# 1 INTRODUCTION

## 1.1 Adsorption of Therapeutic Proteins on Solid Surfaces

The understanding of adsorption of recombinant therapeutic proteins on various kinds of surfaces, e.g. in the course of up-stream and down-stream processing, fill and finish, storage, or administration is crucial in the pharmaceutical industry. In addition, it has been well known for a long time that proteins adsorb to glass and plastic, which can result in a reduced dose reaching the patient [1,2]. Table 1 gives some striking examples of protein loss to a selection of different surfaces. This summary underlines fatal consequences which potentially arise from adsorption, especially from solutions of low concentration. One of the most intensively investigated proteins of clinical relevance in this regard is insulin [2-5].

The main problem arises from the protein loss in solution. Without other optimizations, one approach to handle this problem is an appropriate increase of the starting concentration. Closely related to this is the inevitable financial effort for the surplus of the active ingredient. Moreover, the formulation can be adequately adapted with respect to the parameters pH and ionic strength or by the addition of suitable excipients. However, changes in the formulation are limited by the necessary conservation of protein stability on the one hand and an adequate biocompatibility on the other hand. Only in very rare cases will the protein itself be subject to changes with the intention to reduce its adsorption tendency. Another option is the selection of appropriate container material that has

**Table 1: Selected examples for the adsorptive loss of therapeutic protein to different surfaces utilized during the production process, storage, or administration.**

| Protein | Surface | Concentration | Protein loss | Reference |
|---|---|---|---|---|
| Insulin | Glass bottle | 30 U/l | 52% after 5 min | [2] |
| Secretin | Siliconized glass container | 40 CHR Units/ml | 20% | [6] |
| Cetrorelix | Glass and plastic vials | 0.2 - 0.4 µg/ml | 30% after 2 h | [7] |
| Factor VIII | PVC mini bag | 146 IU/ml | Approx. 60% after 48 h | [8] |
| Interleukin 2 | Silicone rubber catheter tubing | 50 - 100 µg/ml | Approx. 90% activity loss after 24 h | [9] |
| Salmon calcitonin and bovine serum albumin | Glass and polypropylene | 25 - 150 µg/ml | 30 - 75% after 12 h | [10] |

low binding properties for the respective protein. However, a universal and at the same time stable coating countervailing protein adsorption has not been found to this day. Additional problems can arise from the adsorption of proteins on solid surface. One of the major difficulties is the structural instability of the proteins, and adsorption may result in unfolding and aggregation phenomena. Both are critical with regard to an increasing potential of causing immunogenicity problems (see below). It is particularly worrisome that these entities, once formed through contact with the interface, may get back into the bulk solution, a process which may possibly be facilitated by collision with dissolved molecules [11]. Thus, an extensive study of the different factors involved in the adsorption process is essential for selecting the right actions to avoid or at least to reduce the above-mentioned serious consequences.

## 1.2 Recombinant Proteins and their Formulation

Recombinant proteins play an important role in modern pharmacotherapy, and special requirements are needed for the formulation of these protein-based active ingredients. In the majority of cases, proteins are administered parenterally as an aqueous solution. Either the aqueous solution is the stored dosage form or a freeze-dried product is reconstituted prior to administration. In order to preserve the biological activity of the proteins, the formulation must ensure the integrity of the protein conformation while also retaining a wide range of functional groups from degradation. A multiplicity of excipients can be considered, of which buffers, sugars, polyols, amino acids, salts, and surfactants play the most important role. In terms of protein stability, the most critical factors are the pH value and the ionic strength [12]. The mechanism of stabilizing the protein structure by means of general protein stabilizers, such as sugars, is explained by the theory of preferential exclusion/interaction, discussed by Timasheff and coworkers [13-15], in which the protein molecule preferentially interacts with either water or the excipient molecules. Surfactants protect the protein from surface-mediated unfolding or aggregation. They also prevent the protein molecules from reaching the solution/air or the solution/packaging container interface [16]. Through this, concentration-dependent phenomena and the range of the critical micelle concentration (CMC) as a typical lower limit for the formulation concentration can be explained. Direct interactions between surfactant and protein molecules, especially with hydrophobic side chains, are an issue as well [17,18]. In the absence of unfolding, interactions between protein molecules are reduced. However, if the surfactant preferentially binds to the more hydrophobic unfolded state, the free energy of the denatured state would be lowered, and this state would be thermodynamically stabilized by the surfactant. Thus, the addition of surfactant can result in both stabilization and destabilization of a protein. Moreover, a chaperon-like effect of the surfactants, which promotes refolding of protein molecules, is discussed [19,20].

