Expert Opinion

The importance of interfaces in protein drug delivery – why is protein adsorption of interest in pharmaceutical formulations?

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Introduction: In the area of peptide and protein drug products, interfaces are present as part of the basic liquid formulation, when freeze-dried formulations are reconstituted and when particulate delivery systems are prepared. Proteins are known to interact with these interfaces, and the effects seen are often irreversible adsorption and structural changes.

Areas covered: This review focuses on the ways in which peptides and proteins interact with surfaces and interfaces, and the effect these interactions have on the stability and safety of the active protein in pharmaceutical formulations. It illustrates, through examples, what can be determined by an adsorption study, and what can change when either the protein or the interfaces are modified. Last but not least, it addresses the value of these studies. The reader will gain an update on the basics of protein adsorption, with a focus on pharmaceutically relevant interfaces and recent advances in the field.

Expert opinion: Protein adsorption is widely studied; however, a more unified approach is still needed, especially on the adsorption of pharmaceutically relevant proteins, modified proteins and surfaces.

Keywords: interfaces, protein adsorption, protein stability, surfaces

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1. Introduction

The area of protein adsorption is widely studied, and a vast amount of material on the subject has been published. The wide variety of focus points range from chromatographic purification, pharmaceutical drug products and biotechnological design of surfaces to the study of basic physical interactions occurring at the interface. The common ground is interest in the behavior of proteins at interfaces and the effect that interactions with interfaces may have on protein physical stability, biological activity and immunogenicity.

A basic understanding of protein behavior at interfaces has been covered in several research and review papers [1-19]. For a pharmaceutical drug product it is important to consider why the protein molecules prefer to associate with the interface, as the interfacial accumulation driving forces may then be counteracted through formulation. To understand and prevent protein adsorption, information on the type of surface, characteristics of the protein and a thorough understanding of the underlying forces that govern protein adsorption along with an understanding of the relationship between protein adsorption and the overall protein stability are necessary.

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A protein encounters different types of interface, namely hydrophilic, hydrophobic, hard and flexible interfaces, during its lifetime, and these could all affect its stability and not least its biological activity [20-24]. The characteristics of the interfaces, such as hydrophilicity/hydrophobicity [25], morphology/topology [26], roughness [27], flexibility and charge [28], are some of the many parameters that affect protein stability by adsorption (**Table 1**). Effects from modifications of an interface, for example, changing from hydrophilic to hydrophobic interfaces, can cause the formation of more rigid adsorbed protein layers [25], reduce protein biological activity [22], or result in the development of a difference adsorption pattern [29]. Typically, modifications of the interface are introduced to decrease protein adsorption, which can play a part in making surfaces more biocompatible. Interfaces can be subdivided into gas, solid and liquid interfaces, but other more complex interfaces occur in the many emerging delivery systems, particulates, implants and devices [30-32]. The air–water and dissolved gas–water interface is encountered in all liquid formulations, and in this paper it will be referred to as the air–water interface. The characteristics of the protein are also important when adsorption is addressed (**Table 1**), and parameters such as size, surface charge, hydrophobicity, Gibbs free energy of unfolding and shape are important.

The forces driving adsorption of proteins to interfaces are best characterized for adsorption of proteins from aqueous solutions to solid surfaces, but the same forces will apply to other interfaces with the restrictions on flexibility given by the interface in question. The most important driving forces are hydrophobic interactions, electrostatic interactions and protein restructuring, but changes in hydrogen bonding and van de Waals forces should also be considered [2,3,33-34]. In 1990, Arai and Norde introduced a division of proteins into 'hard' or 'soft' proteins depending on the proteins' internal stability, which subsequently affects their ability to adsorb to hydrophilic and hydrophobic solid surfaces [4]. 'Soft' proteins have low internal stability and tend to adsorb well on both hydrophilic and hydrophobic surfaces. Adsorption may lead to a gain in entropy resulting from a loss of secondary structure, thereby counteracting the unfavorable effects of hydrophilic dehydration of the sorbent surface and protein, and electrostatic interactions. More hydrophobic surfaces induce larger perturbations in the conformation of an absorbing protein molecule [4]. The adsorption behavior of protein with a high internal stability ('hard' protein) is governed by hydrophobic and electrostatic interactions between protein and sorbent surfaces. 'Hard' proteins adsorb on hydrophobic surfaces and do not always undergo structural changes on adsorption. On hydrophilic surfaces, 'hard' proteins adsorb only if there are sufficient favorable electrostatic interactions [4,18,33,35].

Protein adsorption to interfaces is addressed in many different contexts, and depending on the interface of interest many different techniques can be used to study protein adsorption (**Table 2**). The information obtained in such studies can be secondary or tertiary structure of the adsorbed protein, amount adsorbed to the interface, flexibility of the adsorbed layer, and packing and activity of the adsorbed protein [22,23,25,29,36-78]. Some resent examples of studies of protein adsorption in pharmaceutically relevant systems include the destabilization of lysozyme after exposure to a silicone oil emulsion [31], aggregation of monoclonal antibodies in the presence of stainless steel [47,79], loss of biological activity and structure resulting from unfolding at the ice–water interface created during freeze-drying [80,81] and fibrillation of insulin induced by micro- or nanoparticulate delivery systems [32,82].

Understanding of the reversibility of structural changes on adsorption and desorption is considered to be of primary interest to determining the significance of adsorption within a formulation and the potential impact on product shelf-life, efficacy and safety.

2. Interfaces in pharmaceutical formulations and delivery systems

2.1 Air–water interfaces

In liquid formulations, a protein will encounter an air–water interface during storage, and this interface will typically be greatly influenced by agitation and movement during transport of the pharmaceutical drug products. This exposure to the air–water interface or the dissolved gas–water interface may have a detrimental influence on the physical stability of dissolved protein, and the exposure is intensified with agitation [83,84]. An example is fibrillation of insulin, which increases with extended exposure to the air–water interface through increased agitation [85,86]. Adsorption of a protein to the air–water interface can be detected by changes in stability in bulk protein, for example fibrillation or aggregation [85,86], or properties of the interface, for example changes in surface tension [57,58,87]. Apart from changes in surface tension, the layer of protein adsorbed at the interface can be characterized by its packing and physical appearance and by its rheological properties [57,59-60]. The layer formed at the air–water interface can also be accompanied by structural changes of the adsorbed protein. A structural rearrangement of lysozyme has been shown with increasing protein adsorption, from an initially flat unfolded lysozyme (mainly containing β-sheet) to a looser second layer (mainly containing random coil) at an air–water interface [88]. Protein adsorption also changes the surface pressure, and it is possible to detect differences between the surface activities of proteins. For example, it takes longer to reach a surface pressure plateau for lysozyme than for β-lactoglobulin and bovine serum albumin (BSA) at the air–water interface [58], which indicates that β-lactoglobulin and BSA are more surface active than lysozyme.

2.2 Solid–liquid interfaces

Protein and peptide drugs may be exposed to a diversity of solid–liquid interfaces during purification, production,

Table 1. Factors that affect protein adsorption.

Protein	Size, shape, charge, pI, Gibbs free energy of unfolding
Solution	pH, ionic strength, specific interaction with excipients
Surface	Charge, hydrophobicity, morphology, roughness, flexibility
Common	Temperature, polarity, shear

storage and use, therefore knowledge about the adsorption behavior of proteins and peptides is crucial in the development of stable protein drugs. Since the 1970s, several papers have described the driving forces for the adsorption and why proteins adsorb to solid–liquid interfaces [2,3,5-9,11-14,16,17,89]. The behavior of proteins at solid–liquid interfaces will depend on the entire system, that is, the solution, the surface and the protein, and numerous studies have addressed the influence of solution, surface and proteins on adsorption. Some general features have been established, namely that most proteins adsorb to hydrophobic surfaces to some extent and that increased hydrophobicity of either the protein or the surface will generally increase the amount adsorbed. The extent of spreading of a protein on a surface is determined by the rate of protein relaxation relative to its rate of arrival at the surface [8] and is therefore inevitably related to the bulk concentration of protein in solution. It has been proposed that the surface becomes covered more quickly at higher protein concentrations, allowing less time for the molecules to relax at the surface [8]. However, the understanding of adsorption is not comprehensive enough to predict the amount of protein adsorbing at different surfaces, and a unified predictive theory is still missing, even though the adsorption of proteins from aqueous solution onto solid surfaces has been studied extensively for 40 years.

2.2.1 *Adsorbed amount at solid surfaces*

Several techniques, such as total internal reflection fluorescence (TIRF) [38,39,71,90-91], ellipsometry [62,66,92], dual polarization interferometry (DPI) [74], quartz crystal microbalance (QCM) [27,42,46,62], reflectometry [22,72], surface plasmon resonance (SPR) [55,71,74] and neutron reflection [61], have proved useful for determining the amount of protein adsorbing per unit surface area to solid surfaces (**Table 2**). Some authors also estimate the number of protein molecules absorbing per unit surface area from isothermal titration calorimetry (ITC) measurements by fitting reversible isotherm equations. However, as pointed out previously by other authors [2], fitting these models, which assume equilibrium reversible binding, to experimental data obtained from irreversibly adsorbed proteins does not yield a meaningful result. The estimated amount of protein adsorbed per unit surface area tends to depend on the experimental set up, and this should be considered when adsorbed amounts determined with different techniques are compared.

The coverage of a surface may change over time if the protein is able to move across a surface, or if the protein reorientates at the surface [29,93-94]. Both symmetric and asymmetric proteins can adsorb with different orientations towards the surface. However, the amount of an asymmetric protein absorbing per unit surface area may be affected much more by changes in the orientation towards the surface than a symmetric protein (**Figure 1**) [29]. This reorientation may also affect the conformation of the adsorbed protein and thereby the amount of protein adsorbing per unit surface area [10].

