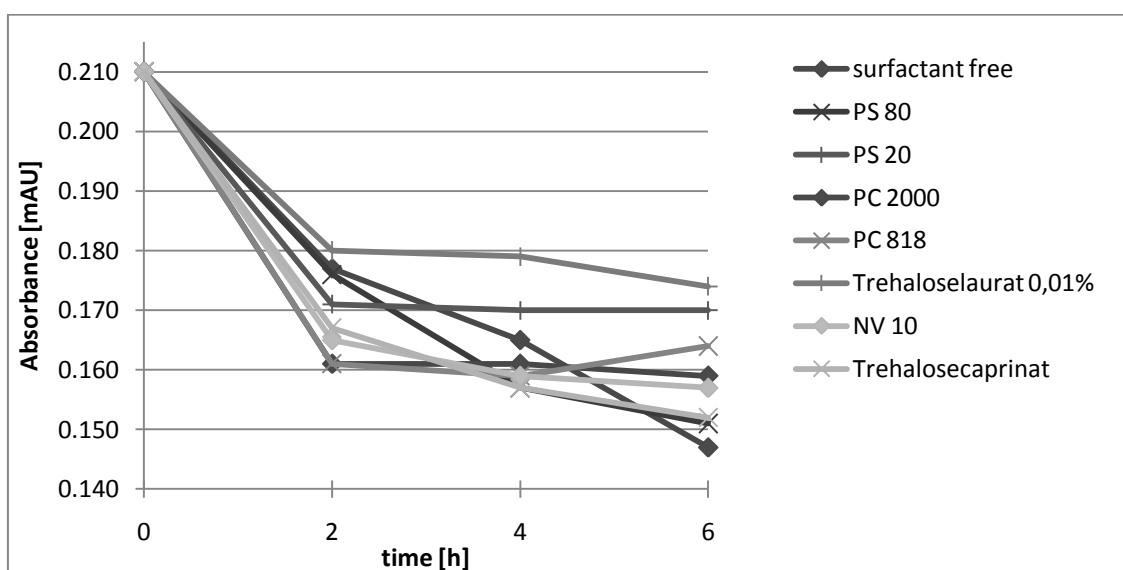
Figure 46 shows hGH adsorption of 0.5 mg/ml solution without surfactant to the container wall of a 2R-vial depending on the history of the container. The autoclaved containers demonstrated the highest rates of adsorption. These containers were further used to evaluate the ability of the surfactants to prevent adsorption. Autoclaving the glass vials made them more prone to adsorption. This is most probably due to the removal of ions from the glass surface or the higher energy intake depolarizing the glass surface (Burke et al. 1992).



**Figure 46: Adsorption of 0.5 mg/ml hGH to three differently prepared types of 2R-vials.**

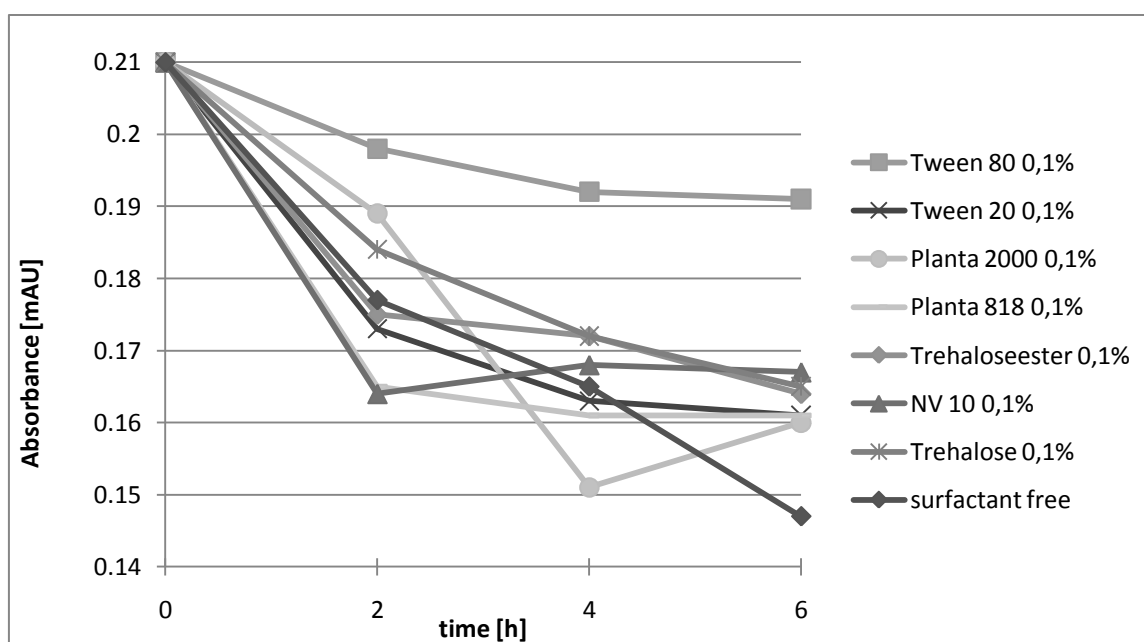


In Figure 47 the adsorption of protein with 0.01 % surfactant in the formulation is shown. The surfactants were not able to reduce adsorption.



**Figure 47: Protein adsorption of 0.2 mg/ml hGH in the presence of 0.01% nonionic surfactant.**

Figure 48 shows the same experiment with a higher surfactant concentration of 0.1%. In this case PS 80 shows the best result. However, no surfactant could effectively reduce adsorption although polysorbates are already described to prevent adsorption better at the applied concentrations (Duncan et al. 1995).



**Figure 48: Protein adsorption of 0.2 mg/ml hGH in the presence of 0.1% nonionic surfactant.**

In all cases approximately 20% loss of protein content was measured after 6 h incubation at ambient temperature. It appears that the best choice in these experiments would be 0.1% PS 80 as this was the only formulation where adsorption could be reduced significantly. However, none of the surfactants showed inferior behavior. Higher amounts of surfactant (1% or higher) might have increased protein contents. Mathes came to a similar conclusion (Mathes 2010). Surfactants can only to a certain extent prevent the adsorption to containers. This is depending on the surface activity and hydrophobicity of the protein, surface tension of the surfactant and several other factors such as unfolding at the interface (Goddard 2002). In this case hGH seems to have a high affinity to the interface which can be adequately addressed with the surfactants.

#### **4.4. Conclusions**

In this chapter II-11 and hGH were exposed to different kinds of stress, thermal, kinetic and interfacial. In order to eliminate the aggregate formation of II-11 during shaking experiments 0.1% surfactant was necessary. This was already too much surfactant for the storage experiments at elevated temperatures as 0.01% performed sufficiently well and showed no differences to 0.001%.

For hGH 0.1% surfactant was not enough to prevent adsorption at container walls. For shaking stress full protection of the protein could be achieved by addition of 0.1% of all applied surfactants. The stresses applied on both proteins in this study could be reduced by the addition of sugar based surfactants. But for different experimental set-ups it was necessary to apply different concentrations of surfactants to prevent proteins from aggregation and adsorption. Other groups also applied agitation stress as predictor for formulation stability and came up with different amounts of surfactant needed for protein stabilization. Recombinant human Interferon-gamma can be stabilized with 0.03% PS 20 (Webb et al. 2002), 0.004% PS 20 stabilizes PEG-GCSF but has inverse effects when stored at higher temperatures (Treuheit et al. 2002), Albutropin can be stabilized protein concentration dependent by PS 20 and PS 80 at a molar ratio greater than 10:1 and 9:1, respectively (Chou et al. 2005) For hGH ratios of 4-5:1 surfactant molecules per protein molecule are described to prevent

aggregation (Bam et al. 1998). For an IgG1 antibody the concentration of 0.01% was not sufficient to fully prevent denaturation against shaking (Mahler et al. 2005). In another shaking experiment 0.1% PS 80 was sufficient to stabilize II-2 against shaking but also destabilized when stored over longer periods (Wang et al. 2008), which is opposite to our findings that more surfactant is needed against shaking stress, than against storage stress.

It could be shown that sugar based surfactants can be applied in the same concentration range as polysorbates to achieve similar results in protein stabilization for all stress methods applied. In the different models the differences between the Plantacare products, the trehalose fatty acid esters and, when tested, NV 10 to polysorbates are minor. For storage stress of II-11 NV 10 showed the best performance, in the adsorption experiments PS 80 of hGH showed the best recovery, and when hGH is shaken the trehalose fatty acid esters were the most promising candidates. Also the concentration range needed for protein stabilization is similar to what can be found in literature. The required concentration of surfactant is strongly protein and model dependent and in this case was between 0.001% and >0.1%. Influencing factors for the amounts needed to prevent protein from aggregation at an interface might be the surface charge (isoelectric point) and amount of hydrophobic amino acids on the protein surface (Ramsden and Prenosil 1994; Larsericsdotter et al. 2001), the unfolding energy and melting temperature (Andersen Kell et al. 2009). Type of buffer and pH value also have an impact on the amount of surfactant needed for stabilization (Ruiz et al. 2005), or the tendency to form aggregates as “planned by nature”, e.g. insulin dimers and hexamers (Brange et al. 1997).

This makes the sugar based surfactants an attractive alternative to polysorbates. Unfortunately, the storage stress model could not prove an advantage over polysorbate, even when higher peroxide contents were produced prior by radiation of the surfactant samples. This should be tested again with another molecule that is more prone to oxidation.

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# 5. Studies on the Binding Interactions between Nonionic Surfactants and Proteins in Liquid Formulations

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## 5.1. Introduction

Surfactants are frequently added stabilizers in protein drug formulations. They are used because they can on the one hand reduce protein adsorption to container surfaces (Hawe and Friess 2008). On the other hand they can help to avoid protein aggregation in solution, specifically when aggregation is interface stress induced (Charman et al. 1993; Bam et al. 1998). It is speculated on the mechanism how the surfactants achieve this effect. There might be the surface competition that leads to physical stable formulations. But also specific interaction was discussed. E.g. coverage of hydrophobic sites on the protein resulting in reduced interaction with interfaces. It has to be kept in mind that hydrophobic interaction is just one minor fraction of the attractive forces.

