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Protein adsorption at solid surfaces plays a key role in many natural processes and has therefore promoted a widespread interest in many research areas. Despite considerable progress in this field there are still widely differing and even contradictive opinions on how to explain the frequently observed phenomena such as structural rearrangements, cooperative adsorption, overshooting adsorption kinetics, or protein aggregation. In this review recent achievements and new perspectives on protein adsorption processes are comprehensively discussed. The main focus is put on commonly postulated mechanistic aspects and their translation into mathematical concepts and model descriptions. Relevant experimental and computational strategies to practically approach the field of protein adsorption mechanisms and their impact on current successes are outlined.

Title:

Understanding Protein Adsorption Phenomena at Solid Surfaces

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Abstract

Protein adsorption at solid surfaces plays a key role in many natural processes and has therefore promoted a widespread interest in many research areas. Despite considerable progress in this field there are still widely differing and even contradictive opinions on how to explain the frequently observed phenomena such as structural rearrangements, cooperative adsorption, overshooting adsorption kinetics, or protein aggregation. In this review recent achievements and new perspectives on protein adsorption processes are comprehensively discussed. The main focus is put on commonly postulated mechanistic aspects and their translation into mathematical concepts and model descriptions. Relevant experimental and computational strategies to practically approach the field of protein adsorption mechanisms and their impact on current successes are outlined.

Key Words

Protein adsorption mechanisms, Cooperative protein adsorption, Overshooting adsorption kinetics, Protein Aggregation, MD- and MC-Simulation

Contents

1 Introduction

Almost one decade ago Nakanishi was assessing the event when a protein adsorbs to a solid interfaces as a "[…] a common but very complicated phenomenon" [1]. To date this concise description is still adequate despite considerable recent advances in the field of protein adsorption. In particular the two aspects of being common and complicated has stimulated a huge research interest in various areas including medicine, pharmaceutical sciences, analytical sciences, biotechnology, cell biology, or biophysics.

Protein adsorption to surfaces is indeed a common event as it is, for instance, the first step in many biological processes such as trans membrane signaling or the blood coagulation cascade [2-5]. On artificial tissue scaffolds protein adsorption is the key factor for a proper vascularization [6] whereas on biomedical implants that are in contact with the blood stream protein adsorption can lead to thrombosis [7, 8]. Further, protein adsorption can trigger adhesion of particles, bacteria or cells possibly promoting inflammation cascades, or fouling processes [9-12]. In the field of analytical sciences non-specific protein adsorption on sensor surfaces, protein chips, or assay platforms is a serious problem degrading the analytical performance of the device [13]. Thus, there is no doubt that protein adsorption is a common phenomenon; wherever proteins come into contact with a solid interface they will most likely adsorb to it [5]. To this end, the second aspect of Nakanishi's formulation characterizing protein adsorption as complicated can be understood as follows: it is complicated to avoid protein adsorption. In fact there is a huge community seeking for biocompatible and protein-resistant materials applicable to biomedical implants or analytical platforms and a number of recent review papers provide a comprehensive overview into this area [14-19]. Advances in this field are in particular due to the introduction of polymer (e.g. PEG, PAA) grafted surfaces or self-assembled monolayers (SAM). However, the principles behind protein rejection are still not fully understood and the long term stability of theses surfaces still needs to be clarified [20-23].

Apart from seeking to hinder protein adsorption processes one can alternatively focus on the complicated behavior of proteins from an opposite perspective, namely when they do adsorb to a solid surface. Important questions arising in this dimension are:

- why and how do proteins adsorb,
- how do adsorbing proteins behave as individual molecules or in an ensemble,
- which general mechanistic rules protein adsorption phenomena adhere to,
- does the adsorption event influence the protein's biological function?

In contrast to small molecules that behave like rigid particles, most proteins do not simply attach to or detach from an interface with certain adsorption and desorption probabilities. Instead, the complex composition and structure of proteins causes by far more exciting phenomena such as structural re-arrangements, changing surface affinities during the adsorption, positive cooperative effects, size exclusion effects, overshooting adsorption kinetics, or surface aggregation.