The random sequential adsorption (RSA) model for adsorption describes the irreversible adsorption of objects to a solid surface, and it assumes that the objects do not overlap and once adsorbed the objects are immobilized on the surface [95]. Computer simulations performed to establish the maximum coverage of a surface using the RSA model for disks in two dimensions found that the maximum coverage of any flat surface is ~ 55% [96-98]. Experimental determination of surface coverage is usually based on measurements of the amount adsorbed form a solution phase, and subsequently correlated to the total surface area and the molecular dimension of the protein. The surface coverage also typically changes if the surfaces are rinsed, as this will potentially remove part of the loosely or reversibly bound protein. The amount of protein adsorbing to most solid surfaces has been found to correspond to values around or below monolayer coverage [14,33]. However, adsorption of protein multilayers has also been reported [68,70,99]. In one study, the adsorption of six proteins with sizes ranging from 15 to 341 kDa from aqueous-buffer solution to solid surfaces was studied using the solution depletion method. It was observed that the proteins with a molecular mass < 100 kDa occupied a single layer at surface saturation corresponding to ~ 55% surface coverage, whereas the proteins with a molecular mass > 100 kDa adsorbed as multilayers [68]. In another studied where adsorption of proteins with sizes up to 1000 kDa were studied with the solution depletion method, it was reported that moles of proteins adsorbing per unit surface area increased with protein molecular mass in a manner that is quantitatively inconsistent with the idea that proteins adsorb as a monolayer covering 55% of the surface. In this paper, the results indicate that proteins with molecular masses > 200 kDa adsorbs in multilayers. It should be noted that the authors in these two papers have looked at protein adsorption in the bulk phase and they have studied adsorption by the depletion method in order to avoid removing the bulk solution. Therefore, data obtained with this technique will differ from data obtained with techniques such as TIRF, ellipsometry, SPR, reflectometry and QCM, where the bulk layer is normally removed after a certain adsorption period in order to remove what is often called loosely adsorbed proteins. An interesting question is whether these high adsorbed amounts corresponding to multilayers are related to aggregation. Stainless steel has been found to induce aggregation of humanized IgG1 antistreptavidin [79]. Moreover, it was shown that no exchange occurred between the adsorbed protein molecules and the

Table 2. Methods for studying protein adsorption, structure and stability in the presence of interfaces.

Method	Information obtained	Type of surface	Examples can be found in Ref.
Atomic force microscopy	Morphology, displacement	Solid, lipid layer	[44,66]
Differential scanning calorimetry	Stability, unfolding	Liquid, particles*	[51,54,68]
Dual polarization interferometry	Thickness, kinetics	Solid, lipid layer	[77]
Ellipsometry	Amount, thickness, kinetics, displacement	Solid, liquid, lipid layer	[43,46,55,57,65,69]
Fluorescence spectroscopy	Tertiary structural changes, possibility of determining orientation (whether protein is adsorbed to a solid surface and contains few Trps)	Liquid, particles*	[50,74]
Fourier transform infrared spectroscopy, circular dichroism, Fourier transform infrared attenuated total reflectance	Secondary structure and in part tertiary structure	Solid, liquid, particles*	[26,68,74,78,81]
Isothermal titration calorimetry	Amount, kinetics	Liquid, particles*	[75]
Langmuir trough	Packing	Air, lipid layer	[56,60,76]
Micropipettes	Adsorption	Liquid, air	[52]
Neutron reflection	Amount	Solid	[64]
QCM, QCM with dissipation monitoring	Amount, kinetics	Solid, lipid layer	[45,49,65,72]
Reflectometry	Amount, thickness	Solid, lipid layer	[25,48,75]
Rheology	Flexibility of the adsorbed layer, kinetics	Liquid, air	[39,59,62-63]
SDS–PAGE	Amount	Solid, particles*	[40,70,71,73]
Surface capacitance using electroanalysis	kinetics	Liquid, solid	[79]
Surface plasmon resonance spectroscopy	Amount	Solid, lipid layers	[47,58,74,77]
Surface tension	Adsorption, exchange, kinetics	Liquid, air	[39,61]
TIRF and TIRF microscopy	Amount, orientation, kinetics, displacement	Solid, lipid layers	[41,42,53,67,74,80]

*Particles can be solid, liposomes, polymeric particles, and so on, and there may be some size limitations for the various methods due to scattering or sedimentation.
QCM: Quartz crystal microbalance; TIRF: Total internal reflection fluorescence.

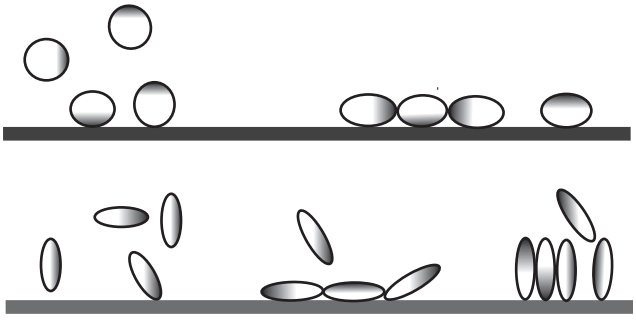

Figure 1. Schematic of protein adsorption of symmetric and asymmetric proteins [29]. Both symmetric and asymmetric proteins can adsorb with different orientations towards the surface. However, the amount of asymmetric protein absorbing per unit surface area may be affected much more by changes in the orientation towards the surface than a symmetric protein.
Adapted in part with permission from [29].

protein molecules in the bulk solution [79], indicating that aggregation occurs at subsequent adsorbed protein layers. In another study, IgG1 was shown to form multilayers at a stainless steel surface until large nucleated particles were formed or until smaller protein-coated particles coagulated [100].

2.2.2 Structural changes in adsorbed proteins and reversibility of adsorption

An important issue related to protein adsorption at solid–liquid interfaces is how the adsorption affects the stability of the protein. Is the secondary and tertiary structure affected on adsorption and will this lead to aggregation in the bulk solution and/or at the surface? Is the adsorption irreversible when the system is diluted with buffer? Can exchanges occur between absorbed protein and protein in bulk solution? If structural changes occur on adsorption, are these structural changes then reversible on desorption? These are very important issues to cover when studying protein adsorption because they will determine how the physical stability of a protein solution is affected by adsorption. At the same time, it is an experimental challenge to address these questions. The effect of adsorption on secondary and/or tertiary structure and the overall physical stability of the protein can be studied using several techniques, such as Fourier transform infrared spectroscopy (FTIR), circular dichroism (CD), fluorescence spectroscopy, nuclear magnetic resonance (NMR) and differential scanning calorimetry (DSC) (**Table 2**).

2.2.2.1 Structural changes on adsorption

After attachment to a surface, a protein molecule will rearrange to optimize its interaction with the surface. During this optimization, also called relaxation of the protein, the protein may expose an increasing number of segments to the

surface [101], which usually leads to some degree of spreading of the protein. It is generally accepted that when proteins adsorb they do so tenaciously, because of the large number of contact segments. Conformational changes in the protein are thought to be the cause of the increasing number of contact points, which also leads to protein adsorption models that do not allow for conformationally changed proteins to desorb spontaneously [102,103]. Surface coverage seems to be important for the extent of structural change induced on adsorption. Several CD studies have shown that the extent of structural change is higher at a low percentage of surface coverage [89,104-105], where the lower coverage gives the protein the opportunity for structural changes owing to fewer structural constraints. In general, proteins undergo more structural changes on adsorption to hydrophobic surfaces than to hydrophilic surfaces. The inherent stability of the adsorbed protein will also influence the extent of structural changes. For example, studies on adsorption of the wild type and two synthetic stability mutants of lysozyme at a hydrophobic silanized silica surface suggest that a protein of lower structural stability would more readily undergo a structural change at the interface, all else being equal [106]. Adsorption of recombinant factor VIII to a hydrophobic silanized silica surface has been shown to cause changes in tertiary structure, whereas less structural change was observed on adsorption to negatively charged hydrophilic surfaces [107]. Conformational changes in the three proteins BSA, β-lactoglobulin and lysozyme on adsorption to the hydrophobic chromatographic material butyl sepharose 4 FF have been studied with total reflectance Fourier transform infrared (ATR FTIR) spectroscopy. Conformational changes occurred in the secondary structure of BSA and the tertiary structure of β-lactoglobulin, whereas no changes occurred in lysozyme [108]. The reversible adsorption of recombinant human platelet-activating factor acetylhydrolase (rhPAF-AH) (pI = 6.7) to hydrophilic silica particles induced no detectable change in protein secondary/tertiary structures, but resulted in rapid and extensive aggregation of rhPAF-AH at pH 6.5 [109]. In addition to conformational changes and aggregation at the surface, adsorption of proteins to surfaces can induce protein aggregation in bulk, for example, fibrillation of insulin or myoglobin after exposure to nanoparticles [24,78,82,110], aggregation of antibodies after exposure to stainless steel, as mentioned above [79], or changes in the stability of the protein by decreasing ΔH and changing T_{max} [65,78,111].

2.2.2.2 *Reversibility of adsorption to solid surfaces*

Reversibility of protein adsorption can be subdivided into reversibility on dilution, changes in solution properties such as pH and ionic strength and reversibility with regard to exchange with other proteins in solution. Most proteins adsorb irreversibly to hydrophobic surfaces with regard to dilution, whereas proteins adsorb to hydrophilic surfaces, where the primary driving forces for the adsorption often are electrostatic interaction, and desorption may occur on changes in the ionic strength or pH of the solution [9]. In pharmaceutical drug products, adsorbed proteins may undergo homomolecular exchange with proteins from the bulk solution. Such exchange may occur because the release can occur gradually, with the newly arrived protein competing for the sites at the surface [101]. Homomolecular exchange can be detected indirectly by measuring structural changes in the protein in the bulk after exposure to sorbent material, whereas labeling of the protein is needed in order to determine the exchange directly. A disadvantage with labeling is that the label itself may influence adsorption and thereby not represent the actual reversibility of adsorption.

Homomolecular exchange has been observed for BSA adsorbed to silica, polystyrene or AgI and IL-2 adsorbed to an infusion pump/catheter tubing system made of borosilicate glass and silicon rubber [8,20,111], whereas humanized IgG1 antistreptavidin adsorbed to stainless steel has been shown not to undergo homomolecular exchange with protein from the bulk [79]. Human albumin and human fibrinogen have both been shown to undergo homomolecular exchange after adsorption to glass, whereas no homomolecular exchange could be observed after adsorption to polystyrene [112]. Furthermore, collagen has been shown to undergo homomolecular exchange after adsorption to polystyrene, whereas collagen did not undergo homomolecular exchange after adsorption to plasma-oxidized polystyrene [113]. These examples show that homomolecular exchange, like the adsorption of a protein, depends on both the protein and the surface. Moreover, homomolecular exchange depends on the solution.