At the same time strong interaction of surfactant with protein may shift the unfolding equilibrium towards the unfolded state, if unfolding is the rate limiting step (Nielsen et al. 2000). The exposed hydrophobic sites and larger surface of the protein provide the energetically basis that the unfolded state is more stabilized than the native. Theories whether surfactants can act as artificial chaperones also exist, but for an active folding process, the measured enthalpies seem to be too low (Bhattacharyya and Das 1999; Garidel et al. 2009). To shed further light into this complex situation we studied the interaction of proteins with surfactants focusing on isothermal titration calorimetry, supported by NMR studies of selected samples.

Isothermal Titration Calorimetry (ITC) is one of the major tools for the determination of thermodynamics in protein science (Liang 2008), especially in protein binding energetics (Leavitt and Freire 2001). The main outcome of ITC-measurements is a binding isotherm reflecting interaction as a function of the amount of injected

substance. If the data can be modeled, ITC provides information on free energy- , enthalpy- entropy- and stoichiometry of binding in one single experiment. By testing at different temperatures the heat capacity change can be tested as well (Simon et al. 2002). Unfortunately determining binding isotherms in a single experiment is only feasible when strong interactions occur or when protein and/or ligand concentrations are extremely high (Chou et al. 2005).

Protein surfactant interactions studied via ITC are described by McAuley et al. who found weak, possibly hydrophobic interactions between lactate dehydrogenase and polysorbates (McAuley et al. 2009) or by Garidel et al. who analyzed the interaction between polysorbates and immunoglobulines and bovine serum albumin (BSA) (Garidel et al. 2009). We disregarded the frequently applied BSA from our studies as this protein might show good binding enthalpies but an not serve as a good model as this interaction is specific to the fatty acid binding pocket of BSA (4) and not transferable to other protein drugs.

A good example for strong protein-surfactant interactions is the lysozyme-sodium dodecylsulfate-system (Jones 1992) as the attracting forces are driven by electrostatic interactions and not only by hydrophobic ones. However, these interactions are not transferable into pharmaceutical formulation work, as for hemocompatibility reasons nonionic surfactants are applied and these cannot interact via attractive charge-charge attractions.

We used ITC to determine the nonspecific interaction between nonionic surfactants and model proteins. As the expected interaction in this study are very weak compared to the enthalpies studied typically in biophysical studies, binding was determined indirectly by analyzing the shift of the critical micelle concentration (CMC) with the addition of proteins in the sample cell (Otzen et al (Andersen Kell et al. 2008)). The shift of the thermogram with increasing protein concentration renders information on the binding stoichiometry.

Further information on direct interaction between proteins and surfactants can in general be gained from Nuclear Magnetic Resonance (NMR) spectroscopy. With the help of two dimensional Nuclear Overhauser Enhancement spectroscopy (NOESY) cross-peaks arising from spins from moieties on different molecules that are spatially close can be identified. This allows determination of interactions and provides information on the site of interaction. The average maximum distance between two



spins that can be detected via NOESY is about 5.5 Å. Ulvenlund et al. (Sjoegren et al. 2005) studied the interaction between polypeptides and alkyl polyglycosides and proved that folding to beta-sheets was improved with the addition of surfactants. Matilainen et al. (Matilainen et al. 2008) used NOESY to determine the interaction between cyclodextrins and glucagon and found inclusion complexes in acidic solution. Wong et al. (Wymore and Wong 1999) applied NOESY to determine the molecular dynamics and the temperature dependence of the partitioning coefficient of substance P peptides in sodium dodecylsulfate (SDS) systems. But to our knowledge the interaction between parenterally applied proteins and surfactants has not yet been studied by NOESY.

The outcome of interaction of three surfactants with three different selected proteins was investigated. Polysorbate 80 (PS 80) was selected as one of the most frequently used surfactants in protein formulation acting as a benchmark. One drawback of PS 80 is its potential to reduce long term stability of formulations due to enhanced oxidation rates of the protein. This adverse effect was related to the presence of side products (Wang et al. 2008). Therefore, additionally two sugar- and fatty alcohol-based nonionic surfactants, Plantacare 818 UP (PC 818) and Plantacare 2000 UP (PC 2000) were implemented in the study (Kuehn and Neubert 2004). They are not yet approved for invasive application but might become an alternative to the established surfactants in the future. They show similar surface behavior to polysorbates (De Grip and Bovee-Geurts 1979; Aveyard et al. 1998) and are environmentally friendly (Garcia et al. 1997; von Rybinski and Hill 1998).

Three proteins were used in this study.  $\alpha$ -Lactalbumin ( $\alpha$ -LA) is known to interact with SDS when studied in the calcium free Apoform (Otzen et al. 2008). The calcium bound Holoform interactions were expected to be smaller as a certain area on the protein's surface is then covered with  $\text{Ca}^{2+}$ -ions. Human and bovine  $\alpha$ -LA are known to act as a cytotoxic agent in the therapy of papilloma, cancer cells or actinic keratosis (Ruffet et al. 2008; Axelsen et al. 2009). Interleukin-11 (IL-11), which is applied to improve platelet recovery after chemotherapy induced thrombocytopenia (Bhatia et al. 2007) was used as a pharmaceutical relevant example as aggregation in formulation can be reduced with the help of surfactants (Page et al. 2000). Furthermore it is oxidation sensitive and consequently the combination with PC 818 / PC 2000 could potentially be of special interest (Yokota et al. 2000). For NOESY

experiments LA and human Growth Hormone (hGH) were applied.

## **5.1. Material and Methods**

### **5.1.1. Material**

PS 80 and bovine  $\alpha$ -LA >95 % calcium depleted was obtained from Sigma-Aldrich (Darmstadt, Germany). The sugar based nonionic surfactants PC 818 and PC 2000 were kindly donated by Cognis KG (Düsseldorf, Germany). Recombinant Interleukin-11 (IL-11) was kindly donated by Wyeth Pharmaceuticals (Andover, USA). Human Growth Hormone (hGH) was kindly donated by Bernina Plus & HDS (Martinsried, Germany).

All buffer salts and pyrene were of analytical grade. Highly purified water was taken from a Millipore system. All materials were used as purchased and not further purified.

In the ITC experiments buffers were used as follows: As a standard buffer 50 mM Sodium EDTA for the apoform of  $\alpha$ -LA was used. For the holoform and for IL-11 a buffer containing 20 mM  $\text{CaCl}_2$  and 20 mM 3-(N-morpholino)propanesulfonic acid was used as standard buffer.

### **5.1.2. Tensiometry**

Determination of CMC via surface tension measurement was performed with a K-100 MK2 tensiometer (Krüss, Hamburg, Germany) in a custom built conical vessel with a platinum-iridium plate and a 765 Dosimat (Metrohm, Filderstadt, Germany). The CMC was calculated using LabDesk 3.1 software (Krüss, Hamburg, Germany).

### **5.1.3. Pyrene Interaction Fluorescence Spectroscopy**

Pyrene fluorescence was applied to alternatively determine the CMC according to (Kalyanasundaram and Thomas 1977). Fluorescence spectra were recorded and the

$I_1/I_3$  ratio was calculated, where  $I_1$  is the emission at 373 nm and  $I_3$  is the emission at 393 nm. Excitation wavelength was 335 nm. The CMC was defined as the surfactant concentration at which the  $I_1/I_3$  ratio becomes smaller than <1 as the fluorescent dye migrates into the micelle core protected from water molecules. Experiments were performed in Quartz cuvettes with 10 mm pathway using a Cary Eclipse spectrofluorimeter (Varian, Palo Alto, USA). 10  $\mu$ l of a 200 mM pyrene solution in ethanol was added to 1990  $\mu$ l surfactant solution (N=3).

#### **5.1.4. Isothermal Titration Calorimetry**

ITC measurements were performed with a VP-ITC calorimeter (Microcal Inc., Northampton, USA). The obtained heat signals were integrated using the Origin software supplied by Microcal Inc. After filling of the sample solution into the sample cell and positioning the stirring syringe, the machine was equilibrated at 5 °C, 25 °C and 37 °C for at least two hours. After equilibration additional 120 sec of initial delay were set before the first injection started. Usually 35 injections of 8  $\mu$ l with an injection speed of 0.5  $\mu$ l/sec were added into the sample cell. Between every injection the heat flow was allowed to equilibrate back to baseline. The reference power was set to 25  $\mu$ Cal/sec for the experiments.

In a typical experiment the reference cell was filled with water, the sample cell contained either buffer or protein solution. The sample cell was titrated with aliquots of nonionic surfactants. Usually, at first the surfactant concentration was set for micelles to occur around the 10<sup>th</sup> titration step in pure buffer. The same titration was repeated with protein solution in the sample cell. Pure buffer titration into the protein solution was performed as a blank control.

#### **5.1.5. Nuclear Magnetic Resonance (NMR) Spectroscopy**

NMR-experiments were performed with a JEOL 500+spectrometer (JEOL, Eching, Germany). For these experiments hGH was dialyzed against distilled water, lyophilized and reconstituted in D<sub>2</sub>O at 35 mg/ml under nitrogen atmosphere.  $\alpha$ -LA powder was reconstituted in D<sub>2</sub>O under nitrogen atmosphere and PC 818 as bulk substance was lyophilized and reconstituted under nitrogen atmosphere to 15 mg/ml

to assure that micelles are present in a mixture of surfactant and protein. Before 2D experiments one dimensional  $^1\text{H}$ -NMR-spectra were recorded to determine the chemical shifts of the aromatic amino acids of the proteins and of the hydrophobic surfactant chain ( $-\text{CH}_2-$  and  $-\text{CH}_3$ ). Consequently two-dimensional NOE-spectra were recorded to determine cross peaks of the above mentioned entities.