Advances of our knowledge of protein adsorption were mainly achieved through experimental approaches. Since the first systematic works in this field which approximately date back to the 1970s, a continuous progress in the development of methods and techniques has remarkably increased the precision of experimental data. Starting from crude measurements of adsorption kinetics at high bulk protein concentrations in the past it is nowadays possible to detect even single molecules at the surface, to measure orientational and structural properties of adsorbed proteins, or to image protein covered surfaces with high resolution. Naturally, experimental observations need to be carefully analyzed to avoid misinterpretations. This is preferably realized by comparing them to theoretical model descriptions whose advantage consists of a very illustrative access to complex processes and mechanisms. The model itself typically provides new hypothesis which can again be tested experimentally and lead to a further refinement until it is justified to call it a valid model. A newly emerging approach to protein adsorption is opened by computational methods aspiring to simulate the behavior of proteins at interfaces. Even though these methods are far from being able to replace the experiment, the enormous potential of computational methods is obvious considering that technical or physical constraints such as the diffraction limit in optical methods do not exist *in silico*.

The intention of the present review is to provide an overview on the current state of our understanding of mechanistic aspects encountered during protein adsorption processes. Included are experimental, computational, and mathematical strategies to explore and describe them. It therefore complements previous monographs or review papers in this field being either of general nature [1, 5, 24-27] or focusing on various aspects like driving forces for protein adsorption [28], experimental methods [29-31], adsorbed protein layers [32], factors ruling protein adsorption [33], or protein-resistant and biocompatible materials [14-19, 34].

2 Factors controlling protein adsorption

2.1 Influence of external parameters on protein adsorption

The conditions under which protein adsorption experiments are conducted have a decisive influence on the adsorption behavior. External parameters are basically temperature, pH, ionic strength, and buffer composition. Certainly, these parameters will be fixed if true physiological conditions are mimicked; however, many experimental studies are conducted at arbitrary conditions.

Temperature has an effect on both, the equilibrium state and the kinetics of protein adsorption. Increased adsorption rates can be expected due to an accelerated diffusivity of proteins towards the sorbent surface. The major driving force of protein adsorption is an entropy gain arising from the release of surface adsorbed water molecules and salt ions and from structural rearrangements inside the protein [28, 32, 35-37]. The amount of surface adsorbed proteins therefore generally increases at elevated temperatures [38].

The pH determines the electrostatic state of proteins. When the pH equals the isoelectric point (pI) of a protein the numbers of negative and positive charges are in balance resulting in a net neutral molecule. At low pH conditions (pH < pI) proteins are positively charged whereas at high pH conditions (pH > pI) proteins are negatively charged. Electrostatic protein - protein repulsions are minimized at the isoelectric point allowing higher packing densities on the surface. Adsorption rates are high when protein and substrate bear opposite charges since electrostatic attractions accelerate the migration towards the surface. However, the total mass load is generally observed to be maximized at the isoelectric point [39-41].

Another parameter controlling protein adsorption processes is the concentration of dissolved ions expressed by the term ionic strength. The ionic strength basically determines the Debye length correlating with the damping distance of the electric potential of a fixed charge in an electrolyte [42]. That means the higher the ionic strength the shorter are electrostatic interactions between charged entities. As a consequence the adsorption of charged proteins or protein domains to oppositely charged substrates is hampered whereas the adsorption to like-charged substrates is enhanced [43]. Such electrostatic effects can be noticed to influence adsorption kinetics. The efficient screening of the electric potential of proteins reduces lateral interactions which are usually of electrostatic nature. This in turn may initiate an increase in packing density, a suspension of cooperative effects, or protein-protein

repulsions [44, 45]. Moreover, high ionic strength conditions increase the tendency of proteins to aggregate [46].

For more than a century it has been known that high salt concentrations promote the precipitation of colloidal substances which is known as the 'salting out' effect due to water absorption by salt ions. In the 1880s Hofmeister recognized that salt ions differ by their ability to precipitate proteins from a solution which lead to the concept of the 'Hofmeister-series' [47]. Ions that promote protein precipitation are called kosmotropes (e.g. SO_4^{2-}, F^-, Mg^{2+}, Ca^{2+}) ions that decelerate protein precipitation are called chaotropes (e.g. ClO_4^-, SCN^-, NH_4^+). It was suggested that this effect correlates with the ability to stabilize (kosmotropic effect) or destabilize (chaotropic effect) the native conformation of proteins which would influence their adsorption tendency [48]. However, predicting the effect that the type of salt ions has on protein adsorption turns out more complicated since recent studies contradict the idea of a simple correlation of the Hofmeister series with the protein adsorption behavior. Anomalies like a stabilizing effect of chaotropic salts on proteins [49], or maximum protein adsorption in between kosmotropic and chaotropic ions [50] were found.