An interesting question related to the stability of pharmaceutical drug products is whether adsorption-induced structural changes are reversible on homomolecular exchanges. This question has been addressed in a few studies. Not surprisingly, the reversibility of the structural changes in BSA has been shown to depend on the surface to which BSA adsorbed, that is, structural changes induced by a hydrophilic silica surface were found to be reversible, whereas adsorption to polystyrene and subsequent desorption caused irreversible changes in the structure of BSA [8]. The reversibility of structural changes induced in BSA after adsorption to AgI particles has been shown to depend on the degree of spreading. At higher surface coverage the homomolecular exchanged BSA molecules refolded into the native structure, whereas at lower surface coverage the homomolecular exchanged BSA molecules evolved to aggregate through surface-induced hydrophobic patches [111].

2.3 Liquid–liquid interfaces

Liquid–liquid interfaces are most commonly encountered as surfaces between oil or organic solvent solutions and water, for example, in formulations of emulsions and microemulsions [114] and in the preparation of drug delivery systems in which the proteins are entrapped in particles [115-117]. Usually, in the latter case, a water-in-oil emulsion is initially created

containing the protein in the aqueous phase, as seen in common methods of preparing liposomes [118], solid lipid [30] and polymeric [115,119-120] micro- and nanoparticles. Oil–water interfaces are also found in syringes for parenteral drug delivery, where silicone oil is used as a lubricant, creating an unintended interface between the oil and the aqueous formulation [31].

The forces governing adsorption at liquid–liquid interfaces are similar to those already discussed for solid–liquid interfaces. Consequently, it is not surprising that many proteins are surface active at liquid–liquid interfaces. This property has long been exploited in their use as emulsifiers in both pharmaceutical and food sciences [121]. However, studies on emulsification properties rarely consider the structural physical stability of the protein, whereas stability is critical in protein drug formulation, for example in the preparation of particulate systems, where the liquid–liquid interface is that between an aqueous protein solution and a volatile organic solvent, such as dichloromethane, chloroform or ethyl acetate [117,122-124]. This liquid–liquid interface might be the main site of protein destabilization in the preparation of particulate systems [117,124-127]. In the case of emulsions used as drug delivery systems, the oil phase is a non-volatile substance, typically vegetable oil. However, the issue of protein stability at such interfaces is equally relevant and should still be assessed.

2.3.1 *Structural changes*

It is suggested that proteins are more prone to destabilization at solid hydrophobic surfaces, as compared with solid hydrophilic surfaces, because of the driving force to expose hydrophobic amino acids from the protein interior to the interface [3]. This tendency is also acknowledged for liquid–liquid interfaces, where it has been observed that recombinant human growth hormone, ovalbumin, tetanus toxoid and urease are destabilized more when emulsification is carried out with the more hydrophobic dichloromethane compared with the less hydrophobic ethyl acetate [122,128-130]. Moreover, thorough studies using a range of oil substances found that the stability of Pa-hydroxynitrile lyase and chymotrypsin at interfaces decreased as the hydrophobicity of the oil phase increased [131,132]. However, increased hydrophobicity of the interface does not necessarily correspond to an increase of destabilization, sometimes the opposite is in fact the case. For example, protein C was denatured to a greater extent on exposure to a water–ethylene acetate interface compared with water–dichloromethane [133]. On the interface of six different oils, urease and ribonuclease showed no general trend of destabilization with increase in hydrophobicity, whereas lipase was destabilized at the most hydrophilic interfaces [132]. Thus, protein adsorption is to be expected at all types of liquid–liquid interface, and the effect on the native protein structure must be evaluated in each case.

There is a consensus that the native structure and thus the biological activity can be retained using the right preparation parameters [130,134-135]. One approach is to prepare particulate drug delivery systems with the intention of avoiding oil–water interfaces, for example to utilize an aqueous dextran solution containing protein and dispersed in water to form an emulsion with water–water interfaces [136,137]. Another approach is to alter the hydrophobicity of the surface, for example by using a more polar solvent [130]. However, it remains to be demonstrated whether a water–water interface generally leads to less denaturation and, as discussed, less hydrophobic interfaces do not always equal higher stability against interfacial denaturation [132,133]. Other approaches, such as the use of excipients to modify protein adsorption, are discussed later.

2.3.2 *Measurement of protein adsorption and stability at liquid–liquid interfaces*

Adsorption has an effect on the macroscopic properties of the liquid–liquid interface, such as surface tension, interfacial rheology and surface charge. Such properties are most easily studied at simple liquid–liquid interfaces, for example, the horizontal interface between two bulk phases of water and oil. The advantages of such systems are ease of measurement and the fact that both the extent and kinetics of adsorption can be quantified at a well-characterized surface. Protein adsorption kinetics has, for example, been obtained by following the change in surface tension at a pendant drop [36,138], the complex viscosity using De Noüy ring method [36] and the surface capacitance using electroanalysis [76] (**Table 2**). At simple liquid–liquid interfaces a clearly visible film at the interface can often be observed when protein adsorption has occurred. Such a film must therefore also exist at the interface in an emulsion, influencing the emulsion properties, and this film can be studied using a micropipette technique [49]. It is also possible to detect directly interactions between the protein and compounds dissolved in the oil phase using mass spectrometry [139]. The main disadvantages of studying such simple interfaces is that only a relatively small total surface area is examined compared with that present in an emulsion system, making it unfeasible for concentration depletion methods and common spectroscopic techniques to be applied.

Physically, the conformational changes in proteins are much more subtle than macroscopic phenomena, and consequently the measurement techniques required to measure possible conformational changes are more advanced. The advantage of a direct measurement in emulsions lies in the possibility of studying the physical stability of adsorbed proteins *in situ* at the interface. However, the light scattering properties of emulsions have a strong limiting effect on the usefulness of spectroscopic techniques in such systems, and all methods must be evaluated with great care with respect to this effect. As an example, CD, a popular technique for evaluating protein conformational changes in solutions, is not directly applicable in emulsions without proper preparation of the sample to obtain comparable refractive index values of the two phases [140]. Manipulation of the refractive index has been obtained by addition of glycerol or

polyethylene glycol to the water phase [140]. Another study measured the adsorption-induced destabilization indirectly by monitoring the turbidity of the aqueous phase, as it changes owing to the formation of aggregates [123]. A procedure that circumvents the problems of directly analyzing emulsions is first to generate the emulsion, incorporating the protein, and subsequently to separate the liquid phases before measurement of protein structure. However, the phase separation procedure may itself compromise the protein stability so that, after separation, the protein is not necessarily identical to the protein in intact emulsion.

2.4 Biological activity and immunogenicity

Adsorption will lead to loss of proteins from a solution, and adsorption from solutions of low concentration may lead to a considerable quantitative loss of the protein and thus reduced therapeutic effect. One of the often discussed issues of protein adsorption is whether the protein is active on adsorption or not. Lipases and enzymes are some of the proteins where this can easily be determined. Reis *et al.* have demonstrated that lipase activity as a function of interfacial composition is attributed more to substrate inaccessibility than to enzyme denaturation or inactivation, as is often hypothesized [141]. The impact of protein adsorption on the conformation is strongly related to the reversibility of protein adsorption and the reversibility of the structural changes. Desorption of proteins that have undergone irreversible structural changes or aggregation may lead to considerable loss of therapeutic effect [20], and the altered protein may be immunogenic [142-144].

2.5 Particulate delivery systems for protein drugs

Proteins are exposed to several interfaces during the preparation of particulate delivery systems. As with other surfaces, adsorption at these interfaces can have a detrimental effect on the stability, and may also affect the release of protein from the delivery system. The choice of materials and excipients and the production method used have a huge influence on the incorporation efficiency and the stability of the protein. The physical properties of the particles, for example, charge, hydrophobicity, morphology, surface roughness, curvature and flexibility, also influence the nature and extent of protein adsorption (**Table 1**) [23,145].

2.5.1 *Changes in adsorption due to curvature*

Changes in curvature (i.e., size of nanoparticles) can influence the amount of protein adsorbed, the structure of the adsorbed protein and also the activity of the released protein. For example, when lysozyme was adsorbed to silica nanoparticles, a monolayer was adsorbed on particles with sizes between 20 and 100 nm. The structure of adsorbed lysozyme contained more α-helix compared with lysozyme in solution, and the content of α-helix was higher for lysozyme adsorbed to the larger particles [23]. On gold particles (used for diagnostic or drug delivery purposes), the binding constants of blood proteins have been reported to increase progressively with increasing particle size (5 – 60 nm), but no major perturbation of secondary structure was observed [145]. The size of the protein as well as its flexibility and shape can influence the adsorbed amount and the structural changes that occur on adsorption to curved surfaces. For example, the adsorptions of BSA and fibrinogen were compared at silica particles ranging from 15 to 165 nm [26]. The adsorption trends, that is, adsorbed amount, were the same for the two proteins; but whereas BSA became increasingly less ordered on the surfaces with low curvature, fibrinogen lost secondary structure to a greater extent when adsorbed onto particles with a high surface curvature [26]. At surfaces with a high curvature, small proteins may be able to interact more with the particle and thereby induce large structural perturbations, whereas for larger particles with a lower surface curvature, that is, more closely resembling a flat surface, the structure that adsorbs can be more restricted and thus allow for less structural rearrangement.

2.5.2 *Changes in adsorption and physical stability due to preparation of particles*

In the preparation of solid lipid nanoparticles (SLN), polymeric particles, lipid microparticles or lipospheres, considerable mechanical and thermal stress occurs, in addition to the generation of numerous interfaces [30,51,115,119]. However, the structural changes in incorporated proteins differ depending on the type of particle or incorporation procedure, for example, depending on whether protein is incorporated into poly (lactic-*co*-glycolic acid) (PLGA) particles, SLN or alginate/chitosan particles [51,119,146]. When the structure of lysozyme was compared on incorporation into a water-in-oil emulsion and SLN, it was shown that although the structure changed after incorporation into both systems, the changes were not the same. For lysozyme the same structural changes can be observed after incorporation in SLN as after mixing of SLN with protein, which indicates that the thermal stress of producing SLN did not cause additional structural changes [51].