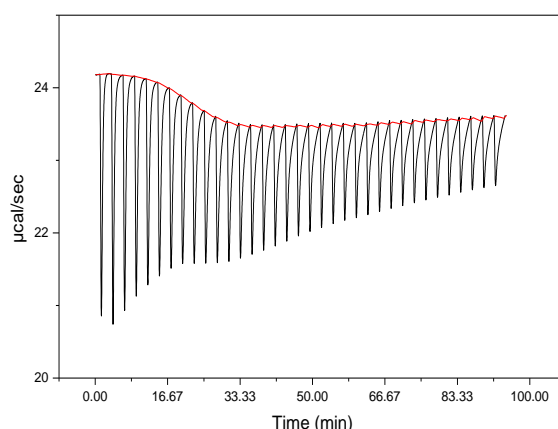
## 5.2. Results and Discussion

### 5.2.1. CMC Determination

In order to analyze protein induced changes in the CMC of the surfactants by ITC experiments it was at first necessary to determine the CMC by other techniques for correlation with the events detected in ITC. The experiments were performed in water and the applied buffers and could not reveal major changes. PS 80 has a CMC of 0.02 mg/ml or 0.015mM. For both alkyl polyglycosides micellization initiates at marginally higher concentration. PC 2000 exhibits a CMC of 0.04 mg/ml or 0.08 mM and PC 818 has a CMC of 0.02 mg/ml or 0.04 mM. The values are in good accordance with literature (Wan and Lee 1974; Boyd et al. 2000). Hill et al. describe this CMC as typical for nonionic surfactant mixtures, especially sugar based surfactant mixtures (von Rybinski and Hill 1998).

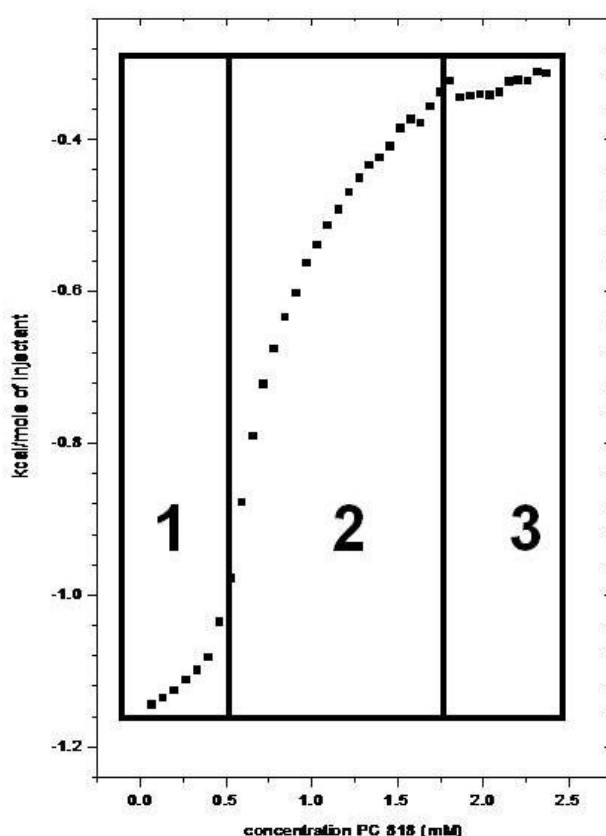
### 5.2.2. ITC Measurements

With the help of CMC-experiments ITC-experiments were planned so that after

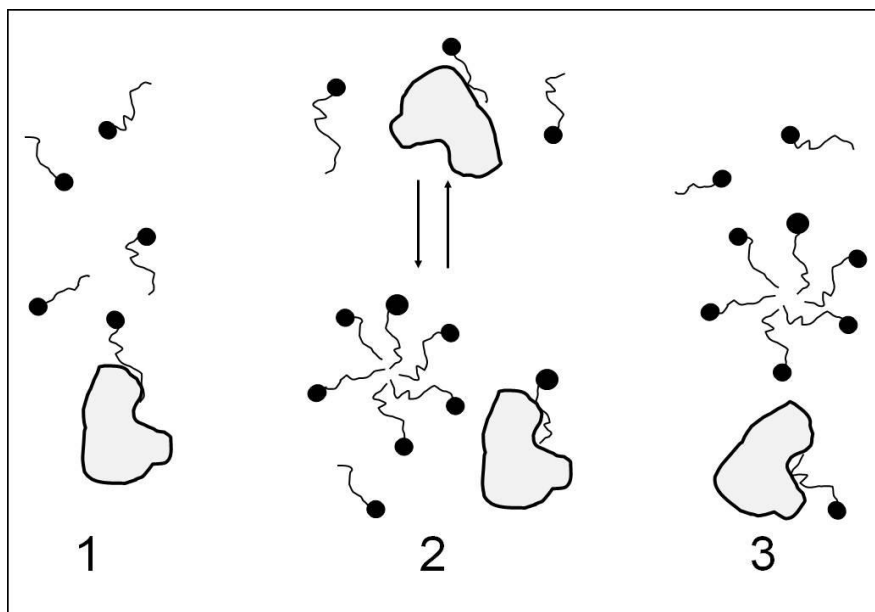


**Figure 49: Raw data of an ITC experiment**

approximately 10 injections the end of complete demicellization should be detectable. Raw data (Figure 49) of the experiments are transformed into thermograms (Figure 50). The experiment can be separated in three phases: 1 and 2 in Figure 50 (as represented as enthalpy per mol injected surfactant) and 3 (schematic picture of the systems at each stage of the experiment). The three phases are also drawn as a figurative sketch in Figure 51. After complete interaction of the protein and the surfactant the CMC is exceeded and phase 3 will be a straight line that only represents heats of dilution. With a cell volume of ~1.4 ml and an injection volume of 5  $\mu$ l per injection 50  $\mu$ l should contain enough surfactant that the first micelles stay intact.

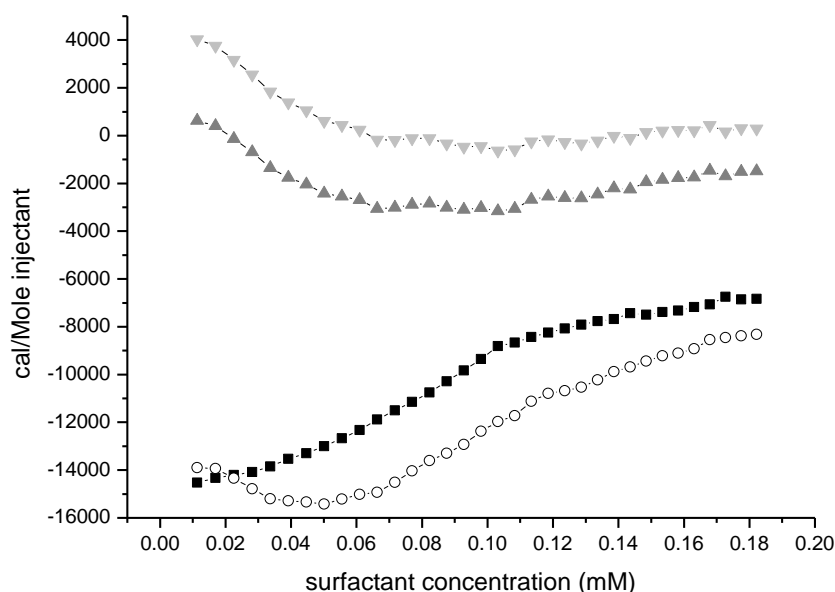


**Figure 50: Typical thermogram of an ITC experiment. Phase 1 represents the titration steps where all micelles are disintegrated in the sample cell. Phase 2 is the represents the CMC as at this concentration some micelles stay intact. In Phase 3 only dilution of intact micelles occur due to concentration differences between syringe and sample cell. When the surfactant interacts with the protein, micellization occurs later, as some molecules are bound to the protein's surface and not available for micelle formation.**



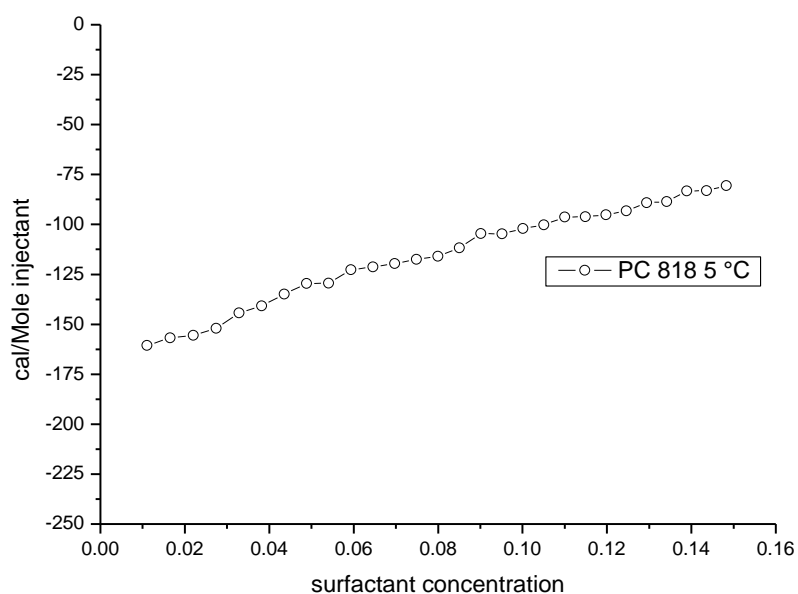
**Figure 51: Graphical demonstration of the three phases occurring during an ITC experiment.**

Consequently CMC was exceeded and the endpoint could be detected as the interval where no or only minor changes in the thermogram occur (see 3 in Figure 50). Figure 52 explains the data processing of the thermograms into the data that are discussed below. The raw data of a titration of surfactant into buffer (blank) and surfactant into protein (sample) were tested. Then a blank subtraction and a baseline parallelizing were performed to diminish background noise as recommended by the International Union of Pure and Applied Chemistry (Schwarz et al. 2008). If the x-axis of the ITC thermograms represents the protein to surfactant ratio all peaks should be similar in their maximum location, if there is specific binding. Another way of displaying the data is to plot against surfactant concentration. In this case, graphs which show a similar shape in binding enthalpies and onset of micellization proportionally increasing with protein concentration (Andersen Kell et al. 2008) demonstrate surfactant-related events in a protein environment (Nielsen et al. 2005). It turned out for our experiments that plotting against surfactant concentration led to better correlation than using the surfactant/protein ratio.