2.2 Influence of protein properties on protein adsorption

Proteins are complex biopolymers composed of 20 naturally occurring amino acids as monomeric units plus possible additional side chains like phosphates, oligosaccharides ore lipids introduced after translation. This unique diversity of basic elements for the build-up of polymeric molecules results in an extraordinary structural and functional complexity which makes simple hypotheses regarding their adsorption behavior difficult. Andrade et al. paraphrases this point with the 'unique molecular personality' of each individual protein [51]. A classification of proteins with respect to their interfacial behavior can be achieved by considering properties like size, structural stability and composition. To this end small and rigid proteins like Lysozyme, β-Lactoglobulin, or α-Chymotrypsin are referred to as 'hard' proteins suggesting a generally little tendency for structural alterations upon surface adsorption [28, 52, 53]. Intermediate size proteins such as the majority of the abundant (> 1 mg/mL) plasma proteins like Albumin, Transferrin, Immunoglobulins, etc. are usually able to undergo conformational reorientations upon surface contact. As a means of simplification the complex structure can be decomposed into individual domains exhibiting specific properties like hydrophilic/hydrophobic, polar/non-polar, or charged/uncharged [51]. One of the experimentally amenable characteristic features of this type of proteins is to exist in two or

more adsorbed states typically differing in adhesion energy. The class of high molecular weight proteins includes polymer-like lipoproteins and glycoproteins whose behavior is essentially dominated by the content of lipids or glycans [54]. Lipoproteins are structurally labile and therefore show a strong affinity to hydrophobic surfaces including significant conformational reorientations. By contrast, adsorption of glycoproteins on hydrophobic surfaces is hindered due to a high content of hydrophilic glycans.

In protein mixtures the adsorption behavior is often a result of an overlap of transport, adsorption, and repulsion processes. Small proteins diffuse faster than large ones and are the dominating species in the early adsorption stage. Larger proteins, however, typically bind stronger to the surface because of a larger contact area and can even repel other pre-adsorbed proteins during spreading on the surface. As a consequence, the total mass of adsorbed proteins can pass through a local maximum during the course of adsorption [24]. This so called Vroman effect will be discussed in more detail further below.

2.3 Influence of surface properties on protein adsorption

Protein-surface interactions are influenced by the protein's properties on one side and by the surface properties on the other side. Important parameters that have to be considered include surface energy, polarity, charge, and morphology [31]. In experimental studies the chosen type of surface typically reflects the scientific context of the project. Model surfaces are required to mimic implant material, cell walls, biosensor surfaces, or filter membranes. There are typically some restrictions imposed by the applied experimental technique such as optically transparent, electrically conducting, or atomically flat surfaces which may affect the flexibility. Although unmodified substrates such as quartz, mica, glass, metals, or graphite are often used in protein adsorption studies it is common to apply surface modifications to arrive at suitable models of relevant surfaces. One of the most frequently applied methods is the silanization of hydroxyl group bearing substrates through chlorosilanes or ethoxysilanes. In this way, substrates consisting of quartz, glass, metal oxides or plasma activated silicon and metals can be efficiently modified with a monolayer of desired functionality without changing optical properties like the transparency. Another method to produce such self-assembled monolayers (SAM) is to expose noble metal substrates, predominantly gold, to alkanethiols which is the method of choice to modify electrically conducting substrates needed for certain analytical techniques like quartz crystal microbalancing (QCM) or surface plasmon resonance

(SPR) [55-57]. Further model surfaces frequently found in protein adsorption studies include polymer coated surfaces (e.g. using dip- or spin coating) [58], poly(ether sulfone) films or membrane filter material [59-61], and Langmuir-Blodgett films [62, 63]. Parameters, such as surface energy, charge, and polarity can be easily tuned with these methods by choosing the appropriate functionalities. Model membranes represented by surface supported lipid bilayers (SLB) are particularly well suited to mimic surfaces present in biological systems. A SLB forms spontaneously when a solution containing unilamellar phospholipid vesicles (ULV) in appropriate buffer is exposed to a hydrophilic surface. Spherical vesicles adhere to the surface, rupture, and spread which in the end leads to a planar bilayer [64]. In a number of studies model membranes were used to investigate protein adsorption phenomena with a realistic background from biological systems [65-67].

