Chapter 1

Introduction and Objectives of the Thesis

Abstract

In the general introduction the formulation development for hydrophobic proteins is discussed. The low solubility of these proteins often combined with a strong tendency to adsorb on different materials during processing or storage are the major challenges during formulation development. Human Serum Albumin (HSA) is frequently used as excipient to overcome the mentioned problems. As HSA is gained from human plasma, its use is always related to the risk of blood born pathogens, as well as batch to batch variations. Furthermore, specific analytics for the active protein are difficult in presence of an excess of HSA. Therefore, ways need to be found to circumvent the use of HSA in protein formulations. Possible approaches for the development of HSA-free formulations for hydrophobic proteins are described in Chapter 1.

Keywords: hydrophobic proteins, solubility, adsorption, HSA

1. Introduction

With the first successful production of recombinant somatostatin, a peptide hormone consisting of 14 amino acids in 1977 [1] and shortly later the production of recombinant insulin [2] the starting point for the tremendous increase of biotechnological products on the pharmaceutical market was set. At about the same time it was accomplished to produce murine monoclonal [3] and later chimeric and humanized antibodies [4,5]. Approved by the FDA in 1982, human insulin was the first genetically engineered consumer health product on the market. Since then, numerous products including recombinantly produced antibodies, proteins and peptides have entered the market and currently about 225 are under development [6].

One critical step for the production of a protein as pharmaceutical product is the development of a stable formulation, as proteins are complex molecules which are susceptible for various degradation mechanisms and instability reactions [7,8]. It is a great challenge to maintain the native and functional structure of a protein during pharmaceutical processing, production, storage and the final application at the patient. The first step is getting insight into the characteristics of the particular protein and identifying possible instability reactions. To achieve this goal the development of analytical techniques which are capable to detect and quantify structural and activity relating changes in the protein is inevitable. In pre-formulation studies the basic knowledge on the protein is gained and the optimum conditions, concerning pH, buffer system, ionic strength and protein concentration have to be determined [9]. Based on this information the further development of a liquid or lyophilized formulation can proceed. Depending on the protein and the type of formulation the addition of excipients and stabilizers has to be evaluated. Thereby, it is a general concept to keep the formulation as simple as possible. The knowledge of the physico-chemical properties of the selected excipients during freezing and in the dried state if using a solid formulation is very important. To avoid failures in protein formulations, one has to be aware of the fact that the physico-chemical properties of excipients can affect protein stability and activity. Therefore, the focus must not be solely set on the protein alone, but on the formulation as a complex system. Protein formulation is an interesting and multifarious field that requires a structured but flexible procedure to achieve the desired goal of a stable formulation.

2. Formulation of Hydrophobic Proteins

2.1 Hydrophobicity of Proteins

Proteins are macromolecules consisting of forty to several hundreds L-amino acids connected via peptide bonds. Both, the size of the protein and the amino acid composition govern the characteristics of the particular protein e.g. folding, hydrophobicity and solubility. When a protein is folded in an aqueous environment about 80% of the hydrophobic amino acids are buried in the interior of the protein without having contact to the surrounding water molecules [10]. Various scales for the classification of amino acids according to their hydrophobicity are available. One example is the scale developed by Cowan and Whittaker (1990) using RP-HPLC which ranges from 0 to 1 (1=high hydrophobicity), with some examples shown in Table 1 [11].

Table 1: Amino acids and hydrophobicity value classified by Cowan and Whittaker (1990) [11].

amino acid	value
Alanine	0.660
Leucine	0.988
Isoleucine	1.000
Methionine	0.846
Tryptophane	0.914
Phenylalanine	0.983

Basically, the residues present on the surface of a protein affect the hydrophobicity of the protein. Each amino acid contributes to the measured surface hydrophobicity in relation to the protein structure as a whole and the composition of the residues on the protein surface [12]. Based on this assumption a more sophisticated classification of the hydrophobicity of amino acids was developed by Berggren et al. (2002), by studying the partitioning of proteins and peptides in an aqueous two-phase system. Generally, the classification of the hydrophobicity of amino acids strongly depends on the experimental setup and therefore the various scales often differ in the prediction of the hydrophobicity of the different amino acids [13]. A novel approach is the prediction of the average surface hydrophobicity of a protein by mathematical models which are based on the amino acid composition and the three-dimensional structure of the protein [14,15].