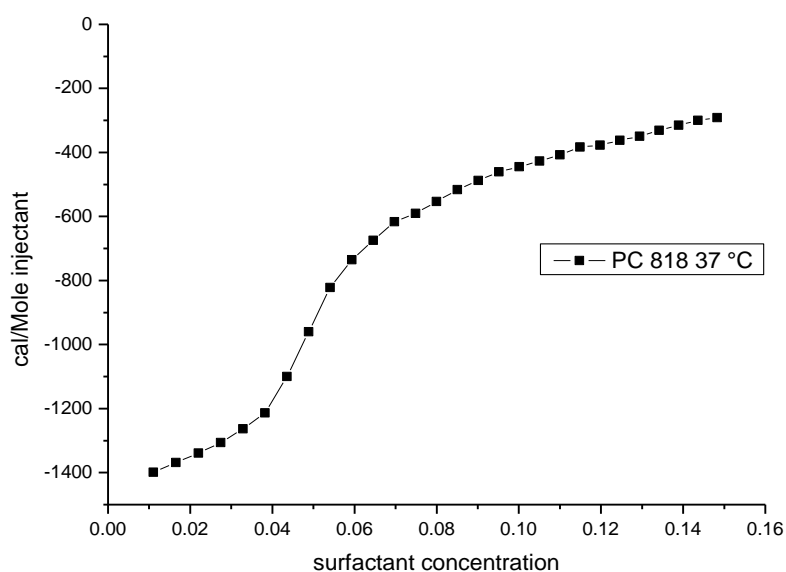


**Figure 52: Data processing.** The two experiments surfactant into pure buffer (white circles) and surfactant into protein solution (black squares) are subtracted from each other (inverted light grey triangles) and parallelized. Additionally a correction to the baseline is done for better comparability of the experiments (dark grey triangles).

The first experiment was carried out with PS 80 Na-EDTA buffer at a concentration of 50 mg/l at 25 °C. The run showed remarkably low enthalpies and reflected only a dilution thermogram (data not shown). Also in a broader concentration range was a region in which a clear transition from demicellization to dilution of micelles upon contact with the sample material (in this case buffer) occurs. However, this transition could not be identified. Obviously, the energy of demicellization and micelle formation respectively is so small at room temperature that the event cannot be detected. Additionally, neither at 5 °C, nor at 37 °C the transition phase (phase 2) could be determined. Thus, in the pharmaceutically relevant temperature demicellization of PS 80 renders only minimal enthalpy, which is smaller than the dilution enthalpy and below the sensitivity of the calorimeter.

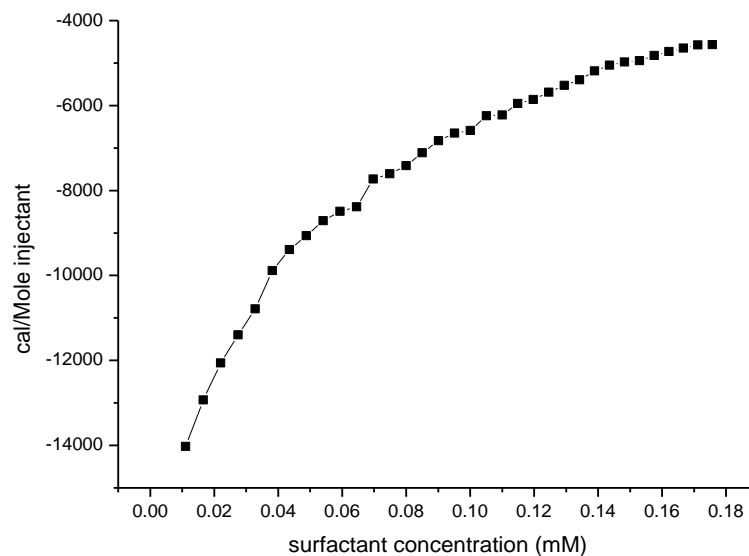


**Figure 53: PC 818 into buffer at 5 °C. The parallel lines are for visualization of the differences in enthalpy before and after the CMC.**



**Figure 54: PC 818 into buffer at 37 °C. The parallel lines are for visualization of the differences in enthalpy before and after the CMC.**



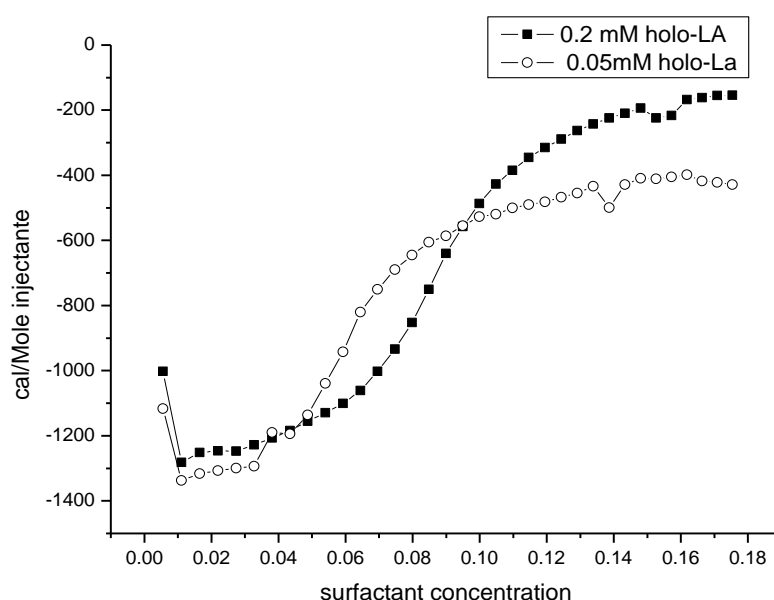


**Figure 55: PC 818 into 0.01  $\mu$ M Apo- $\alpha$ -LA at 5 °C.**

For PC 818, the experiments carried out at 5 mg/mL at 25 °C as well did not allow to follow the process of the demicellization. However, a transition from phase 1 to phases 2 and 3, respectively, could be observed at 5 °C and even more pronounced enthalpy change occurred at 37 °C (see Figure 53 and Figure 54). The demicellization process is driven by entropy and is strongly dependent on the temperature as it is characteristic for hydrophobic interactions (Nielsen et al. 2000). After reproducing the transition and optimization of the injection procedure with respect to injection time and spacing between two injections (300 s) 0.1  $\mu$ M calcium free  $\alpha$ -LA was added into the EDTA containing buffer providing the Apoform of  $\alpha$ -LA. Injection of PC 818 into Apo- $\alpha$ -LA solution resulted a marked exothermic interaction at 5 °C (Figure 55), although the pure demicellization process was hardly exothermic at 5 °C (Figure 53). Otzen et al. found a similar effect for Apo- $\alpha$ -LA with other nonionic surfactants (Otzen et al. 2008). They claimed that already low amounts of surfactant are sufficient to destabilize the protein in its calcium-free form. The low melting temperature of Apo- $\alpha$ -LA of 20-32 °C indicates that unfolding might occur already at 5 °C (Hiraoka et al. 1980; Stuart et al. 1986; Vanderheeren and Hanssens 1994). Thus the interaction between  $\alpha$ -LA in its apoform and the alkyl polyglycosid-surfactant can clearly be demonstrated and results in drastic destabilization of the protein. The results also demonstrates that the presence of surfactant may pull the equilibrium of unfolding for unfolding prone proteins towards the non-native state due

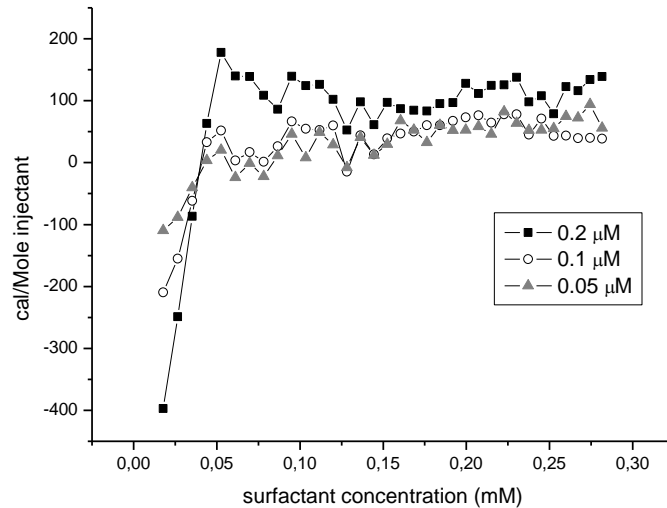
to direct interaction. Due to the low melting temperature the experiments with the apoform of  $\alpha$ -LA at 25 °C and 37 °C were not performed.