Interaction between proteins and particulate surfaces may not only alter the release, but also influence characteristics of the particle itself, for example, surface charge and morphology. Adsorption and incomplete release of an incorporated protein may not pose a safety issue, as long as the same amount is adsorbed to the polymer each time and no structurally altered and non-reversible structural changes are induced. In the case of PLGA microparticles, many have reported incomplete protein release and an initial burst release due to surface adsorption. However, by modifying the interaction between protein and surface, through PEGylation of the protein [147], this retention of protein in the polymeric particles could be avoided.

Interaction between phospholipids (e.g., in liposomes) and proteins is a normal event in the lipid membrane of cells, therefore these interactions do not necessarily lead to instability, loss of function or irreversible structural changes.

However, most cellular or membrane proteins have a different structure from pharmaceutical proteins. The interaction is guided by the same forces as for other interfaces, and Zhdanov and Kasemo have introduced a model to describe the interaction between lipid layers and proteins and provide guidelines as to when adsorption and desorption can occur [148,149]. The interaction is based mainly on electrostatic forces, which is also illustrated through the interaction between BSA and a lecithin layer [75], where more is bound in the presence of NaCl than $CaCl_2$.

An example of an alternative preparation procedure that does not cause structural changes of the incorporated protein is the preparation of alginate/chitosan particles using non-organic solvents and gelation for their formation [146]. Another alternative preparation procedure for polymeric particle is interfacial polymerization, where the particles are formed to include the protein. An example is poly(alkylcyanoacrylate) (PACA) particles, where insulin can be incorporated and released, and which can show *in vivo* activity after oral administration [116,150]. The activity of protein in these particles can be compromised by crosslinking the protein with the polymer monomer, as has been shown for D-Lys6-GnRH [151].

2.5.3 *Adsorption as a mechanism to control protein release or activity*

Adsorption of proteins can be utilized in vaccine delivery as a way of controlling the release and modifying the antigens' immunogenicity. For example, 80% adsorption to adjuvants such as Adju-Phos® or Alhydrogel® is required for approval according to EMA guidelines, and this adsorption can be maximized by controlling the phosphate content of the adjuvant [152]. This adsorption renders proteins such as lysozyme, ovalbumin and BSA less physically stable, which is expected to constitute part of the induction of the immune response [153]. For recombinant botulinum neurotoxin (BoNT), the tendency to adsorb to Alhydrogel and aluminum hydroxide adjuvant salts changes during storage of the formulation, but with unknown effects on the efficacy of the vaccine [154]. The adsorption of antigen to adjuvants would be expected to influence the strength of an immune response. Although most often adsorption of therapeutic protein is not wanted and constructs are designed to minimize protein adsorption, it can also be beneficial, for example, for immobilization of antibodies as part of an ELISA assay, where orientation is important [155].

The challenge for preparing particulates lies in devising methods of preparation that do not cause structural changes (i.e., adsorption or other types of stress), but also in understanding what occurs at the interface and adjusting the preparation procedure and excipients accordingly to minimize interfacially induced structural changes in the protein. If problems from protein adsorption are identified, an alternative method of preparation should be devised, either by changing the solvents or by altering excipients; or it should be accepted that adsorption and structural changes occur and safety actions should be taken accordingly.

3. Effects from modifications of protein, addition of excipients or modification of the surfaces

Protein adsorption can be modified by changes in protein structure, interface characteristics or the addition of a more surface-active excipient.

3.1 Effects of modification of proteins

Most of the early protein drugs that were approved were murine monoclonal antibodies or replacements proteins with amino acid sequences identical to those of native proteins [156]. However, over the last 15 years a second generation of protein therapeutics, modified by genetic engineering, has been marketed. These are typically first-generation proteins with an altered amino acid sequence, or proteins to which oligosaccharides (glycosylated), polyethylene glycol chains (PEGylated) or fatty acid (acylated) chains have been covalently attached. The primary rationale for the second-generation protein drugs was to improve the proteins' pharmacokinetic profile, for example by prolonging the plasma half-life. Such modifications alter the overall characteristics of the protein and are therefore likely to affect their adsorption behavior by steric alteration of their interaction with surfaces (**Figure 2**). As adsorption may alter the overall stability of the proteins, it is important to establish the influence of modifications in the structure on protein adsorption behavior.

When the primary structure in a protein is altered, the Gibbs free energy of unfolding and/or the hydrophobicity and thus the surface activity may change. Kato and Yutani illustrated that less physically stable mutants were also the most surface active, that is, they were rapidly adsorbed and/or more readily unfolded or otherwise rearranged at hydrophobic interfaces [157]. In another study, insertions of Trp-repeats were introduced, and an increased adsorption was observed as more Trp residues were introduced [158]. These Trp-repeats were also modified to include changes in charge, where negatively charged repeats did not show adsorption, whereas extensive adsorption was observed for positively charged repeats at a hydrophilic surface. At hydrophobic (and negatively charged) surfaces, the adsorption correlated with peptide stretch charge, where adsorption was lowest from the protein with the more negative insert, as shown in the following sequence: $(AlaTrpTrpAspPro)_n$ < $(AlaTrpTrpPro)_n$ < $(AlaTrpTrpLysPro)_n$ [159]. These examples illustrate that through alternations in charge and hydrophobicity adsorption can be manipulated at any surface. Also, special constructs such as tethered proteins force the protein through their structure, to orientate at the interface in a predetermined manner [160]. This introduces new knowledge; by identifying these tethered constructs and potentially incorporating them into protein structures, adsorbed protein could be forced

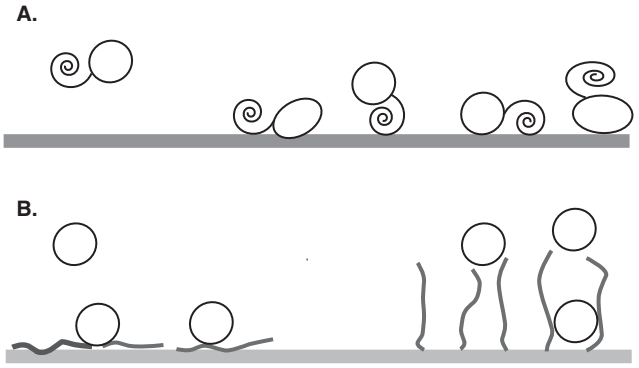

Figure 2. Schematic of protein adsorption. A. Adsorption of protein modified by attachment of a PEG chain or an acyl chain may affect the interaction between the protein and the surface [183]. **B.** Modification of the surface may affect the interaction between the protein and the surface and thereby influence the adsorption.
Adapted in part with permission from [183].

into a predetermined orientation at interfaces [160]. This would indicate that certain kinds of adsorption behavior can be introduced into proteins. In reality, however, the modifications introduced into protein structure are for purposes other than to affect the proteins' adsorption behavior, as in the case of the modification of insulin introduced to modify the association behavior and circulation time. When dealing with these modified proteins it is important to remember that physical properties, such as adsorption behavior, are likely to have been altered from the parent protein. The adsorption pattern of monomeric and hexameric insulin has been studied at hydrophilic and hydrophobic surfaces. At hydrophobic surfaces adsorption behavior was the same for both forms, whereas the monomeric insulin did not adsorb to the hydrophilic surface and hexameric insulin adsorbed only at high solution concentrations [161]. The differences in adsorption of insulin variants were based on association degree [92,161], that is, changes in hydrophobicity. It would be tempting to correlate this change in hydrophobicity to the overall stability, as the hexamer is considered more physically stable than the monomer, and thereby also correlates increased stability to decreased adsorption. This may be the case for some proteins, but not for all. For example, in lipases the polypeptide chain flexibility appears to influence surface adsorption more greatly. The more flexible, less stable and less charged lipase variants adsorb more readily with changes in structure occurring on adsorption to a solid surface, whereas other less flexible and more stable lipases do not adsorb as readily [69]. This indicates that flexibility and charge in parts of the protein can influence adsorption more than the overall structural stability.

Examples of the effect that modifications such as glycosylation, PEGylation and acylation can have on adsorption are included below. Modifications such as glycosylation also alter the charge and hydrophobicity of proteins. The effects on adsorption to solid surfaces have been compared for a glycosylated lipase (with zero, one (the wild type) and five added glycans), and the adsorption to hydrophilic surfaces depended on the number of glycosylations whereas the adsorption to hydrophobic surfaces did not [71], meaning that adsorption depends more on the structure of the inherent protein and surface than on glycosylation. Glycosylation can increase the thickness of the adsorbed layer by altering the packing of the protein at the surface [162], and thereby alter the possibility of structural changes and reversibility of adsorption.

One of the modifications in several marketed protein drug products is PEGylation [163], where a polyethylene glycol chain is covalently attached to the protein. This attachment of PEG to a protein alters the overall size and hydrophobicity of the protein and PEGylation has been shown to decrease the amount of a given protein that adsorbs to hydrophobic surfaces [39,72,90]. PEGylation has also been shown to alter the desorption pattern of lysozyme. For PEGylated lysozyme it has been shown that addition of the PEG allowed desorption to occur more readily from solid surfaces than for the parent non-PEGylated lysozyme [39,90]. For glucagon, a smaller peptide, the introduction of linear or branched PEG chains was compared. From this study it was shown that a difference in adsorbed amount between PEGylated glucagon and non-PEGylated glucagon occurred, but there was no difference between linear or branched PEG [72].

Another more recent modification used in pharmaceutical products is acylation, where a fatty acid chain is added resulting in a more hydrophobic protein. For acylated insulin, the association behavior (ability to form hexamer structures) and the adsorption to hydrophobic surfaces was modified. When insulin was mainly monomers and dimers, more of the acylated insulin adsorbed to the surface, but when insulin and acylated insulin was a mixture of monomer, dimer and hexamer, no differences were observed [164]. Altering the hydrophobicity of a protein can also change its surface activity; the surface activity of IgG increased when it was modified by covalently binding a C8-C16 fatty acid [87], which will also influence its adsorption behavior.

These examples of altered adsorption of chemically modified proteins to surfaces clearly indicate that when changes are made to a protein structure it is important to consider the impact on physical properties such as interactions with interfaces and solid surfaces. The chemical modifications affect protein surface charge, hydrophobicity and polypeptide chain flexibility, and these properties have been shown to be important in the driving forces underlying protein's physical interactions with interfaces.

3.2 Use of excipients

Excipients used to prevent or reduce protein adsorption are either more surface active than the active protein itself (i.e., surfactants), which preferentially adsorbs at the interface, or other proteins are added in excess concentrations to act as

competitive inhibitors of adsorption, for example, serum albumin [165,166].