The adhesion energy of proteins to surfaces varying in surface tension, polarity, charge and wettability can be directly measured using atomic force microscopy or the surface force apparatus [58, 59, 61, 68-70]. Proteins tend to adhere more strongly to non-polar than to polar, to high surface tension than to low surface tension and to charged then to uncharged substrates. Belfort et al. postulates that non-polar surfaces destabilize proteins and thereby facilitate conformational reorientations leading to strong inter protein and protein-surface interactions [57]. This explains the rather general experimental finding that in most cases the affinity of proteins to surfaces increases on hydrophobic substrates and decreases on hydrophilic substrates [24]. A prominent exception to this rule of thumb is the adsorption behavior of glycoproteins in which the hydrophobic domains are buried inside a shell of glycans. Glycoproteins adsorb extensively on hydrophilic planar surfaces and sparsely on hydrophobic surfaces [71].

In contrast to substrates modified with thin monolayers the class of polyelectrolyte multilayers constitutes a somewhat exceptional type of surface. It has been known for a long time that poly(ethylene oxide) (PEO) or poly(ethylene glycol) (PEG) (both are chemically the same) grafted surfaces are protein-resistant due to steric repulsion and excluded volume effects [22, 72, 73]. Consequently, these surfaces have great potential for the design of medical devices or implants which are exposed to blood. In recent years the favorable protein adsorption properties of poly(acrylic acid) (PAA) brushes have been reported [74-76]. At low salt concentration these polyelectrolyte layers retain proteins in their native state, i.e. they hinder denaturation processes. Czeslik et al. have shown that the activity of the enzyme horseradish peroxidase is not affected after adsorption to PAA brushes in contrast to adsorption to a bare silica surface [77]. Moreover, the protein insulin does not transform into

amyloid fibers when attached to PAA brushes even at conditions at which fibrillization is normally observed [75, 78]. In contrast to low salt concentrations PAA brushes become highly protein resistant at high salt concentrations as these conditions suppress the process termed ‘counterion evaporation’ which is the major driving force for protein adsorption to polyelectrolytes [79, 80].

3 The individual behavior of proteins at interfaces

When proteins approach a solid interface they typically do not behave like symmetric rigid particles that can either adsorb on or desorb from a surface. These two processes were the only ones that Irving Langmuir considered when he developed a theory for the adsorption of gas molecules to surfaces, nowadays widely known as the *Langmuir adsorption isotherm* [81]. Although this theory is still a kind of starting point serving for the development of theoretical descriptions of protein adsorption events, it is obviously too simplistic to match the complex behavior of proteins. Remarkably, Langmuir himself was absolutely aware of the limits of his equation. He commented: “Considering the nature of the simplifying assumptions made in its derivation it should, of course, not be looked upon as a general equation of the adsorption isotherm” (Langmuir, 1932) [81]. Unlike small and rigid gas molecules proteins exist in a large variety of structural properties, size, and shape. Due to a defined folding into their secondary and tertiary structure proteins contain a specific distribution of hydrophobic, hydrophilic, positively charged and negatively charged side chains which has a major impact on their adsorption characteristics. Things become even more complex considering that many properties like the folding state or the number of positive and negative charges inside proteins can vary in different environmental conditions depending on pH, ionic strength, or temperature.

3.1 Side-on or end-on? The protein’s orientation

Proteins are typically asymmetric and complex molecules of a few nanometers in size. Only in exceptional cases they exhibit a spherical shape; more common are elliptical, rod-like, or even more sophisticated shapes such as heart-like (BSA), or Y shaped (IgG) etc. In solution proteins rotate freely whereas on a surface each protein will adapt a certain orientation which

determines which part of the molecule interacts with the surface and which part is exposed to the bulk solution. Clearly, this is an important issue when adsorbed proteins are receptors, enzymes, or have any other specific bioactivity that is exerted by a certain part of the molecule. Structural hindrance of the paratope of an antibody due to an inappropriate orientation can impair its antibody recognition efficiency [82, 83].