After changing the buffer to MOPS+CaCl<sub>2</sub> (20 mM)  $\alpha$ -LA is present in the Holo-form. Three concentrations of Holo- $\alpha$ -LA, 0.05  $\mu$ M, and 0.2  $\mu$ M were titrated with PC818 at an injecting concentration of 5 mg/mL at 5 °C. A change in the thermograms compared to the blank run could be detected with the presence of Holo- $\alpha$ -LA in the sample cell. This is indicating interactions between excipient and protein. This interaction was found to level off at 0.1 mM for both protein concentrations (Figure 56). The interaction was exothermic at a very low level compared to the apoform experiments pointing out that the interaction do probably not result in unfolding of the protein.

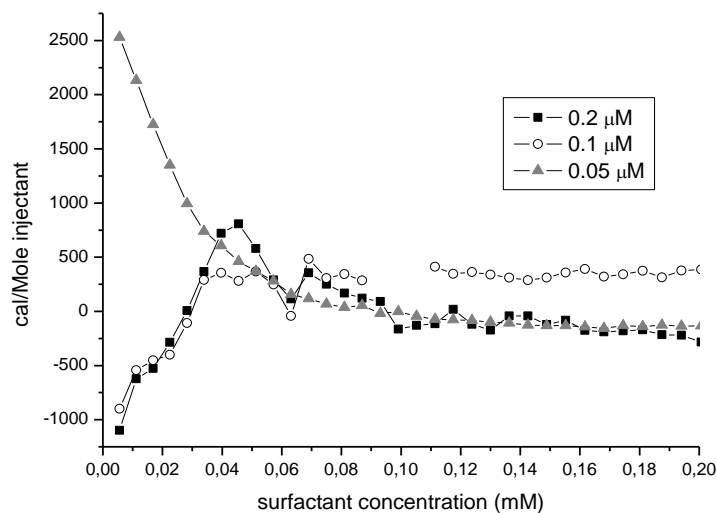


**Figure 56: PC 818 into Holo- $\alpha$ -LA at 5 °C at 0.05 mM (white circles) and 0.2 mM (black squares) protein concentration.**

For PC 2000 as titrant again the transition around CMC at 0.075 mM was in accordance to the CMC values determined via fluorescence spectroscopy and surface tension measurements. Finally, interaction of PC 2000 with Holo- $\alpha$ -LA was studied. At 5 °C PC 2000 also exhibited an interaction with Holo- $\alpha$ -LA up to a concentration of 0.06 mM surfactant (Figure 57).



**Figure 57: PC 2000 into Holo- $\alpha$ -LA at 5 °C at 0.05 mM (grey triangles), 0.1mM (white circles) and 0.2 mM (black squares) protein concentration.**



**Figure 58: PC 2000 into Holo- $\alpha$ -LA at 37 °C at 0.05 mM (grey triangles), 0.1mM (white circles) and 0.2 mM (black squares) protein concentration.**

At 37 °C a similar change in the thermograms was detectable up to approx. 0.6 mM surfactant for concentrations of 0.05, 0.1 and 0.2  $\mu$ M Holo- $\alpha$ -LA (Figure 58). The enthalpy of the interaction is low. The temperature change furthermore led to a conversion from endothermic to exothermic enthalpy. This leads to the conclusion that the observed effect is a form of hydrophobic hydration. Entropy driven effects are strongly temperature dependent as described in (Nielsen et al. 2000). In contrast to the experiments with Apo- $\alpha$ -LA no unfolding is observed as the

enthalpies are several orders of magnitude smaller. The sustaining baseline shift results from slightly different pH values in the protein and surfactant solution. PC 818 as well as PC 2000 are formulated without any preservative but are rather set to a low pH to inhibit microbial growth. The protein solution could not perfectly match this salt/pH system. Another impact resulting in disturbing of a clear baseline is the salt content in the surfactant raw material.

The last set of experiments was conducted with IL-11 with concentrations of 0.2, 0.4 and 0.8  $\mu\text{M}$  protein. An interaction could be determined for both surfactants, PC 2000 and PC 818, but the enthalpies were very weak, decreasing with rising the temperature from 5 to 37 °C by up to one order of magnitude. Interaction could be detected up to a surfactant concentration of 0.05 mM for PC 818 and 0.17 mM for PC 2000, respectively.

In general, these small temperature dependent enthalpies point to nonspecific interactions as shown for example with salting out effects by smaller organic substances, possibly without unfolding of the protein (Hvidt and Westh 1998). The small enthalpies and the strong temperature dependence direct towards hydrophobic hydration as the driving process of the interaction between Holo- $\alpha$ -LA or IL-11 and the surfactants. IL 11 can be protected from shaking induced stress or stresses at surfaces during spray drying by nonionic surfactants (Friess and Fitzner 2001). We conclude that for these systems the stabilizing effect must originate from the interface coverage by the surfactants. Direct interactions are only marginal and should not play a major role.

In literature some approaches towards determination of protein surfactant interaction via ITC have been made. Bam et al. found little interaction between the more hydrophobic interaction hGH and PS 80 (Bam et al. 1998) and proposed the stabilizing effect of the surfactant would derive from covering hydrophobic patches on the protein surface. Garidel et al. found no interaction between PS 80 and lysozyme as well as a monoclonal antibody (Garidel et al. 2009). For the fusion protein albutropin Manning et al. could only detect significant interaction between the albumin that has a specific fatty acid binding part on the protein surface and PS 20 and PS 80 (Chou et al. 2005).

This surface coverage effect of protein stabilizing surfactants has been proven for different other proteins and processes (Burke et al. 1992). Lysozyme adsorption onto

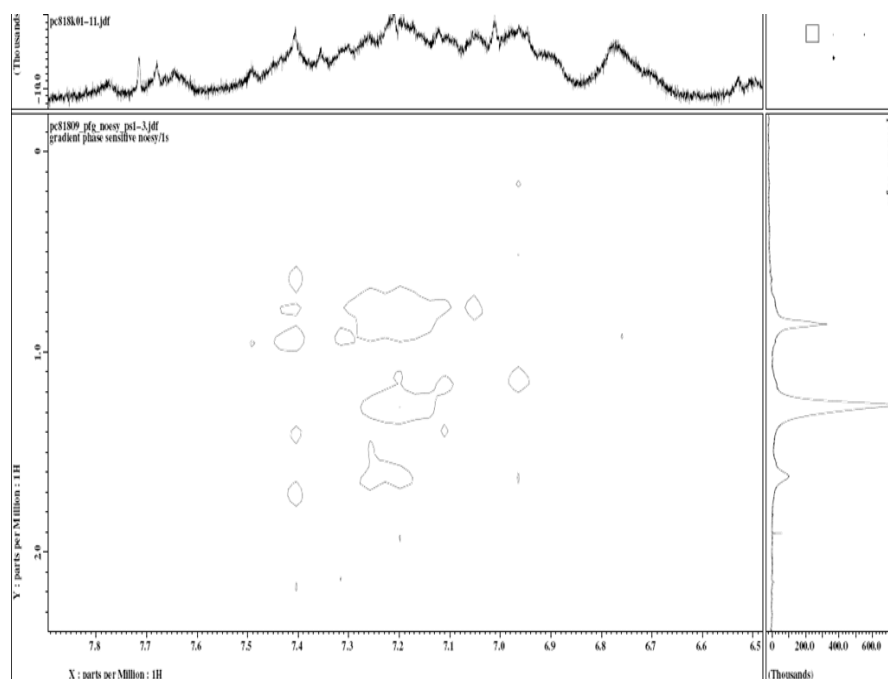
hydrophobic silica can be prevented by the addition of PS 80 (Joshi and McGuire 2009), n-Octyl- $\beta$ -glucopyranoside and other nonionic surfactants reduce agitation induced aggregation of insulin (Sluzky et al. 1992) and recombinant human factor XIII could also be protected from agitation and freeze-thaw induced aggregation by PS 20 addition (Kreilgaard et al. 1998). Replacement of protein from the surface by the surfactant were studied with the help of surface tension analytics (McAuley et al. 2009; Serno et al. 2010), atomic force microscopy (Gunning et al. 2004), electron spectroscopy for chemical analysis (Adler and Lee 1999) and contact angle measurements (Zhang and Ferrari 1997).

### **5.3. NMR - Spectroscopy**

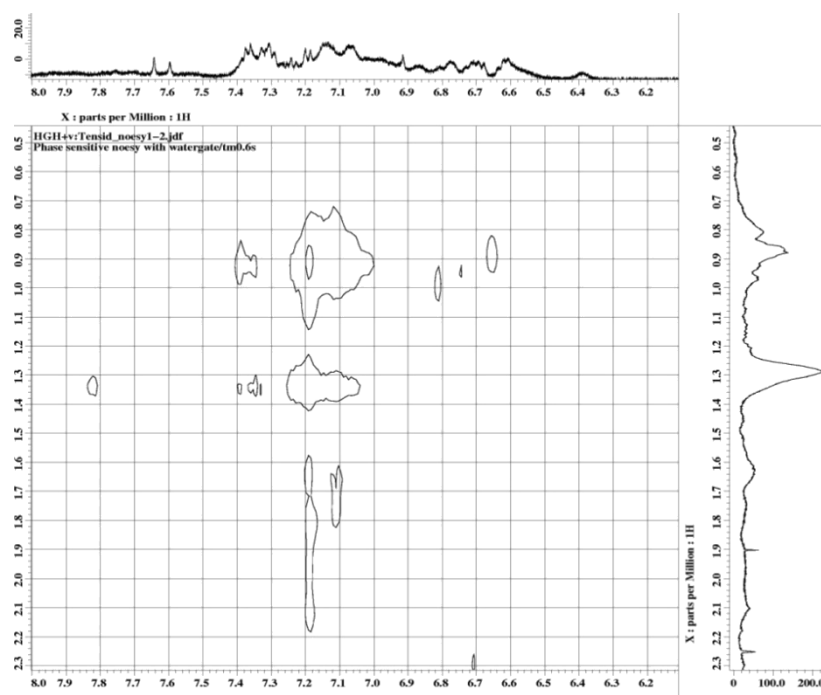
In order to obtain further understanding of potential direct interactions of surfactants and proteins, NOESY-NMR experiments were performed. Besides LA in the Holo-form, hGH was selected since it is characterized by a rather high content of hydrophobic, specifically aromatic amino acid side chains and PC 818 was used as a model surfactant. Figure 59 and Figure 60 show the NOE spectra of Holo- $\alpha$ -LA and PC 818 and hGH with PC 818, respectively. Cross peaks can be found between the chemical shifts at 0.9 ppm and 1.3 ppm in the surfactant spectrum and the chemical shifts at 7.2 ppm and 7.4 ppm in the protein spectrum. The latter two represent the aromatic amino acids of the proteins and cannot be found in the surfactant spectra. As expected these signals for hGH show a slightly higher intensity due the higher number of aromatic amino acids in the sequence. For both Holo- $\alpha$ -LA and hGH interactions could be found between the aromatic side chains and the hydrophobic fatty acid chain of PC 818. In this experiment interactions between aliphatic hydrophobic amino acid side chains of the protein sequence and the surfactant fatty acid chain could neither be proven nor ruled out as intermolecular signals overlay potential cross peaks between both molecules. The same holds true for interactions with the carbohydrate part of the surfactant.