The conventional surfactants used in pharmaceutical preparations are typically small non-ionic surfactants, and they act by forming surface-active complexes that occupy the interface or compete for space at the interface with already adsorbed protein [19,40,167-170]. It is rarely discussed what mechanism is desired for preventing protein adsorption to interfaces. The issues that should be addressed are: whether the surfactant can displace protein from the interface; whether adsorption can be prevented; whether surfactant–protein complexes are formed in the bulk or at the interface; whether the surfactant–protein complex adsorbs to the interface; and last but not least whether the protein refolds after displacement. Hence, experiments are typically set up in different ways depending on the overall objective of the study. Engel *et al.* have shown that bovine α-lactalbumin can be removed (up to 90%) from a solid polystyrene surface by addition of CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), and that desorbed protein refolded to its native form [171].

Displacement of protein from interfaces has been termed orogenic displacement and it is shown to occur through nucleation and growth of surfactant-rich domains in the adsorbed protein layer. This displacement alters the mechanical properties of the protein layer and is found to be independent of the type of surfactant, which indicates that binding between surfactant and protein does not necessarily influence displacement. For example, non-ionic surfactants (e.g., Tweens) are found to displace protein (β-lactoglobulin and β-casein) at an air–water interface through orogenic displacement, whereas the ionic surfactants appear to influence protein adsorption through mechanisms dependent on electrostatics [172]. Joshi and co-workers and Mollmann and co-workers have studied the efficiency of Tween 80 in displacing lysozyme, Factor VIII and insulin from solid or air–water interfaces, and they all show that Tween 80 was effective below its critical micelle concentration (CMC), but its effectiveness in preventing/removing adsorbed protein increased above the CMC [52,64,107,173]. When Tween 80 was added in high concentration to insulin before adsorption, adsorption was prevented, which could indicate that Tween 80 either adsorbs to the surface to sterically inhibit protein adsorption or that a complex was formed between Tween 80 and the protein in the bulk solution that is less surface active and remains in solution [64].

At the oil–water or organic–water interface generated during the formation of particulate systems, surfactants are used either to displace protein or even to prevent it from adsorbing. For example, adding bulky surface-active excipients to the aqueous phase, which strongly adsorb to the interface and sterically block access to the interface, has been shown to diminish structural changes of the incorporated protein [40,48,174-175]. Also, in the preparation of PLGA particulate systems, surfactant concentration and type can be optimized to increase encapsulation and insulin release [176,177], most probably through modification of the adsorption to the interface. Another strategy to prevent interfacial protein adsorption during PLGA particle preparation is to make it more favorable for the protein to stay in the solution by changing the internal aqueous solution solvent properties, for example, by addition of cyclodextrins [125,134], Tweens or other excipients, such as trehalose, sucrose and mannitol [122,130].

Often, the detergency of surfactants is compared based on their concentration, that is, critical micelle concentration. However, this comparison may not be directly correlated to its efficacy in preventing adsorption unless the CMC is determined in the exact system as the presence of protein will alter the CMC [178]. The results still show a dependency of concentration, with concentrations below CMC rarely being very efficient at displacing proteins from interfaces; but in conjunction with free protein or complex formation it can be enough to prevent adsorption, even below the CMC.

3.3 Modification of the surface

A sorbent surface (and especially the solid) can be modified to make it more biocompatible, anti-fouling or non-fouling, or merely to decrease its potential for protein adsorption. Examples of modified surfaces that are less prone to protein adsorption are surfaces coated with a thin layer of hydrophilic polymers or modified by PEGylation [179-181]. These surface modifications alter the interaction between the surface and the protein by sterically hindering or altering the interaction (**Figure 2**) [181-183]. Some surfaces are specifically designed as anti-fouling surfaces, where the surface is able to resist protein adsorption [182,184]. The biocompatibility of surfaces is often tested by determining the competitive adsorption between plasma proteins, for example, ubiquitin, human serum albumin, prothrombin, IgG and fibrinogen at solid interfaces [37], where a well-designed biocompatible surface controls adsorption of protein [185].

4. Expert opinion

Protein drugs encounter interfaces during purification of the protein drug, preparation of the drug product, processing into delivery systems, storage and use. As protein adsorption is related to the stability, efficacy and safety of the drug product, protein adsorption must be evaluated during formulation of a drug product. It is especially important to study whether the secondary and tertiary structures are affected on adsorption, whether the adsorption is irreversible, whether exchange can occur between absorbed protein and protein in bulk solution, whether structural changes occur on adsorption and, if they do, whether these structural changes are then reversible on desorption, and finally whether aggregation can be initiated at the surface.

Protein adsorption has been studied comprehensively over recent years and a lot of new information has been obtained. However, it is difficult to link the knowledge obtained about adsorption of individual proteins to

general knowledge about pharmaceutically relevant protein adsorption. It is desirable to be able to predict the adsorption of a protein in a given system, but also to be able to predict the impact protein adsorption in a certain system would have on protein stability, efficacy and immunogenicity. Moreover, as more antibodies are entering clinical trials [186], it would be desirable to be able to predict these effects for large proteins as well.

Meanwhile, a comprehensive protein adsorption theory is still missing; the complexity of proteins, interfaces and delivery systems keeps increasing. In the pharmaceutical field, the focus for future protein adsorption studies should be on effects of modification of the protein backbone and attachment of PEG, acyl chains or glycan moieties, and on the composition of the variety of delivery systems where effects from adsorption is one of the issues that is often not addressed. Also, the effect of modifications of substrates with the aim of reducing adsorption and minimizing structural changes on adsorption or biocompatibility of implantable devices should be in focus. An example of recent advances of interest is the design of bio-inspired surfaces in nanodevices, where the activity of a protein is retained even after immobilization on a surface [187]. This could be especially interesting in the context of ensuring activity of proteins in devices, implants or in assay technology.

Protein adsorption is to be expected at all types of interface. However, the effect on the native structure as well as the reversibility of the adsorption and hence the effect on stability, efficacy and safety must be evaluated in each case.

In future research on protein interactions with surfaces, it will be important to look for correlations between surface effects and the structural properties of proteins. One of the most promising attempts at protein classification has been into 'hard' or 'soft' proteins. These terms have specific meaning that relates to the potential of the protein to interact in specific ways with surfaces. Within each of these groups, there is likely to be a possible ranking of proteins, and perhaps available proteins can be placed along a spectrum from 'hard' to 'soft'.

Declaration of interest

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Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Rabe M, Verdes D, Seeger S. Understanding protein adsorption phenomena at solid surfaces. Adv Colloid Interface Sci 2011;8:87-106

•• **Very recent review on adsorption to solid surfaces.**

2. Haynes CA, Norde W. Globular proteins at solid/liquid interfaces. Colloids Surf B Biointerfaces 1994;2:517-66

3. Norde W. Adsorption of proteins from solution at the solid-liquid interface. Adv Colloid Interface Sci 1986;25:267-340

4. Arai T, Norde W. The behaviour of some model proteins at solid-liquid interfaces 1. adsorption from single protein solutions. Colloids Surf 1990;51:1-15

5. Norde W. Energy and entropy of protein adsorption. J Dispersion Sci Technol 1992;13:363-77

6. Norde W. Driving forces for protein adsorption at solid surfaces. In: Malmsten M, editor, Biopolymers at interfaces. Marcel Dekker, New York; 2003. p. 21-43

7. Norde W, Buijs J, Lyklema J. Adsorption of globular proteins. Fundam Interface Colloid Sci 2005;5:1-59

8. Norde W, Giacomelli CE. BSA structural changes during homomolecular exchange between the adsorbed and the dissolved states. J Biotechnol 2000;79:259-68

9. Norde W, MacRitchie F, Nowicka G, et al. Protein adsorption at solid-liquid interfaces: reversibility and conformation aspects. J Colloid Interface Sci 1986;112:447-56

10. Ramsden JJ. Puzzles and paradoxes in protein adsorption. Chem Soc Rev 1995;24:73-8

• **Illustration of the puzzling task of addressing protein adsorption.**

11. MacRitchie F. Proteins at interfaces. Adv Protein Chem 1978;32:283-326

12. Hlady V, Buijs J. Protein adsorption on solid surfaces. Brain Res 1996;7:72-7

13. Gray JJ. The interaction of proteins with solid surfaces. Curr Opin Struct Biol 2004;14:110-15

14. Wahlgren M, Arnebrant T. Protein adsorption to solid surfaces. Trends Biotechnol 1991;9:201-8

15. Sadana A. Protein adsorption and inactivation on surfaces. Influence of heterogeneities. Chem Rev 1992;92:1799-818

16. Malmsten M. Formation of adsorbed protein layers. J Colloid Interface Sci 1998;207:186-99

17. Norde W, Lyklema J. Why proteins prefer interfaces. J Biomater Sci Polym Ed 1991;2:183-202

•• **Excellent review on protein adsorption.**

18. Norde W. My voyage of discovery to proteins in flatland. and beyond. Colloids Surf B Biointerfaces 2008;61:1-9

•• **Excellent review on basic protein adsorption.**

19. Kotsmar C, Pradines V, Alahverdjieva VS, et al. Thermodynamics, adsorption kinetics and rheology of mixed protein-surfactant interfacial layers. Adv Colloid Interface Sci 2009;150:41-54

•• **Excellent review on protein adsorption to liquid–liquid interfaces.**

20. Tzannis ST, Hrushesky WJM, Wood PA, et al. Irreversible inactivation of interleukin 2 in a pump-based delivery environment. Proc Natl Acad Sci U S A 1996;93:5460-5