2.2 Hydrophobic Proteins used as Pharmaceuticals

Many proteins that are used as pharmaceuticals, e.g. interferons, interleukins or growth factors are considered as hydrophobic. The hydrophobicity of these proteins is further increased, when they are recombinantly produced in Escherichia coli (E. coli) as host cells, as glycosilation is not possible in E. coli. Human Interferon beta-1b (INF-ß-1b) for example is glycosilated at a single site at Asn 80 at the end of helix C [16]. The lack of glycosilation in the recombinant human INF-ß-1b (rHINF-ß-1b), produced in E. coli increases the hydrophobicity of the protein, which is reflected in the retention time in RP-HPLC with a C18 column [17]. Two major issues that have to be overcome during formulation development for hydrophobic proteins are the low solubility and the adsorption of the active protein to surfaces, e.g. filters, tubes, pumps or primary packaging materials.

2.3 Solubility of Hydrophobic Proteins

The low solubility of hydrophobic proteins becomes an issue, when the target concentration for the formulation cannot be achieved. Already during preformulation studies the point of solubility needs to be addressed. For a protein it is not possible to determine one definite solubility value, as solubility is a function of pH, ionic strength and the presence of further excipients. Generally, solubility is lowest at the isoelectric point (pI) of a protein, due to the zero net charge of the molecules. Shaw et al. (2002) produced variants of ribonuclease Sa with isoelectric points from 4.6 to 10.2 (pI of the wild type: 3.5) by exchanging individual amino acids [18]. They showed that the minimum solubility of the variants lies within one pH unit around the pI and the solubility increases at higher and lower pH values [18]. Another example is insulin, with a maximum solubility below 0.1 mg/ml at its pI at pH 5.4, while the solubility is higher than 30 mg/ml at pH below 4.0 or above 7.0 [19]. Hydrophobic proteins often show a solubility below 1.0 mg/ml at physiological pH, e.g. 0.8 mg/ml for G-CSF [20] or 0.05 mg/ml for rhINF-ß-1b [21].

The solubility of a protein needs to be tested as function of the pH, generally in the range of pH 3.0 to 10.0. However, at the acidic and alkaline end of the pH-range physical and chemical instability reactions are more likely to occur. Table 2 summarizes chemical instabilities that are characteristic for acidic and alkaline pH conditions.

Table 2: Chemical instability reactions of proteins at low and high pH [7,22].

acidic pH-range	alkaline pH-range
deamidation of asparagine and glutamine	deamidation of asparagine and glutamine via a cyclic imid or hydrolysis (pH 7-12)
oxidation of methionine (< pH 4)	oxidation of cysteine
proteolysis of aspartic acid (dilute acid)	ß-elimination
cleavage of aspartic acid-X (very acidic)	shift of disulphide bonds

Chemical and physical instability reactions of proteins, as well as potential analytical techniques to determine these reactions are discussed extensively in literature for example in the reviews of Manning et al. (1989) [7], Wang (1999 and 2005) [22,23], Chi et al. (2003) [8] and Reubsaet et al. (1998) [24]. For the selection of the appropriate formulation pH a balance between sufficient solubility and stability has to be found.

Besides the selection of an optimum formulation pH, there are several approaches available to achieve the target concentration. The structural modification of a protein is one way to increase its solubility, with the conjugation with polyethylene glycol (PEG) being the most relevant method. Besides the low solubility, PEGylation can overcome several other problems related to the use of proteins as pharmaceuticals e.g. immunogenicity, susceptibility to enzymatic degradation, rapid kidney clearance and the related a short circulating half-live in vivo [25-27]. For the covalent conjugation PEG derivatives with activated functional groups are linked to reactive amino acids, e.g. lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, treonine, tyrosine respectively the N-terminal amino group or the C-terminal carboxylic acid [28].