Overall, the cross peak signals are significant, but very low. This confirms the ITC results. Hillgren et al. could prove an interaction between PS 80 and lactate dehydrogenase by using Pulse-Field-Gradient Spin-Echo NMR (Hillgren et al. 2002). They found out that interactions between surfactants and proteins do take place, but

extent and related enthalpies are rather small. Taking into account that a very high concentration of both surfactant and protein is needed to obtain an adequate one dimensional spectrum, the overall interaction is most likely undetectable at pharmaceutically applied surfactant concentrations.



**Figure 59: 2D-NOE spectrum of the system PC 818 and Apo- $\alpha$ -LA.**



**Figure 60: 2D-NOE spectrum of the system PC 818 and hGH.**

Interaction between a smaller artificial “protein” and alkyl polyglycosides could be proven by Sjoegren et al. (Sjoegren et al. 2005). In systems containing  $\beta$ -cyclodextrin and glucagon more hydrophobic protein moieties are packed into the hydrophobic cavity of the excipient (Qin et al. 2002; Matilainen et al. 2008).

In our experiments it could be clearly demonstrated that the proteins interact with surfactants via the hydrophobic chain of the surfactant and the aromatic amino acids of the protein. An interaction of the hydrophobic aliphatic amino acids is very likely, but could not be proven with this experimental set-up. These interactions are very weak and could be detected only at high concentrations. This has two reasons. First, the signal of the aromatic amino acids is rather faint even at higher protein concentrations. Secondly, the interaction itself is delicate compared to the other signals that were detected via NOESY experiments. Consequently, these studies would have to be performed for all protein-surfactant combinations but this work was not practicable due to the enormous material consumption. To the best of the authors’ knowledge, this is the first description of interaction proof by NOESY for the system pharmaceutical protein and nonionic surfactant. The method must be developed for enhanced sensitivity but nevertheless gives evidence for the direct vicinity of both substances in liquid formulations.

It is open, whether this direct interaction leads to stabilization of proteins as proposed for hGH and PS 80 through shielding of the hydrophobic patches at the proteins surface (Bam et al. 1995). Alternatively, the interaction might lead to increased unfolding as described for alpha-helical bovine acyl-coenzyme-A-binding protein and SDS due to destabilization of the folded state of the protein (Andersen Kell et al. 2009) shifting the equilibrium to unfolding. On the other hand surface competition of surfactants is a driving force for protein stabilization during the shelf life of a formulation and is widely described in literature (Maa and Hsu 1997; Zhang and Ferrari 1997; Gunning et al. 2004; Matheus 2006; Mahler et al. 2009; McAuley et al. 2009; Serno et al. 2010).

## **5.4. Conclusions**

In this study nonspecific interactions between sugar based nonionic surfactants and protein are clearly detectable, but no fixed binding numbers could be calculated via

ITC experiments. Holo- $\alpha$ -LA and IL11 do not show specific binding or unspecific binding with fixed binding ratios when titrated with PC 818 and PC 2000. The interaction seems to be more dependent on surfactant concentration than on a specific protein/surfactant ratio. As the enthalpy of interaction is strongly dependent on temperature hydrophobic hydration might be the driving force of interaction. Additionally, NOE spectroscopy points to hydrophobic interaction between aromatic amino acid side chains of Holo- $\alpha$ -LA and hGH and the hydrophobic chains of PC 818. These interactions are rather weak in nature. Future NMR-studies might take the effect of different surfactant concentrations below and above CMC into account as well as different protein states, folded vs. unfolded or partially unfolded. This study is one the few providing proof for direct interaction of protein drugs and surfactants. However, the fact that proteins and surfactants show direct interaction does not answer the question whether this phenomenon is beneficial for protein stability. The protein molecules may either be stabilized by shielding of the hydrophobic patches of the protein's surface or unfolding of the protein molecules may be induced by preferred interaction and stabilizing the unfolded state. But as surfactants already proved their suitability as stabilizers in protein formulations, the main stabilizing effect might not be derived from weak direct intermolecular contact, but from the surface competition between surface active proteins and the surfactant molecules.



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# 6. Determination of the Interaction between Nonionic Surfactants and human Growth Hormone with the Help of Fluorescence Correlation Spectroscopy

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## 6.1. Introduction

The technique of Fluorescence Correlation Spectroscopy (FCS) is already described in 1.2.2.4.3. Takakuwa et al. used the autocorrelation function and the resulting diffusion constants to characterize the interaction between a fluorescent labeled protein and liposomes similar (Takakuwa et al. 1999). In a similar way we tried to investigate the interaction between hGH and polysorbate 80 or two different sugar based surfactants.

For this kind of experiments a low labeling degree was needed, i.e. one molecule of fluorescent dye per molecule protein would be desirable. For analysis of this system multicomponent fit was applied, fitted for two to four different species to differentiate between labeled protein, labeled aggregates, labeled protein surfactant complexes and to diminish background fitting also for free dye. An increase of diffusion times would give a hint of an interaction of nonionic surfactant micelles and proteins as described by Schellekens et al. in the context of immunogenicity of different erythropoietin (Epo) formulations (Berland 1997; Hermeling et al. 2003). Further sample properties like counts per particle (cpp) describing the brightness of the fluorescent conjugate and the total intensity of the sample were determined. Parallel to regular FCS measurements a scanning for intensively fluorescent targets (SIFT) was also performed (due to set-up of the machine, the first and last sample could not be scanned). During a SIFT experiment the focus moves throughout the cell which increases the odds of tracking extremely large and hence slowly diffusing entities (Bertsch et al. 2005). This technique has been applied e.g. to scan for large fibrils of labeled protein of alpha-synuclein plaques as marker for Parkinson's and Alzheimer's disease (Giese et al. 2005) and might show larger oligomers of hGH that would have

been not detected by FCS with a fixed focus.

## **6.2. Material and Methods**

### **6.2.1. Material**

PS 80 of technical grade was obtained by Sigma-Aldrich (Darmstadt, Germany). The sugar based nonionic surfactants PC 818 and PC 2000 were kindly donated by Cognis KG (Düsseldorf, Germany). The water used in all experiments was deionized water from the university supply purified in a PURELAB Plus system (ELGA labwater, Celle, Germany) with a conductivity of  $<0.055 \mu\text{S/cm}$ . Buffer salts and other reagents were of analytical grade or higher. hGH was kindly donated by Pharmacia & Upjohn and processed as described below for the nuclear magnetic resonance experiments. hGH was dialyzed over a time period of 24 h against 100 mM PBS pH 7.4 at 4°C using a 3500 Da MWCO Slide-a-Lyzer dialysis cassette with a nominal volume from 3-12ml (Thermo Scientific, Rockford, USA). The dialysis was performed to remove glycine, which is present in the original formulation. The glycine would otherwise react with the fluorescent probes. After dialysis, the protein concentration was determined via size exclusion chromatography. The protein was adjusted to a concentration of 1.00 mg/ml. Fluoroprobe 488 was obtained as staining kit from KMF Laborchemie (Lohmar, Germany). And Alexa Fluor 488 dye was also obtained as a ready-to-use kit from Invitrogen (Carlsbad, USA).

### **6.2.2. Methods**

#### **6.2.2.1. Size Exclusion Chromatography**

The monomer content of the protein formulation as well as the dimer formation was investigated via SEC. A HP 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector and a 3000 SWXL gel filtration column (Tosoh Bioscience, Tokyo, Japan) was used for this experiment. 50  $\mu\text{l}$  of a centrifuged protein sample was injected into the HPLC. Within the HPLC a 0.2  $\mu\text{m}$  online filter was used. The running buffer was composed of 50 mM  $\text{NaH}_2\text{PO}_4$  and

150 mM NaCl at a pH of 7.2. The analytics were performed at a flow-rate of 0.5 ml/min with UV-detection at 216 nm.

#### **6.2.2.2. Optimization of the Binding Protocol**

At first 100 µl of Fluoroprobe 488 with 2 mg/ml in DMSO were mixed with 100 µl of a 1 mg/ml hGH solution and 1 ml sodium carbonate buffer supplied with the labeling kit. After incubation and stopping the reaction with 10 µl hydroxylamine solution the dye was separated from the protein by centrifuging in Vivaspin columns with a nominal MWCO of 5000 Da (Sartorius Stedim biotech, Aubagne Cedex, France). Consequently, the purification step was changed to PD-10 gel sepharose separation columns (Sephadex<sup>TM</sup> G-25M, GE Healthcare, Buckinghamshire, United Kingdom). The column was equilibrated with 25 ml PBS before adding the sample. The reaction solution was added on the column and 10 ml PBS buffer pH 7.2 was added as eluent. 500 ml fractions were collected. These fractions were separated then for a second time.