## 1.3 Immunogenicity of Proteins

The risk of immunogenicity reactions associated with therapeutic proteins should not be underestimated. By breaking the immune tolerance, therapy may fail, or an autogenic protein with an essential biological activity may be inactivated. A dramatic example in this regard is the sudden appearance of erythroblastopenia in erythropoietin-medicated patients, which occurred in temporal relation to a formulation change [21]. This crisis has encouraged scientific researchers, companies, and administration agencies (FDA, EMEA) to elucidate such relationships.

Sources affecting the immune response can be roughly categorized by either treatment or processing-related factors [22]. Treatment-related factors involve the immune tolerance of the patient as well as the dosing schedule, the route of administration, and treatment duration. Processing-related factors include intrinsic protein properties, such as sequence, three-dimensional structure, and glycosylation pattern but also the whole manufacturing process, container closure, as well as storage and handling [23,24]. The presence of aggregates, which are typically formed during processing and storage, has received particular attention [25]. Especially protein aggregates have shown to increase immunogenicity due to their size together with newly formed recognition patterns, analogous to virus-like arrays, which may be specifically recognized by the immune system [25].

## 1.4 Glass – a Primary Packaging Material for Parenteral Dosage Forms

From the beginning of the 20th century, primary packaging (vials, ampoules, carpules, and syringes) for liquid parenteral dosage forms or lyophilisates mainly consists of borosilicate glass because of its high chemical resistance, formability, and tightness. This circumstance has hardly changed with regard to contemporary protein pharmaceuticals. Hence, today's packaging materials were virtually developed several decades ago for low molecular weight active pharmaceutical ingredients. For some time, polymeric materials have been gaining ground in the primary packaging sector because of their high break resistance, their excellent drainability, and solvent resistance [26]. However, these advantages are accompanied by a considerable permeability for oxygen and humidity [27].

Overall, vials, ampoules, and syringes are, for the most part, made out of glass tubes. Due to the manufacturing process of both the glass tube and the primary packaging container, the surface of the glass exhibits a different composition than the bulk material [28]. The surface of the glass resembles a fire-polished material, featuring roughness in the sub-nanometer scale. An influence of surface roughness on protein adsorption, which should be taken into consideration when in the nanometer scale, is therefore assumed to be negligible [29]. However, defects from the manufacturing process as well as corrosion reac-

tions may lead to a significant roughening of the surface [30,31]. Furthermore, it has to be considered, that in the pharmaceutical field, glass containers are commonly cleaned, rinsed, and then subjected to a heat sterilizing or depyrogenizing step at 180 - 350°C directly before filling. This treatment can have a significant impact on the nature of the outermost glass surface due to the removal of contaminants or the alteration of the chemical glass composition. Although glass basically features high resistance, its surface chemically reacts with the liquid formulation. Thereby, the pH value is of particular importance. In an acidic medium, an exchange of $H^+$ or $H_3O^+$ with the mobile cations $Na^+$ or other network modifiers, such as $K^+$, $Mg^{2+}$, or $Ca^{2+}$, occurs. As a consequence of the alkali or earth alkali release, the pH value in the solution increases. This can appreciably affect the stability of the respective biomolecule. On the other hand, hydroxyl ions of a basic solution are able to break up siloxane bonds, leading to a degradation of the glass matrix. Thereupon, $Si(OH)_4$ or larger moieties all the way up to glass particles, as well as all other glass components such as boron or aluminum, can get into the drug solution. Through the autoprotolysis of water, the contact of glass with a solution at pH 7 equals a combined mechanism comprising a simultaneous acidic and alkaline attack. For kinetic reasons, the acidic attack predominates in the beginning and leads to an increase in the $OH^-$ concentration, whereupon an alkaline degradation can be triggered.