Another way to enhance protein solubility is the addition of excipients with surfactants, cyclodextrins, amino acids and salts being most effective. For parenteral protein formulations non-ionic surfactants, mostly polysorbate 20 and 80 are used. Besides the increase of solubility, surfactants can prevent adsorption of protein on surfaces and aggregation, which can be induced by the formation of interfaces e.g. during freeze-thawing or agitation [8]. Several mechanisms for the stabilizing effect of surfactants are postulated. One is that surfactants can directly bind to the protein surface, which was shown for example for human growth factor (hGH) [29,30]. The binding of surfactant to a protein can lead to a stabilization or destabilization depending on the protein. Additionally, the surface tension of a protein solution is lowered by surfactants and with it

the driving force for aggregation and adsorption is decreased [22]. Surfactants compete with proteins for the adsorption to surfaces, which leads to a stabilization of the protein, which was shown by Kreilgaard et al. (1998) for recombinant human factor XIII and polysorbate 20 [31]. Polysorbate 80 protected lactate dehydrogenase (LDH) from denaturation during freeze-thawing by hindering the interaction of LDH with ice and the amount of polysorbate 80 required for the protection correlated with the ice crystal surface area [32].

Cyclodextrins, which are cyclic oligosaccharides composed of six to eight dextrose units, can be used to enhance the solubility of proteins. For i.v. application chemically modified ß-cyclodextrins, e.g. 2-hydroxypropyl-ß-cyclodextrins (HPCD) were used to increase the solubility of Interleukin-2 (IL-2), ovine Growth Factor (O-GH) and Bovine Insulin [33]. Other examples are the use of cyclodextrins for a nasal formulation [34] or a parenteral formulation of Interferon-ß [35]. Furthermore, cyclodextrins can reduce aggregation and precipitation of proteins, but on the other hand thermal degradation can be favored [36,37].

Protein solubility is further a function of the ionic strength and the used salt type. Salts, respectively anions and cations can be classified by their chaotrope effect (salting in), respectively cosmotrope (salting out) effect [38]. A stabilizing or salting out effect is achieved when macromolecules result in preferential hydration of the protein, whereas binding of salts to the protein surface often leads to a destabilizing, salting in effect [39,40]. In some cases, the solubility of a protein can be enhanced at its isoelectric points when low salt concentrations are added [41]. Especially in early developed formulations, HSA was frequently used to stabilize hydrophobic proteins used in low concentration, which is further discussed in section 3 of the introduction.

2.4 Protein Adsorption

Hydrophobic proteins, which are often used at low concentration, are susceptible to surface adsorption resulting in a reduced protein concentration in the final product. For recombinant human Interleukin-11 (rhIL-11), used at a concentration of 1 µg/ml a reduction of activity by more than 40% after three hours at room temperature was caused by the adsorption of the protein on the glass container [42]. Adsorption can occur on all kinds of surfaces, e.g. filling equipment, tubes, filters or packaging materials. Especially during filtration processes, when the protein solution gets in contact with large filter surfaces the risk of protein adsorption is very high. Therefore, protein adsorption needs to be considered during production and formulation development. The selection of the container material can also impact the degree of protein adsorption. Schwarzenbach et al. (2002) demonstrated with atomic force microscopy (AFM) that the adhesion force

of Interferon-α-2a (INFα-2a) was reduced by 40% when using glass type I+, which has a specially treated surface instead of glass type I [43].

Norde (1995) reviewed the principles of protein adsorption on solid surfaces and described the different stages of the process [44]. The mechanism of adsorption is shown in Figure 1. Before a protein can adsorb to a surface, it needs to be transported to the surface (1). The basic mechanisms are diffusion and convective transport by laminar or turbulent flow. While the velocity of transport is increasing with protein concentration, the attachment of the protein to the surface is concentration independent. After binding to the surface (2) the protein can undergo structural reorientations (3). Adsorption is usually an irreversible process. However, desorption of protein (4) can be achieved by surface active ingredients [45]. Desorption of lysozyme from a polysulfone (PES) membrane with surfactants was shown by Kaplan et al. (2002) [46]. Finally the protein is transported away from the surface again by diffusion or convective transport (5).