When Alexa 488 was applied, the first labeling was performed according to the manual. For coupling reaction, 2 ml of the protein solution (1 mg/ml) were added to 200 µl 1 M sodium carbonate and 10 µl dye solution. The sample was incubated 1 h at room temperature (200 rpm on a horizontal shaker). At the end of the incubation, the sample was purified with a PD-10 column. The eluate was collected in 12 fractions of 500 µl. Only the fractions 2 to 6 were retained and combined. Concentrations were determined using SEC. The dye concentration was reduced to one fifth of the original concentration to reduce the amount of free dye and finally by changing the collected fractions after separation to the fractions 7 to 12 the protocol was finalized.

#### **6.2.2.3. Fluorescence Correlation Spectroscopy**

FCS and Fluorescence Intensity Data analysis (FIDA) measurements were carried out with an Insight Reader (Evotec Technologies, Düsseldorf, Germany) with the excitation wavelength set to 488 nm, using a 40X 1.2 NA microscope objective

(Olympus, Japan) and a pinhole diameter of 70  $\mu\text{m}$  at FIDA setting. Excitation power was 100  $\mu\text{W}$ . Each sample was measured in triplicate during 10 s at room temperature. FCS plate reader applicable NUNC 96-well plates (Nalgene, Denmark) were used with a sample volume of 20  $\mu\text{l}$ . The fluorescence data were analyzed by autocorrelation analysis using the FCSPEvaluation software version 2.0 from Evotec technologies. Fluorescence intensity data were acquired and summed over time intervals of constant length (bins) of 40  $\mu\text{s}$  using the Insight Reader software. The frequency of specific combinations of resulting photon counts per bin of Alexa 488 or Fluoprobe 488 was recorded in a two dimensional intensity distribution histogram with intensity values from 0 - 255 photons/bin or in a one dimensional autocorrelation function applying one-, two- or three-species fits, respectively. The protein concentration for all experiments was set to 0.5 mg/ml protein. The ratio of labeled protein to native protein was 1 to 1000. Surfactant concentrations ranged from 1% to 0.00001%. All experiments were performed in triplicate.

## **6.3. Results and Discussion**

### **6.3.1. Optimization of the Binding Protocol**

The results from this experiment are summarized in Table 10. When the Fluoroprobe 488 kit was used as described in the user's manual 93.1% free dye was detected in the solution by FCS measurement. As this was far from acceptable the purification step was changed from Vivaspin centrifuge tubes to PD-10 columns. This reduced the amount of unbound dye to 35.5% which was still not acceptable. A second purification step resulted only in dilution of the protein as tested by SEC but not in a reduction of the amount of free dye. Adjusting the stoichiometry from 10:1 to 2:1 did not lead to significant reduction of free fluorophore, either. Interestingly, although the amount of labeled dye was decreased an additional increase in the counts per particle distribution could be detected. That means the ratio of labeled dye per molecule protein was increasing, which was also not desired. As the use of a size exclusion column as well as centrifugation should result in complete separation of dye and protein, the dye is probably non-covalently linked to



the hydrophobic proteins surface and could not be removed. Hence we changed the fluorescent dye to Alexa Fluor 488. From the 500 µl fractions that were collected from the final separation step, only the fractions 2 to 6 were retained and combined. Concentrations were determined using SEC. The resulting amount of free dye was 45.7 %.

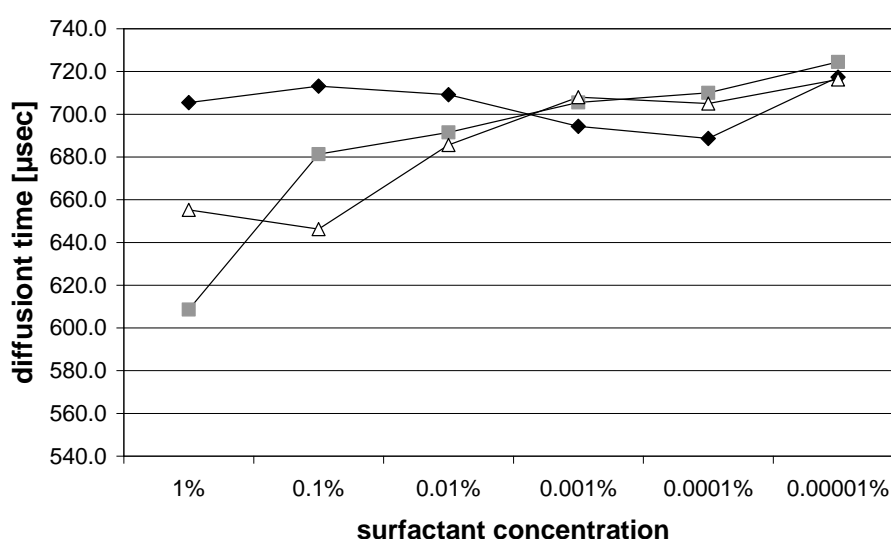
#	Protocol	Amount of free dye [%]	Amount of labeled protein [%]	Counts per particle
1	Standard protocol Fluoroprobe 488	93.1	6.9	24.8
2	#1 + use of PD 10 columns and change stoichiometry to 1:2	35.5	64.5	32.6
3	# 2 + 2 <sup>nd</sup> purification step	35.5	64.5	32.6
4	Alexa 488 and PD 10 columns	45.7	54.3	23.4
5	#4 + different stoichiometry (1:2)	38.3	61.7	23.3
6	#5 + 2 <sup>nd</sup> purification step; fractions 7-12	11.7	88.3	29.8

**Table 10: Optimization of the ratio of free fluorescent dye to labeled dye and degree of labeling as described by particle intensity (counts per particle)**

As this protocol was adapted from an antibody labeling kit with the aim of a high degree of labeling to optimize the binding reaction the dye concentration was reduced to one fifth of the original concentration. This resulted in 38.3 % free dye. And by changing the collected fractions after separation to the fractions 7 to 12 the amount of free surfactant could be reduced to 11.7 % which was acceptable for further experiments. Although these fractions showed a higher count per particle rate ( $1 < \text{labeling degree} < 1.3$ ), this was acceptable as the amount of free dye was decreased so drastically. A third purification step led not to significantly lower amounts of free dye but of strong dilution of the protein.

### 6.3.2. Fluorescence Correlation Spectroscopy

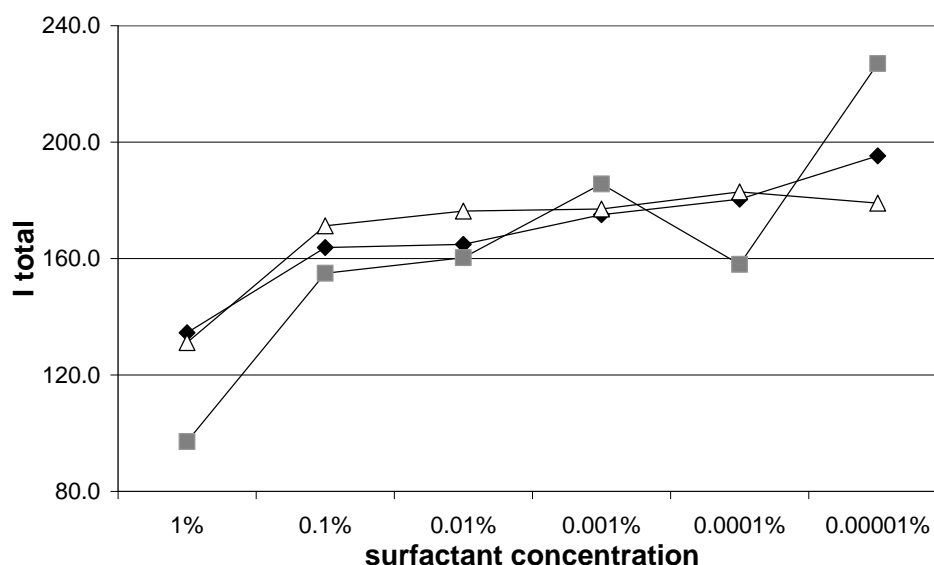
In Figure 61 the diffusion times are plotted against the surfactant concentration. hGH in a concentration of 0.1 mg/ml after labeling and purification has a diffusion time 539.4  $\mu$ s. After mixing labeled and unlabeled protein to a concentration of 0.5 mg/ml the diffusion time increased by 10%, which is viscosity dependent. When concentrations of 0.00001% to 0.01% were applied the diffusion time for all surfactants was in a range of 700  $\mu$ s. Increasing the concentration to higher values in the PS 80 and PC 818 containing formulations made the protein diffuse faster (611  $\mu$ s and 656  $\mu$ s, respectively for 1%). If there would have been an inclusion of protein aggregates into micelles the opposite would have been expected.