## 1.5 Surface Modifications of Glass Containers for Parenteral Pharmaceuticals

There are numerous ways to modify a glass surface, such as coatings or chemical modifications pursuing protective or different functional goals [32]. However, only a few are applied in the pharmaceutical industry. Among them, primarily the coating with silicon oil (siliconization) is of higher relevance [33]. This kind of hydrophobization is often applied to prefilled syringes as a lubricant for the rubber plunger in order to facilitate ease of movement within the barrel. Moreover, by adjusting the surface hydrophobicity, the drainability of the containers is improved and the glass surface stability with regard to the aforementioned corrosion effects is increased. However, siliconization, which is inevitably associated with increased hydrophobic interactions, may be associated with an increased protein adsorption on the container surface [34]. Furthermore, despite the application of a thermal baking process, silicon oil may partially detach from the surface and get into the solution [35]. It is discussed that the formed droplets induce protein aggregation [36]. A recently developed plasma polymerization step could improve the deposition of hydrophobic layers on glass [37]. Another surface modification is a quartz-like inner coating, which drastically reduces the amount of ions dissolved from the glass [38]. Several other surface coatings, among them coatings on the basis of polyethylene

glycol (PEG), are described in literature to reduce protein adsorption on a wide range of materials, e.g. glass, plastic, and metal [39,40]. PEG chains form an extremely polar and well-hydrated surface that is free of charges in aqueous media. Hence, van-der-Waals interactions and (possibly) electrostatic and hydrophobic interactions are minimized. Polysaccharides and phospholipids are discussed as alternatives [41].

## 1.6 Protein Adsorption at the Solid Liquid Interface

Proteins are intrinsically surface-active and tend to accumulate at interfaces. The individual steps involved in the adsorption process of a protein molecule at solid liquid interface, as well as its detachment, are schematically depicted in Figure 1. One can differentiate between:

① The transport of the protein molecule from the solution towards the surface by diffusion and convection, influenced by the electrostatic potential of the solid surface.

② The interaction of the protein with the surface. Protein attachment is driven by a decrease of the Gibbs energy in the system. Theoretically, the adsorption of a protein per se is a reversible step, whereas in practice, mostly irreversibility is observed. The reason behind this phenomenon is that proteins usually interact with the solid surface through a plethora of contact regions at the same time, depending on, for example,