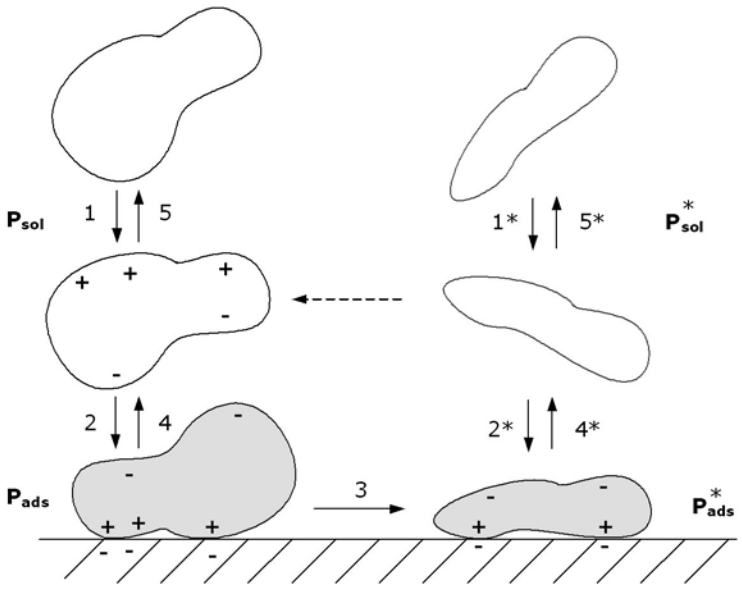

Figure 1: Protein adsorption mechanism of a protein on a surface. P_{sol} and P_{ads} are the native state of the protein in solution and after adsorption. P* are structurally perturbed states of the protein [44].

Numerous factors can impact the kinetics and the degree of adsorption of the particular protein on surfaces, e.g. protein concentration, temperature, pH, ionic strength and the presence of further excipients [44,47,48]. The hydrophobic or hydrophilic properties of the surface further govern the adsorption process. Spontaneous adsorption can occur when the change in Gibbs Energy ($\Delta_{ads}g$) is negative, by a decrease in enthalpy ($\Delta_{ads}h$) or increase in entropy ($\Delta_{ads}s$) at a constant temperature as shown in equation (1) [46].

$$\Delta_{ads}g = \Delta_{ads}h - T\Delta_{ads}s \qquad (1)$$

Generally, the adsorption process is driven by hydrophobic interactions and hydrogen bonding [49]. In addition, electrostatic interactions between charged surfaces and proteins, which are surrounded by counter ions to form electrical double layers, play an important role. The charged surface attraction occurs when protein and surface exhibit opposite charges. The electrical double layers can overlap and potential differences in net charge are balanced by the incorporation of counter ions between protein and surface [50]. The highest affinity of a protein to a surface is achieved, when the opposite charges of protein and surface result in a net charge of zero.

Adsorption is associated with a structural reorientation of the protein on the surface, which often leads to irreversible denaturation. Vermeer et al. (1998) identified an increase in α-helical structures from 0.0% to 17% and a decrease of ß-sheet structure from 76% to 32% for a mouse monoclonal immunoglobulin (isotype 1) with circular dichroism (CD) after adsorption to Teflon particles [51]. In a formulation containing 0.05 to 0.2 mg/ml Interleukin-2 (IL-2) more than 97% of the initial activity was lost after 24 hours circulation in silicone rubber tubing, whereas only 20% to 30% of the activity loss can be attributed to adsorbed protein [52]. This indicates that activity loss is often more pronounced than the decline of the concentration after adsorption and associated with structural changes [52]. The tremendous activity loss is especially a problem when protein solutions are delivered via continuous infusions or when delivery devices e.g. micro pumps are applied. Tzannis et al. (1997) analyzed the time course of the adsorption process for IL-2 and found rapid adsorption after 10 minutes combined with a loss α-helical in favor of ß-sheet structures. After five hours the residual concentration in solution decreased not significantly anymore. However, the adsorbed molecules undergo structural changes and the ß-sheet structures disappear in favor of the original α-helix elements, as well as random structural elements [52]. Jørgensen et al. (1999) showed that already after 20 minutes the concentration of four peptide epidermal growth factor (EGF) receptor ligands was reduced to 33% to 73% on polyethylene, 15% to 46% on polystyrene and 12% to 29% on glass tubes due to adsorption [53].