21. Zoungrana T, Findenegg GH, Norde W. Structure, stability, and activity of

adsorbed enzymes. J Colloid Interface Sci 1997;190:437-48

22. Koutsopoulos S, Patzsch K, Bosker WT, et al. Adsorption of trypsin on hydrophilic and hydrophobic surfaces. Langmuir 2007;23:2000-6
23. Vertegel AA, Siegel RW, Dordick JS. Silica nanoparticle size influences the structure and enzymatic activity of adsorbed lysozyme. Langmuir 2004;20:6800-7
24. Bellezza F, Cipiciani A, Quotadamo MA, et al. Structure, stability, and activity of myoglobin adsorbed onto phosphate-grafted zirconia nanoparticles. Langmuir 2007;23:13007-12
25. Anand G, Sharma S, Dutta AK, et al. Conformational transitions of adsorbed proteins on surfaces of varying polarity. Langmuir 2010;26:10803-11
26. Roach P, Farrar D, Perry CC. Surface tailoring for controlled protein adsorption: effect of topography at the nanometer scale and chemistry. J Am Chem Soc 2006;128:3939-45
27. Rechendorff K, Hovgaard MB, Foss M, et al. Enhancement of protein adsorption induced by surface roughness. Langmuir 2006;22:10885-8
28. Kato K, Sano S, Ikada Y. Protein adsorption onto ionic surfaces. Colloids Surf B Biointerfaces 1995;4:221-30
29. Roach P, Farrar D, Perry CC. Interpretation of protein adsorption: surface-induced conformational changes. J Am Chem Soc 2005;127:8168-73
30. Almeida AJ, Souto E. Solid lipid nanoparticles as a drug delivery system for peptides and proteins. Adv Drug Deliv Rev 2007;59:478-90
31. Ludwig DB, Carpenter JF, Hamel JB, et al. Protein adsorption and excipient effects on kinetic stability of silicone oil emulsions. J Pharm Sci 2010;99:1721-33

• **An interesting study addressing an interesting issue in protein formulation.**

32. Nayak A, Dutta AK, Belfort G. Surface-enhanced nucleation of insulin amyloid fibrillation. Biochem Biophys Res Commun 2008;369:303-7
33. Kleijn M, Norde W. The adsorption of proteins from aqueous solution on solid surfaces. Hetero Chem Rev 1995;2:157-72
34. Lu DR, Lee SJ, Park K. Calculation of solvation interaction energies for protein adsorption on polymer surfaces. J Biomater Sci Polym Ed 1991;3:127-47
35. Liu SM, Haynes CA. Energy landscapes for adsorption of a protein-like HP chain as a function of native-state stability. J Colloid Interface Sci 2005;284:7-13
36. Baldursdottir SG, Fullerton MS, Nielsen SH, et al. Adsorption of proteins at the oil/water interface-Observation of protein adsorption by interfacial shear stress measurements. Colloids Surf B Biointerfaces 2010;79:41-6

• **A detailed study on the use of rheology to study protein adsorption.**

37. Barnthip N, Parhi P, Golas A, et al. Volumetric interpretation of protein adsorption: kinetics of protein-adsorption competition from binary solution. Biomaterials 2009;30:6495-513
38. Daly SM, Przybycien TM, Tilton RD. Adsorption of Poly(ethylene glycol)-modified lysozyme to silica. Langmuir 2005;21:1328-37
39. Daly SM, Przybycien TM, Tilton RD. Aggregation of lysozyme and of poly (ethylene glycol)-modified lysozyme after adsorption to silica. Colloids Surf B Biointerfaces 2007;57:81-8

• **Adsorption of PEGylated protein to solid surfaces.**

40. Day JPR, Pudney PDA, Bain CD. Ellipsometric study of the displacement of milk proteins from the oil-water interface by the non-ionic surfactant C10E8. Phys Chem Chem Phys 2010;12:4590-9
41. Denis FA, Hanarp P, Sutherland DS, et al. Protein adsorption on model surfaces with controlled nanotopography and chemistry. Langmuir 2002;18:819-28
42. Dolatshahi-Pirouz A, Rechendorff K, Hovgaard MB, et al. Bovine serum albumin adsorption on nano-rough platinum surfaces studied by QCM-D. Colloids Surf B Biointerfaces 2008;66:53-9
43. Freij-Larsson C, Nylander T, Jannasch P, et al. Adsorption behaviour of amphiphilic polymers at hydrophobic surfaces: effects on protein adsorption. Biomaterials 1996;17:2199-207
44. Green RJ, Davies MC, Roberts CJ, et al. Competitive protein adsorption as observed by surface plasmon resonance. Biomaterials 1999;20:385-91
45. Green RJ, Hopkinson I, Jones RAL. Unfolding and intermolecular association in globular proteins adsorbed at interfaces. Langmuir 1999;15:5102-10
46. Guicai L, Xiaoli S, Ping Y, et al. Investigation of fibrinogen adsorption on solid surface by quartz crystal microbalance with dissipation(QCM-D) and ELISA. Solid State Ion 2008;179:932-5
47. Hoehne M, Fauna S, Dong A, et al. Adsorption of monoclonal antibodies to glass microparticles. J Pharm Sci 2010;100(1):123-32
48. Jorgensen L, van de Weert M, Bjerregaard S, et al. Probing structural changes of proteins incorporated into water-in-oil emulsions. J Pharm Sci 2004;93:1847-59
49. Jorgensen L, Kim DH, Vermehren C, et al. Micropipette manipulation a technique to evaluate the stability of water-in-oil emulsions containing proteins. J Pharm Sci 2004;93:2995-3003
50. Jorgensen L, Wood GK, Rosenkrands I, et al. Protein adsorption and displacement at lipid layers determined by total internal reflection fluorescence (TIRF). J Liposome Res 2009;19:99-104
51. Jorgensen L, Martins S, van de Weert M. Analysis of protein physical stability in lipid based delivery systems – the challenges of lipid drug delivery systems. J Biomed Nanotechnol 2009;5:401-8
52. Joshi O, McGuire J. Adsorption behavior of lysozyme and Tween 80 at hydrophilic and hydrophobic silica-water interfaces. Appl Biochem Biotechnol 2009;152:235-48
53. Kamilya T, Pal P, Talapatra GB. Interaction of ovalbumin with phospholipids langmuir-blodgett film. J Phys Chem B 2007;111:1199-205
54. Kapilashrami A, Malmsten M, Eskilsson K, et al. Ellipsometric studies of nonionic block copolymers adsorbed at the solid/water and oil/water interfaces. Colloid Surf A Phys Chem Eng Aspects 2003;225:181-92
55. Karlsson M, Ekeroth J, Elwing H, et al. Reduction of irreversible protein adsorption on solid surfaces by protein

engineering for increased stability. J Biol Chem 2005;280:25558-64

56. Knudsen JC, Ogendal LH, Skibsted Droplet surface properties and rheology of concentrated oil in water emulsions stabilized by heat-modified b-lactoglobulin B. Langmuir 2008;24:2603-10

57. Kudryashova EV, de Jongh HHJ. Modulation of the adsorption properties at air-water interfaces of complexes of egg white ovalbumin with pectin by the dielectric constant. J Colloid Interface Sci 2008;318:430-9

58. Lad MD, Birembaut F, Matthew JM, et al. The adsorbed conformation of globular proteins at the air/water interface. Phys Chem Chem Phys 2006;8:2179-86

59. Lee MH, Reich DH, Stebe KJ, et al. Combined passive and active microrheology study of protein-layer formation at an air-water interface. Langmuir. 2009;26:2650-8

60. Leick S, Degen P, Kohler B, et al. Film formation and surface gelation of gelatin molecules at the water/air interface. Phys Chem Chem Phys 2009;11:2468-74

61. Lu JR, Zhao XB, Yaseen M. Protein adsorption studied by neutron reflection. Curr Opin Colloid Interface Sci 2007;12:9-16

62. Lundin M, Elofsson UM, Blomberg E, et al. Adsorption of lysozyme, [beta]-casein and their layer-by-layer formation on hydrophilic surfaces: effect of ionic strength. Colloids Surf B Biointerfaces 2010;77:1-11

63. Makie AR. Structure of adsorbed layers of mixtures of proteins and surfactants. Curr Opin Colloid Interface Sci 2004;9(5):357-61

64. Mollmann SH, Elofsson U, Bukrinsky JT, et al. Displacement of adsorbed insulin by Tween 80 monitored using total internal reflection fluorescence and ellipsometry. Pharm Res 2005;22:1931-41

65. Mollmann SH, Jorgensen L, Bukrinsky JT, et al. Interfacial adsorption of insulin - Conformational changes and reversibility of adsorption. Eur J Pharm Sci 2006;27:194-204

66. Nejadnik MR, Garcia CD. Staining proteins: a simple method to increase the sensitivity of ellipsometric measurements in adsorption studies. Colloids Surf B Biointerfaces 2011;82:253-7

67. Noh H, Vogler EA. Volumetric interpretation of protein adsorption: mass and energy balance for albumin adsorption to particulate adsorbents with incrementally increasing hydrophilicity. Biomaterials 2006;27:5801-12

68. Noh H, Vogler EA. Volumetric interpretation of protein adsorption: partition coefficients, interphase volumes, and free energies of adsorption to hydrophobic surfaces. Biomaterials 2006;27:5780-93

69. Otzen D. Differential adsorption of variants of the Thermomyces lanuginosus lipase on a hydrophobic surface suggests a role for local flexibility. Colloids Surf B Biointerfaces 2008;64:223-8

70. Parhi P, Golas A, Barnthip N, et al. Volumetric interpretation of protein adsorption: Capacity scaling with adsorbate molecular weight and adsorbent surface energy. Biomaterials 2009;30:6814-24

71. Pinholt C, Fano M, Wiberg C, et al. Influence of glycosylation on the adsorption of Thermomyces lanuginosus lipase to hydrophobic and hydrophilic surfaces. Eur J Pharm Sci 2010;40:273-81

- **Effect from glycosylation on adsorption to solid surfaces.**

72. Pinholt C, Bukrinsky JT, Hostrup S, et al. Influence of PEGylation with linear and branched PEG chains on the adsorption of glucagon to hydrophobic surfaces. Eur J Pharm Biopharm 2011;77:139-47

- **Effect from PEGylation on adsorption to solid surfaces.**

73. Sankaranarayanan K, Dhathathreyan A, Miller R. Assembling fibrinogen at air/water and solid/liquid interfaces using langmuir and langmuir-blodgett films. J Phys Chem B 2010;114:8067-75

74. Sonesson AW, Callisen TH, Brismar H, et al. A comparison between dual polarization interferometry (DPI) and surface plasmon resonance (SPR) for protein adsorption studies. Colloids Surf B Biointerfaces 2007;54:236-40

75. Tantipolphan R, Rades T, McQuillan AJ, et al. Adsorption of bovine serum albumin (BSA) onto lecithin studied by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. Int J Pharm 2007;337:40-7

76. Thomsen AE, Jensen H, Jorgensen L, et al. Studies on human insulin adsorption kinetics at an organic-aqueous interface determined using a label-free electroanalytical approach. Colloids Surf B Biointerfaces 2008;63:243-8