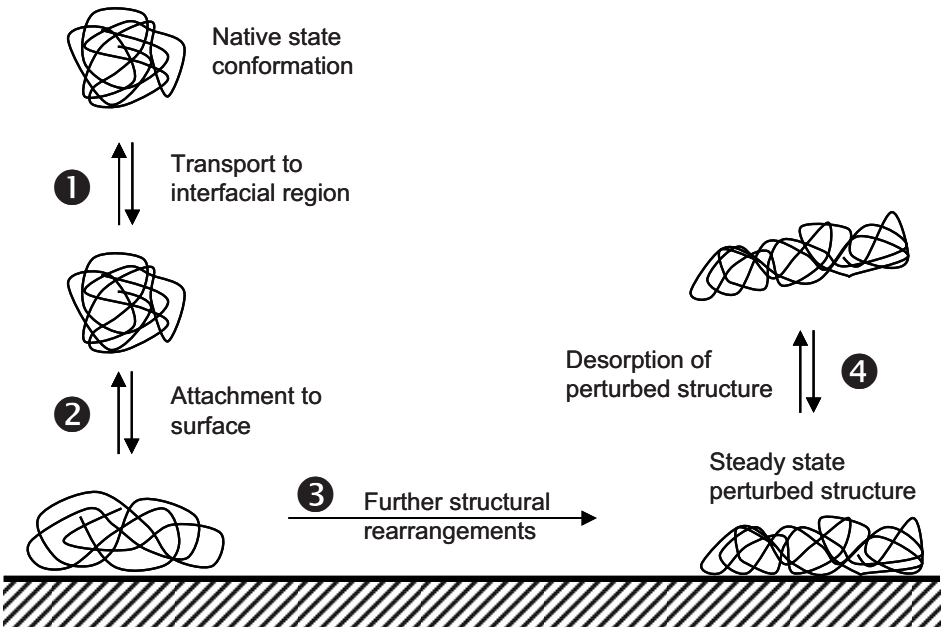


**Figure 1: Schematic representation of the mechanism of protein adsorption on a solid surface (adapted from Norde and Haynes [42]).**

their amino acid composition, their size, and their overall physical and chemical properties. Thereby, already changes in the proteins' secondary or tertiary structure can emerge.

③ The optimization of the protein - surface binding. Over time, the number of interaction points is further increased with the possibility of simultaneous molecular restructuring. Especially on hydrophobic surfaces, alterations of the proteins' secondary and tertiary structure often arise, caused by hydrophobic interactions.

④ Desorption and the diffusion back into the solution. This is less probable for unfolded proteins than for native ones due to a high number of interaction points with the surface and a more stable binding after protein unfolding.

The driving forces which facilitate protein adsorption were discussed by many authors [43-45]. They are basically equivalent to the forces that also account for the formation and the persistence of the proteins' three-dimensional structure. Regardless of the mechanism and the kinetics of the adsorption step, protein adsorption can only take place if the Gibbs energy $G$ of the system decreases, provided that the temperature and pressure are constant [43]. The relation is depicted in Equation 1.

$$\Delta_{ads} G = \Delta_{ads} H - T \cdot \Delta_{ads} S < 0 \quad (1)$$

Therein, $H$, $S$, $T$, and $\Delta_{ads}$ equal the enthalpy, the entropy, the absolute temperature, and the change in each thermodynamic function through the adsorption process, respectively. For a basic understanding of the adsorption process, it is important to know how different kinds of interactions affect $\Delta_{ads}G$ [45]. In the following, the most important interaction types are outlined briefly.

*(a) Interaction between electrical double layers (electrostatic interactions)*

Both the protein molecules and the sorbent surface are electrostatically charged. In an aqueous medium, they are surrounded by counter ions which neutralize surface charges and by which means an electrical double layer is formed. Electrostatic interactions basically follow the Coulomb law. For systems that consist of multiply charged biomolecules and solid substrates, the resulting total electrostatic energy is equivalent to the sum of every single Coulomb pair [44].

*(b) Changes in the hydration state (hydrophobic interactions)*

Nonpolar groups are generally forced back from the aqueous system since favorable interactions such as hydrogen bonding towards water molecules are largely or completely missing. The overall hydrophobic surface area of proteins with water contact

decreases through the adsorption of such groups to solid surfaces. Furthermore, the entropy in the system increases because the ordered orientation of water molecules in the proximity of the hydrophobic areas is also forced back. Thus, dehydration resembles a driving force for adsorption. The principle of hydrophobic interactions is also reflected in the three-dimensional structure of proteins. While hydrophilic patches are directed outwards, i.e. towards the aqueous phase, hydrophobic residues largely shield themselves in the interior of the protein from hydrophilic interactions. As a final result, the adsorption tendency dramatically increases with increasing hydrophobicity of the protein surface [46] or with an increasing share of the hydrophobic protein interior in interaction with the solid surface after a possible protein unfolding step.