Protein adsorption can be minimized by the addition of surfactants to the formulation. Zhang and Ferrari (1999) showed that Albumin adsorption onto silicon surfaces was reduced by polysorbate 20 [54]. Adsorption of three model proteins (hen egg white lysozyme, bovine serum albumin and ribonuclease A type IIA) onto different surfaces was reduced up to 30% by increasing the sugar concentrations, with trisaccharides being more effective than disaccharides and monosaccharides [48]. The addition of an excess of Human Serum Albumin to the active protein is another common approach to reduce the loss of the active protein due to adsorption.

3. Human Serum Albumin as Stabilizer for Proteins

Human Serum Albumin is the most abundant protein in human plasma, where it is the major transport protein for fatty acids, as well as for different metabolites, drugs and organic compounds. After synthesis in the liver the non-glycosilated HSA is exported into the blood, where it is present at a concentration of about 0.6 mM. HSA is composed of 585 amino acids and contains 17 disulphide bonds within each molecule. The dominating secondary structural elements are alpha-helices with about 67%. The heart-shaped structure consists of three repeating subdomains I-III, which contain two subdomains each [55]. Several binding sites in the different subdomains of HSA are characterized. The interaction of HSA with fatty acids and other components is extensively discussed in literature [56-58].

In the pharmaceutical field HSA is used as drug substance and as excipient for the stabilization of other proteins. Generally, HSA is extracted from human plasma and therefore implicates problems associated with human blood derived products like the risk of blood born pathogens and batch to batch variations. Figure 2 shows a scheme of the production process for commercial HSA.

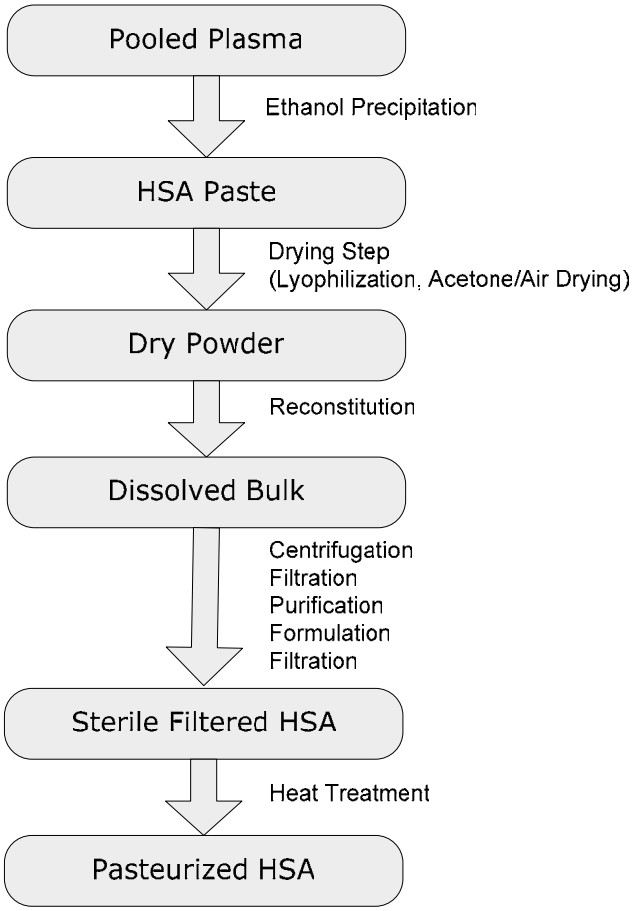

Figure 2: Scheme for commercial production of HSA out of human plasma [59].