77. Zareh SK, Wang YM. Single-molecule imaging of protein adsorption mechanisms onto surfaces. Microsc Res Tech 17 Nov 2010. [Epub ahead of print]

78. Jorgensen L, Bennedsen P, Hoffmann VN Sr, et al. Adsorption of insulin with varying self-association profiles to a solid Teflon surface–Influence on protein structure, fibrillation tendency and thermal stability. Eur J Pharm Sci 2011;42(5):509-16

79. Bee JS, Davis M, Freund E, et al. Aggregation of a monoclonal antibody induced by adsorption to stainless steel. Biotechnol Bioeng 2010;105:121-9

80. Schwegman JJ, Carpenter JF, Nail SL. Evidence of partial unfolding of proteins at the ice/freeze-concentrate interface by infrared microscopy. J Pharm Sci 2009;98:3239-46

81. Cochran T, Nail SL. Ice nucleation temperature influences recovery of activity of a model protein after freeze drying. J Pharm Sci 2009;98:3495-8

82. Linse S, Cabaleiro-Lago C, Xue W-F, et al. Nucleation of protein fibrillation by nanoparticles. Proc Natl Acad Sci U S A 2007;104:8691-6

83. Clarkson JR, Cui ZF, Darton RC. Protein denaturation in foam - II. Surface activity and conformational change. J Colloid Interface Sci 1999;215:333-8

84. Clarkson JR, Cui ZF, Darton RC. Effect of solution conditions on protein damage in foam. Biochem Eng J 2000;4:107-14

85. Nielsen L, Khurana R, Coats A, et al. Effect of environmental factors on the kinetics of insulin fibril formation: elucidation of the molecular mechanism. Biochemistry 2001;40:6036-46

86. Sluzky V, Klibanov AM, Langer R. Mechanism of insulin aggregation and stabilization in agitated aqueous-solutions. Biotechnol Bioeng 1992;40:895-903

87. Kamyshny A, Magdassi S, Relkin P. Chemically modified human immunoglobulin G: hydrophobicity and surface activity at air/solution interface. J Colloid Interface Sci 1999;212:74-80

88. Yano YF, Uruga T, Tanida H, et al. Driving force behind adsorption-induced protein unfolding: a time-resolved x-ray reflectivity study on lysozyme adsorbed at an air/water interface. Langmuir 2008;25:32-5

89. Norde W, Favier JP. Structure of adsorbed and desorbed proteins. Colloid Surf 1992;64:87-93

•• **One of a few studies addressing structure of desorbed protein.**

90. Pai SS, Przybycien TM, Tilton RD. Protein PEGylation attenuates adsorption and aggregation on a negatively charged and moderately hydrophobic polymer surface. Langmuir 2010;26:18231-8

91. Daly SM, Przybycien TM, Tilton RD. Adsorption of poly(ethylene glycol)-modified ribonuclease A to a poly (lactide-co-glycolide) surface. Biotechnol Bioeng 2005;90:856-68

92. Mollmann SH, Bukrinsky JT, Frokjaer S, et al. Adsorption of human insulin and Asp(B28) insulin on a PTFE-like surface. J Colloid Interface Sci 2005;286:28-35

93. Michaeli I, Absolom DR, Van Oss CJ. Diffusion of adsorbed protein within the plane of adsorption. J Colloid Interface Sci 1980;77:586-7

94. Daly SM, Przybycien TM, Tilton RD. Coverage-dependent orientation of lysozyme adsorbed on silica. Langmuir 2003;19:3848-57

95. Brosilow BJ, Ziff RM, Vigil RD. Random sequential adsorption of parallel squares. Phys Rev A 1991;43:631-8

96. Feder J. Random sequential adsorption. J Theor Biol 1980;87:237-54

97. Hinrichsen EL, Feder J, Jossang T. Geometry of random sequential adsorption. J Stat Phys 1986;44:793-827

98. Tanemura M. Random complete packing by disks. Ann I Stat Math 1979;31:351-65

99. Holmberg M, Hou X. Competitive protein adsorption- multilayer adsorption and surface induced protein aggregation. Langmuir 2009;25:2081-9

100. Tyagi AK, Randolph TW, Dong A, et al. IgG particle formation during filling pump operation: a case study of heterogeneous nucleation on stainless steel nanoparticles. J Pharm Sci 2009;98:94-104

101. Andrade JD. Principles of protein adsorption. InAndrade JD, editor, Surface and interfacial aspects of biomedical polymers. Plenum Press, New York; 1985. p. 1-80

102. Soderquist ME, Walton AG. Structural-changes in proteins adsorbed on polymer surfaces. J Colloid Interface Sci 1980;75:386-97

103. Lundstrom I, Elwing H. Simple kinetic-models for protein exchange-reactions on solid-surfaces. J Colloid Interface Sci 1990;136:68-84

104. Kondo A, Oku S, Higashitani K. Structural-changes in protein molecules adsorbed on ultrafine silica particles. J Colloid Interface Sci 1991;143:214-21

105. Wu XY, Narsimhan G. Effect of surface concentration on secondary and tertiary conformational changes of lysozyme adsorbed on silica nanoparticles. Biochim Biophys Acta Proteins Proteomics 2008;1784:1694-701

106. Singla B, Krisdhasima V, McGuire J. Adsorption kinetics of wild type and two synthetic stability mutants of T4 phage lysozyme at silanized silica surfaces. J Colloid Interface Sci 1996;182:292-6

107. Joshi O, McGuire J, Wang DQ. Adsorption and function of recombinant Factor VIII at solid-water interfaces in the presence of Tween-80. J Pharm Sci 2008;97:4741-55

108. Ueberbacher R, Haimer E, Hahn R, et al. Hydrophobic interaction chromatography of proteins - V. Quantitative assessment of conformational changes. J Chromatogr A 2008;1198:154-63

109. Chi EY, Weickmann J, Carpenter JF, et al. Heterogeneous nucleation-controlled particulate formation of recombinant human platelet-activating factor acetylhydrolase in pharmaceutical formulation. J Pharm Sci 2005;94:256-74

110. Cabaleiro-Lago C, Quinlan-Pluck F, Lynch I, et al. Inhibition of amyloid b protein fibrillation by polymeric nanoparticles. J Am Chem Soc 2008;130:15437-43

111. Vermonden T, Giacomelli CE, Norde W. Reversibility of structural rearrangements in bovine serum albumin during homomolecular exchange from AgI particles. Langmuir 2001;17:3734-40

112. Nonckreman ClJ, Rouxhet PG, Dupont-Gillain CC. Dual radiolabeling to study protein adsorption competition in relation with hemocompatibility. J Biomed Mater Res 2007;81A:791-802

113. Gurdak E, Dupont-Gillain CC, Booth J, et al. Resolution of the vertical and horizontal heterogeneity of adsorbed collagen layers by combination of QCM-D and AFM. Langmuir 2005;21:10684-92

114. Ho HO, Hsiao CC, Sheu MT. Preparation of microemulsions using polyglycerol fatty acid esters as surfactant for the delivery of protein drugs. J Pharm Sci 1996;85:138-43

115. Bilati U, Allemann E, Doelker E. Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microspheres. Eur J Pharm Biopharm 2005;59:375-88

116. Graf A, McDowell A, Rades T. Poly (alkycyanoacrylate) nanoparticles for enhanced delivery of therapeutics - is there real potential? Expert Opin Drug Deliv 2009;6:371-87

117. van de Weert M, Hoechstetter J, Hennink WE, et al. The effect of a water/organic solvent interface on the structural stability of lysozyme. J Control Release 2000;68:3-9

• **A good study showing the effect of liquid–liquid interfaces on protein conformation.**

118. Vemuri S, Rhodes CT. Preparation and characterization of liposomes as therapeutic delivery systems: a review. Pharm Acta Helv 1995;70:95-111

119. van de Weert M, Hennink WE, Jiskoot W. Protein instability in poly (lactic-co-glycolic acid) microparticles. Pharm Res 2000;17:1159-67

120. Watnasirichaikul S, Davies NM, Rades T, et al. Preparation of biodegradable insulin nanocapsules from biocompatible microemulsions. Pharm Res 2000;17:684-9

121. Dickinson E. Proteins at interfaces and in emulsions - stability, rheology and interactions. J Chem Soc Faraday Trans 1998;94:1657-69

122. Cleland JL, Jones AJS. Stable formulations of recombinant human growth hormone and interferon-gamma

for microencapsulation in biodegradable microspheres. Pharm Res 1996;13:1464-75

123. Kwon YM, Baudys M, Knutson K, et al. In situ study of insulin aggregation induced by water-organic solvent interface. Pharm Res 2001;18:1754-9

124. Sah H. Protein behavior at the water/methylene chloride interface. J Pharm Sci 1999;88:1320-5

125. Morlock M, Koll H, Winter G, et al. Microencapsulation of rh-erythropoietin, using biodegradable poly(,-lactide-co-glycolide): protein stability and the effects of stabilizing excipients. Eur J Pharm Biopharm 1997;43:29-36

126. Raghuvanshi RS, Goyal S, Singh O, et al. Stabilization of dichloromethane-induced protein denaturation during microencapsulation. Pharm Dev Technol 1998;3:269-76

127. Uchida T, Shiosaki K, Nakada Y, et al. Microencapsulation of hepatitis B core antigen for vaccine preparation. Pharm Res 1998;15:1708-13

128. Sah H. Protein instability toward organic solvent/water emulsification: implications for protein microencapsulation into microspheres. PDA J Pharm Sci Technol 1999;53:3-10

129. Alonso MJ, Gupta RK, Min C, et al. Biodegradable microspheres as controlled-release tetanus toxoid delivery systems. Vaccine 1994;12:299-306

130. Sturesson C, Carlfors J. Incorporation of protein in PLG-microspheres with retention of bioactivity. J Control Release 2000;67:171-8

131. Hickel A, Radke CJ, Blanch HW. Role of organic solvents on Pa-hydroxynitrile lyase interfacial activity and stability. Biotechnol Bioeng 2001;74:18-28

132. Ghatorae AS, Guerra MJ, Bell G, et al. Immiscible organic-solvent inactivation of urease, chymotrypsin, lipase, and ribonuclease - separation of dissolved solvent and interfacial effects. Biotechnol Bioeng 1994;44:1355-61