**Figure 61: Diffusion time of labeled hGH as a function of the surfactant concentration (♦ PC 2000, ■ PC 818, and Δ PS 80).**

It is generally possible to tell between small differences in the diffusion time and hence size as shown by Chattopadhyay (Ghosh et al. 2009) who could distinguish between the native, the molten globule and the unfolded state of bovine serum albumin using FCS. Takakuwa et al. could prove the interaction of phospholipid liposomes with the very hydrophobic membrane protein 4.1 (Takakuwa et al. 1999). Thus, it seems very unlikely that micelles protein complexes occur for this system with nonionic surfactants and hGH. In contrast, the total intensity of the sample decreases with higher amounts of surfactant as shown in Figure 62 from values of about 200 to 120 when surfactant concentrations of 0.00001% and 1% were

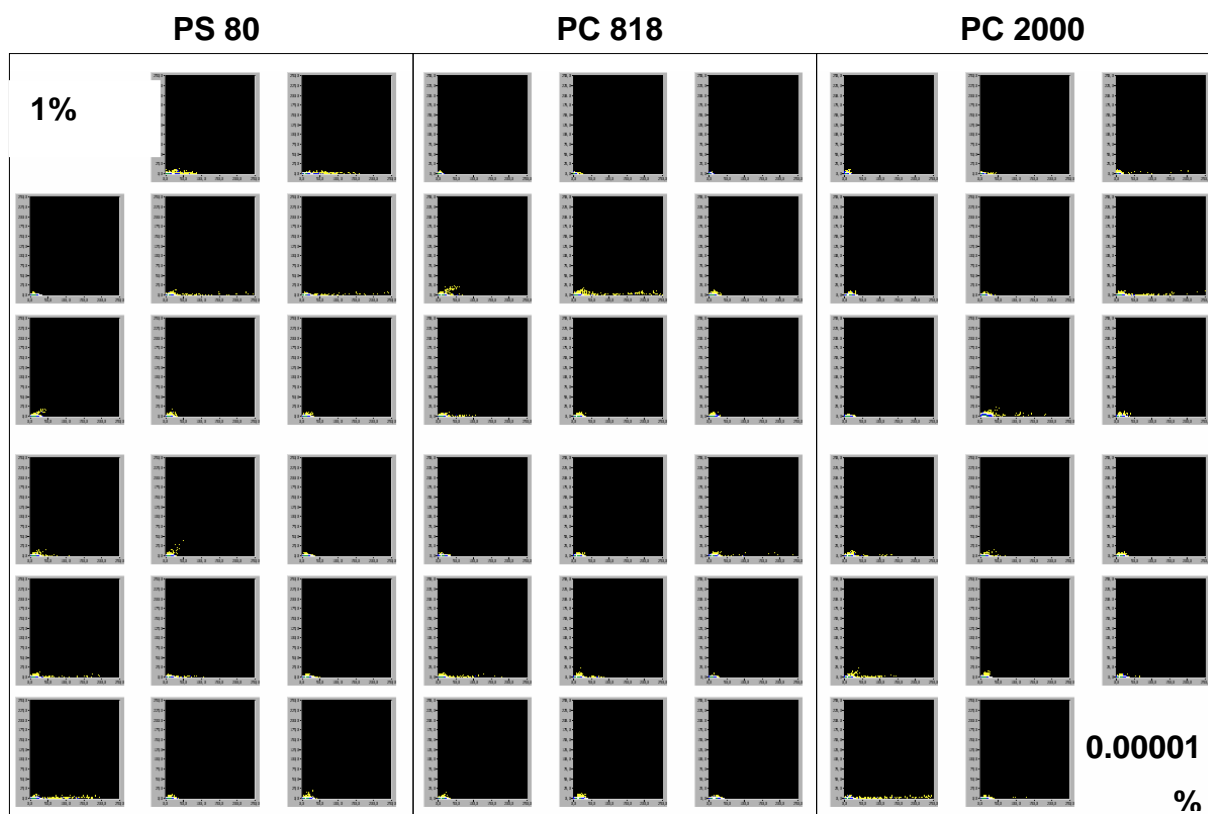
compared. A decrease in total intensity might come from quenching effects of the surfactant in the solution, but nothing indicates inclusion of the protein into micelles. On the other hand, the cpp rate, which remained unaltered for all concentrations, would represent the brightness of a fluorescent labeled protein or the inclusion complex of one or more protein molecules in a micellar “carrier” as these systems would appear as one particle through the laser beam (data not shown).



**Figure 62: Total intensity of surfactant samples (♦ PC 2000, ■ PC 81, and Δ PS 80)**

In additional SIFT experiments aggregates could be detected in some formulations as indicated as wider distributions in Figure 63, but no correlation to the type of surfactant present nor to SEC results could be made.

For all measurements the fixed parameters for unlabeled dye were used in the multicomponent fit. No third fluorescent species could be detected, which also indicates that unlabeled dye did not interact with surfactant micelles.



**Figure 63: SIFT measurements of hGH and surfactants.**

## 6.4. Conclusions

The results from FCS and SIFT measurements both direct into the direction of no interaction on a micellar level. Neither the diffusion times of the micellar solutions nor the diffusion times of surfactant formulations, containing surfactants below CMC, differ significantly from protein in buffer. The only detectable changes arising from surfactants are related to total intensity. These results are a hint that no micellar inclusion happens. A complex between aggregated proteins within a micellar construct must have about three times larger volume than hGH monomers. Assuming that a polysorbate micelle has a diameter of 3-5 nm (Croy and Kwon 2005) and the same holds true for a folded hGH molecule (5 nm) (Maa and Hsu 1996) a third species with a significantly larger diffusion time would have been expected. But no third species with a higher diffusion time could be found in all formulations with better correlation values as for two species (i.e. pure dye and labeled protein). Binding of protein into a micellar complex core could not be detected. Due to changes of the

fluorescence intensity in the presence of surfactant one could argue interaction might occur. The mode of this possible interaction is not be due to inclusion of protein aggregates or completely unfolded monomers and micellar surfactant molecules, but due to interaction of monomeric surfactant with protein molecules or pure quenching effects from the surfactant itself as no changes in diffusion rates could be detected. We could not confirm the proposed interaction from Schellekens et al. between Epo and nonionic surfactants (Hermeling et al. 2003) to our applied system with hGH.

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## 7. Summary of the thesis

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The aim of this thesis was to find alternative nonionic surface active substances that are able to protect pharmaceutical proteins as good as the already approved excipients. The search was focused on substances that on the one hand show similar behavior in regards to physico-chemical properties as the gold standards polysorbate 20 and polysorbate 80. On the other hand the drawbacks of polyethylene glycol derived products, such as peroxide residues, should be circumvented.

Sugar-based surfactants were hypothesized as promising alternatives. In this thesis, first a screen for suitable sugar-based surfactant products from pharmaceutical, cosmeceutical and food industry was performed. These substances were tested on their basic physico-chemical properties, such as critical micelle concentration, solubility, micelle size, viscosity. With the help of mass spectrometry composition of the products was analyzed. Testing for peroxide equivalents, even after UV-radiation, revealed that these products are not forming any oxidizing species. Additionally, trehalose fatty acid esters were synthesized and physico-chemically characterized. The most promising candidates were Plantacare 818, Plantacare 2000, Ryoto Monoester P and two of the trehalose esters (capril and lauryl monoester). In regards to critical micelle concentration, effect on surface tension, hemolytic activity and micelle size the substances were in comparable range with the polysorbates.

With the most promising surfactants stress tests in pharmaceutical protein formulations were performed. Especially the trehalose esters and the Plantacare products showed quite similar behavior to the polysorbates. Different stress tests were performed, mainly aiming for mechanical stress. What was unexpected, was that also under thermal stress the sugar-based surfactants could not show superiority to the polysorbates. Although lacking peroxide residues in the sugar-based surfactant formulations, the formation of oxidized methionine of Interleukin 11 was almost unaffected compared to high peroxide containing polysorbates. But, when put under mechanical stress some of the sugar-based surfactants were better stabilizers, e.g. in shaking experiments of human Growth Hormone, than the approved polysorbates. Another question that should be answered in this thesis is whether pharmaceutical

proteins are stabilized due to interactions with the outer shell of the protein or simply by surface coverage of the interfaces between the aqueous protein formulation and e.g. container walls or the container head space. Three different biophysical methods have been applied for this. Isothermal Titration Calorimetry revealed a very small interaction enthalpy between surfactants and proteins. That interaction was unspecific, i.e. without a fixed binding ratio, and temperature dependent. Due to the experimental set-up this interaction could only be shown for sugar-based surfactants as for the polysorbates the demicellization enthalpy was too low at temperatures between 5 °C and 37 °C to be applied in this system. Nuclear Overhauser Enhancement NMR revealed that the hydrophobic chains of the surfactants are in close spatial distance to the hydrophobic aromatic amino acids of the protein. This was proven at higher excipient and protein concentration than would normally be applied to overcome a high noise signal in the NMR. Fluorescence Correlation Spectroscopy revealed no increase in diffusion times when surfactant molecules are present in the formulation. Hence, it seems unlikely that proteins migrate into a micelle and unfold there as was hypothesized by others. The main outcome of these experiments was that the direct interaction between protein and surfactant is very low. It is very likely that competition at hydrophobic interfaces is the main stabilizing property of nonionic surfactants.

Summarizing, trehalose fatty acid ester and Plantacare 818 and Plantacare 2000 are suitable alternatives to polysorbates in the applied field of low concentration protein formulations. The direct interaction between nonionic surfactants and pharmaceutical proteins is very low and unspecific.

In future studies, one might have a look on higher concentrated formulations of e.g. monoclonal antibodies and gain a better knowledge if these surfactants are able to prevent protein oxidation that would appear in the presence of polysorbates. Furthermore, the application of biophysical methods on high concentrated formulations might be interesting, as the protein at some point needs enough space that direct interaction might also have an impact on the formulation stability.





## **Presentations and Publications associated with this thesis:**

November 10	Synthesis, Characterization and Assessment of Suitability of Trehalose Fatty Acid Esters as Alternatives for Polysorbates in Protein Formulation published in Eur. J Pharm Biopharm 2010 76 (3); 342-350
November 09	Poster presentation at the DPhG Graduate Student Symposium, Pichlarn, Austria
July 09	Poster presentation at the Colorado Protein Stability Conference, Breckenridge, USA
July 09	Poster presentation at the AAPS National Biotech Conference, Seattle, USA
July 08	Poster presentation at the AAPS National Biotech Conference, Toronto, Canada
March 08	Poster presentation at the World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Barcelona, Spain

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