(c) *Dispersion interactions*

Dispersive interactions, also known as London forces, are attractive forces. They are based on the permanent electron density fluctuation of an atom species which, in turn, polarizes the electron system of another species. Dispersive forces cannot be saturated but are rather additive in nature. These forces are the dominating ones among the three van-der-Waals force components: London forces, Keesom forces (interactions between permanent electric moments), and Debye forces (interactions between permanent and induced dipoles) [44].

The actual binding energies of the above interaction components vary significantly. According to Auterhoff, ion-ion interactions amount to 5 - 80 kJ/mol, depending on the permittivity of the surrounding medium, whereas dipole - dipole interactions and H-bonds add up to 1 - 30 kJ/mol. In comparison, dispersive interactions and hydrophobic interactions amount to approx. 2 kJ per mol methylene group [47]. According to Norde, the contribution from the hydrophobic amino acid dehydration inside the proteins is approx. 9.2 kJ/mol/nm$^2$ with regard to hydrophobic interactions [43].

It was described above that hydrophobic protein structures are largely located inside the proteins, although hydrophobic amino acids on the surface area of proteins are not exceptional. Nevertheless, hydrophilic areas are mainly located at the protein's interface to the aqueous medium. This stabilizes the ordered α-helical and β-sheet structures. When the protein comes in contact with a hydrophobic surface, the hydrophobic interactions inside the proteins lose their influence on the three-dimensional structure, and hydrophobic patches turn out from the interior of the protein towards the sorbent surface [43]. The promotion of protein unfolding and aggregation through hydrophobic surfaces was shown for insulin [48] and β-lactoglobulin [49]. Also the wetting behavior of the surface, which is strongly associated with its contact angle and its hydrophobicity, directly affects the adsorbed amount of protein and the extent of structural alterations [50]. But ordered protein structures may also get notably lost when new hydrogen bonds are built

towards a polar interface. As a result, the conformational entropy of the protein increases and, as a consequence, adsorption increases as well [51]. In this regard, Arai and Norde observed appreciable adsorption of the structurally less stable α-lactalbumin on a hydrophilic surface of the same electrical charge [52]. If both electrostatic attraction and hydrophobic interactions were missing, adsorption was mainly mediated through the entropy gain of adsorption-induced unfolding.

## 1.7 Factors Influencing the Adsorption of Therapeutic Proteins on Solid Surfaces

Protein adsorption is a highly complex process. With regard to therapeutic protein pharmaceuticals, the extent of adsorption, as well as the structural stability and the irreversibility, predominantly depend on three key components, which are the protein, the solid surface, and the formulation composition. Norde classified proteins into "hard proteins" and "soft proteins" according to their adsorption behavior [53]. The former adsorb on hydrophilic surfaces only under electrostatic attraction. On the contrary, soft proteins are structurally more labile and adsorb on hydrophilic surfaces, even in the case of electrostatic repulsive conditions under structural reorientation. Besides stability factors, basic chemical properties of the proteins are of particular importance (see Table 2). The factors on the part of the sorbent surface chemistry, which influence the adsorption process, were for the most part already mentioned above in connection with the adsorption driving

**Table 2: A selection of important factors influencing the adsorption behavior of proteins on solid surfaces.**

| Protein | Sorbent surface | Formulation |
|---|---|---|
| • Surface distribution of amino acids<br>• Molecule size<br>• 3D-structure in solution<br>• (Net) charge and sign / location of protein IEP<br>• Charge distribution on the protein surface<br>• Protein stability | • Chemical composition<br>• Hydrophilicity / hydrophobicity<br>• Interfacial energy<br>• Charge (sign)<br>• Charge density<br>• Electron donator and acceptor potentials<br>• Sterical influences (surface roughness) | • pH value<br>• Buffer type<br>• Ionic strength<br>• Polarity / dielectric constant<br>• Excipients (like sugars, polyols and surfactants) |