133. Zambaux MF, Bonneaux F, Gref R, et al. Preparation and characterization of protein C-loaded PLA nanoparticles. J Control Release 1999;60:179-88

134. Sah H. Stabilization of proteins against methylene chloride water interface-induced denaturation and aggregation. J Control Release 1999;58:143-51

135. Ghaderi R, Carlfors J. Biological activity of lysozyme after entrapment in poly (d,l-lactide-co-glycolide)-microspheres. Pharm Res 1997;14:1556-62

136. Jin T, Zhu HH, Wu F, et al. Preparing polymer-based sustained-release systems without exposing proteins to water-oil or water-air interfaces and cross-linking reagents. J Control Release 2008;128:50-9

137. Stenekes RJH, Franssen O, van Bommel EMG, et al. The preparation of dextran microspheres in an all-aqueous system: effect of the formulation parameters on particle characteristics. Pharm Res 1998;15:557-61

138. Beverung CJ, Radke CJ, Blanch HW. Protein adsorption at the oil/water interface: characterization of adsorption kinetics by dynamic interfacial tension measurements. Biophys Chem 1999;81:59-80

•• **Excellent overview of protein adsorption to liquid–liquid interfaces.**

139. Hartvig RA, Mendez MA, van de Weert M, et al. Interfacial complexes between a protein and lipophilic ions at an oil-water interface. Anal Chem 2010;82:7699-705

140. Husband FA, Garrood MJ, Mackie AR, et al. Adsorbed protein secondary and tertiary structures by circular dichroism and infrared spectroscopy with refractive index matched emulsions. J Agric Food Chem 2001;49:859-66

141. Reis P, Holmberg K, Watzke H, et al. Lipases at interfaces: a review. Adv Colloid Interface Sci 2009;147-48:237-50

142. Kessler M, Goldsmith D, Schellekens H. Immunogenicity of biopharmaceuticals. Nephrol Dial Transpl 2006;21:9-12

143. Rosenberg AS. Effects of protein aggregates: an immunologic perspective. AAPS J 2006;8:E501-7

144. Carpenter JF, Randolph TW, Jiskoot W, et al. Overlooking subvisible particles in therapeutic protein products: gaps that may compromise product quality. J Pharm Sci 2009;98:1201-5

145. Lacerda SHD, Park JJ, Meuse C, et al. Interaction of gold nanoparticles with common human blood proteins. ACS Nano 2009;4:365-79

146. Sarmento B, Ferreira DC, Jorgensen L, et al. Probing insulin's secondary structure after entrapment into alginate/chitosan nanoparticles. Eur J Pharm Biopharm 2007;65:10-17

147. Pai SS, Tilton RD, Przybycien TM. Poly (ethylene glycol)-modified proteins: implications for poly(lactide-co-glycolide)-based microsphere delivery. AAPS J 2009;11:88-98

148. Zhdanov VP, Kasemo B. Protein adsorption and desorption on lipid bilayers. Biophys Chem 2010;146:60-4

149. Zhdanov VP, Kasemo B. Adsorption of proteins on a lipid bilayer. Eur Biophys J 2010;39:1477-82

150. Graf A, Rades T, Hook SM. Oral insulin delivery using nanoparticles based on microemulsions with different structure-types: optimisation and in vivo evaluation. Eur J Pharm Sci 2009;37:53-61

151. Kafka AP, Kleffmann T, Rades T, et al. Histidine residues in the peptide D-Lys (6)-GnRH: potential for copolymerization in polymeric nanoparticles. Mol Pharm 2009;6:1483-91

152. Iyer S, HogenEsch H, Hem SL. Effect of the degree of phosphate substitution in aluminum hydroxide adjuvant on the adsorption of phosphorylated proteins. Pharm Dev Technol 2003;8:81-6

153. Jones LS, Peek LJ, Power J, et al. Effects of adsorption to aluminum salt adjuvants on the structure and stability of model protein antigens. J Biol Chem 2005;280:13406-14

154. Vessely C, Estey T, Randolph TW, et al. Stability of a trivalent recombinant protein vaccine formulation against botulinum neurotoxin during storage in aqueous solution. J Pharm Sci 2009;98:2970-93

155. Pereira Arias-Bouda LM, Kuijper S, Deutekom HV, et al. Enzyme-linked immunosorbent assays using immune complexes for the diagnosis of tuberculosis. J Immunol Methods 2003;283:115-24

156. Walsh G. Second-generation biopharmaceuticals. Eur J Pharm Biopharm 2004;58:185-96

157. Kato A, Yutani K. Correlation of surface properties with conformational stabilities of wild-type and six mutant tryptophan

synthase a subunits substituted at the same position. Protein Eng 1988;2:153-6

158. Malmsten M, Veide A. Effects of amino acid composition on protein adsorption. J Colloid Interface Sci 1996;178:160-7

159. Malmsten M, Burns N, Veide A. Electrostatic and hydrophobic effects of oligopeptide insertions on protein adsorption. J Colloid Interface Sci 1998;204:104-11

160. Knotts TA IV, Rathore N, de Pablo JJ. An entropic perspective of protein stability on surfaces. Biophys J 2008;94:4473-83

161. Nilsson P, Nylander T, Havelund S. Adsorption of insulin on solid surfaces in relation to the surface properties of the monomeric and oligomeric forms. J Colloid Interface Sci 1991;144:145-52

162. Leaver J, Brooksbank DV, Horne DS. Influence of glycosylation and surface charge on the binding of [kappa]-casein to latex particles. J Colloid Interface Sci 1994;162:463-9

163. Kang JS, DeLuca PP, Lee KC. Emerging PEGylated drugs. Expert Opin Emerg Drugs 2009;14:363-80

164. Pinholt C, Hostrup S, Bukrinsky JT, et al. Influence of acylation on the adsorption of insulin to hydrophobic surfaces. Pharm Res 2011;28(5):1031-40

•• **Recent example of adsorption of modified proteins to solid surfaces.**

165. Hawe A, Frieß W. Formulation development for hydrophobic therapeutic proteins. Pharm Dev Technol 2007;12:223-37

166. Jorgensen L, Hostrup S, Moeller EH, et al. Recent trends in stabilising peptides and proteins in pharmaceutical formulation - considerations in the choice of excipients. Expert Opin Drug Deliv 2009;6:1219-30

167. Pradines V, Fainerman VB, Aksenenko EV, et al. Adsorption of protein-surfactant complexes at the water/oil interface. Langmuir 2010;27:965-71

168. Pradines V, Kragel J, Fainerman VB, et al. Interfacial properties of mixed b-Lactoglobulin-SDS layers at the water/air and water/oil interface. J Phys Chem B 2008;113:745-51

169. Miller R, Fainerman VB, Makievski AV, et al. Dynamics of protein and mixed protein/surfactant adsorption layers at the water/fluid interface. Adv Colloid Interface Sci 2000;86:39-82

170. Fainerman VB, Lucassen-Reynders E, Miller R. Adsorption of surfactants and proteins at fluid interfaces. Colloid Surface A Phys chem Eng Aspects 1998;143:141-65

171. Engel MFM, Visser AJWG, van Mierlo CPM. Refolding of adsorbed bovine alpha-lactalbumin during surfactant induced displacement from a hydrophobic interface. Langmuir 2003;19:2929-37

172. Gunning PA, Mackie AR, Gunning AP, et al. The effect of surfactant type on protein displacement from the air-water interface. Food Hydrocoll 2004;18:509-15

173. Joshi O, Chu LP, McGuire J, et al. Adsorption and function of recombinant Factor VIII at the air-water interface in the presence of Tween 80. J Pharm Sci 2009;98:3099-107

174. Taluja A, Bae YH. Role of a novel excipient poly(ethylene glycol)-b-poly(L-histidine) in retention of physical stability of insulin at aqueous/organic interface. Mol Pharm 2007;4:561-70

175. Pean JM, Boury F, Venier-Julienne MC, et al. Why does PEG 400 co-encapsulation improve NGF stability and release from PLGA biodegradable microspheres? Pharm Res 1999;16:1294-9

176. Rosa GD, Iommelli R, La Rotonda MI, et al. Influence of the co-encapsulation of different non-ionic surfactants on the properties of PLGA insulin-loaded microspheres. J Control Release 2000;69:283-95

177. Hamishehkar H, Emami J, Najafabadi AR, et al. The effect of formulation variables on the characteristics of insulin-loaded poly (lactic-co-glycolic acid) microspheres prepared by a single phase oil in oil solvent evaporation method. Colloids Surf B Biointerfaces 2009;74:340-9

178. Ruiz-Pena M, Oropesa-Nunez R, Pons T, et al. Physico-chemical studies of molecular interactions between non-ionic surfactants and bovine serum albumin. Colloids Surf B Biointerfaces 2010;75:282-9

179. Farrell M, Beaudoin S. Surface forces and protein adsorption on dextran- and polyethylene glycol-modified polydimethylsiloxane. Colloids Surf B Biointerfaces 2010;81:468-75

180. Fristrup CJ, Jankova K, Hvilsted S. Surface-initiated atom transfer radical polymerization-a technique to develop biofunctional coatings. Soft Matter 2009;5:4623-34

• **Recent example on modification of surfaces.**

181. Malmsten M, Van Alstine JM. Adsorption of poly(ethylene glycol) amphiphiles to form coatings which inhibit protein adsorption. J Colloid Interface Sci 1996;177:502-12

182. Holmberg M, Hou X. Competitive protein adsorption of albumin and immunoglobulin G from human serum onto polymer surfaces. Langmuir 2009;26:938-42

183. Satulovsky J, Carignano MA, Szleifer I. Kinetic and thermodynamic control of protein adsorption. Proc Natl Acad Sci U S A 2000;97:9037-41

184. Schroen CGPH, Roosjen A, Tang K, et al. In situ quantification of membrane foulant accumulation by reflectometry. J Membr Sci 2010;362:453-9

185. Ratner BD. Reducing capsular thickness and enhancing angiogenesis around implant drug release systems. J Control Release 2002;78:211-18

186. Walsh G. Biopharmaceutical benchmarks 2010. Nat Biotechnol 2010;28:917-24

• **Review on marketing trends of biologicals**

187. Ishihara K, Takai M. Bioinspired interface for nanobiodevices based on phospholipid polymer chemistry. J R Soc Interface 2009;6:S279-91

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