The favored orientation of a protein on the surface can be tracked back to its free energy minimum resulting from attractive coulomb and van-der-Waals interactions, hydrogen bonds, and the entropy gain of solvent molecules or counter ion release. Because of their complex structure proteins typically exhibit different affinities in different regions of their surface depending on the local composition of amino acid residues. An often applied concept is to divide the protein's surface into different patches or domains that can be of hydrophobic, hydrophilic, positively, or negatively charged nature [84-86]. Thus, on hydrophilic interfaces proteins predominantly expose those patches toward the surface that are rich of hydrophilic residues and on hydrophobic surfaces proteins direct their hydrophobic patches to the surface. Analogously, proteins adsorbing at positively or negatively charged interfaces tend to expose oppositely charged regions to the surface. This concept helps to explain the frequent observation that net positively or net negatively charged proteins can adsorb to a like-charged surface, i.e., on the 'wrong' side of their isoelectric point [87-90].

If a protein is structurally stable its orientation on the surface can be characterized by 'side-on' or 'end-on' orientation, referring to an elliptically shaped particle that is respectively attached with its long or short axis to the surface [91-93]. Clearly, the final layer thickness of a protein monolayer in its saturation state is higher in the case of 'end-on' oriented proteins than in the case of 'side-on' oriented proteins which has been exploited by Lu et al. [94-97] and Su et al. [98-100]. These authors have used neutron reflectance measurements to determine the orientation of Lysozyme and BSA adsorbed in various systems.

Although the rotational diffusion of adsorbed proteins is generally hindered they still can alter their orientation if the local surrounding conditions change. This was in particular observed when electrostatic interactions are involved, i.e., when both, adsorbent and surface are electrically charged [93, 101]. Due to the increasing protein density on the surface in the course of continuous adsorption protein-protein interactions may become increasingly dominating and create a different free energy minimum state to which the adsorbed proteins adapt. It can be assumed that adsorbing proteins initially adapt an orientation such as to maximize favorable surface-protein interactions resulting from electrostatic attraction. The interactions between neighboring adsorbed proteins, by contrast, may be unfavorable if like

charged patches are facing each other. This repulsion is negligible at low protein surface densities when inter protein distances are rather large. Upon rising surface densities, however, protein-protein distances become smaller and the initial orientation becomes more and more unfavorable compared to a specific other orientation that allows less repulsive forces. As a consequence an orientational change manifested by a rotation of the surface bound proteins takes place once a critical packing density is reached. Experimental studies on β-Lactoglobulin and Fibronectin adsorption to charged surfaces have shown that such a re-orientation goes hand in hand with a loss of binding energy leading to an increased desorption rate [102, 103]. Fig. 1 schematically illustrates such a situation in the case of β-Lactoglobulin adsorption to a negatively charged surface (referring tor ref. [102]). In total this protein bears several tens of the potentially charged amino acids Lysine, Arginine, Histidine, Aspartic acid, and Glutamic acid. To achieve a more comprehensible representation the protein is strongly abstracted through a composition of positive and negative domains (Fig. 1, top). With this simplistic scheme the change of the favorable orientation of an adsorbed protein as function of the surface density is readily understood. Adsorbed proteins change their orientation from the initial state to a new state in which the proteins lose free energy by reducing attractive forces towards the surface and gain free energy due to less repulsive forces between neighboring proteins. Even though the real situation is much more complex the underlying effects can be assumed to be analogous.

3.2 Conformational changes

It is now generally accepted that many proteins undergo conformational changes upon adsorption to a solid interface. This is because the conformation of a protein that corresponds to the free energy minimum in solution typically does not correspond to the free energy minimum of this protein once it is in contact with the surface. The surface-protein contact area induces a gain in free energy and hence proteins tend to maximize their footprint through a conformational re-organization as was shown in numerous experimental works [104-110]. The extend to which proteins can adopt a new conformation is tightly connected to their structural ability which can be characterized with the help of the concept of ‘hard’ and ‘soft’ proteins [28, 52, 53]. Similar to a preferred orientation proteins have a favorable conformational state on the surface which often differs from the native conformational state in the buffer solution since additional protein-surface interactions start to play a role. Initially

proteins approach the surface in their native state and bind through some initial contact sites to the surface. Subsequently, structural re-organizations take place driven by favorable protein-surface interactions and an entropy gain due to a loss of ordered secondary structure inside the molecule plus the release of counter ions or solvation molecules. To this end the conformational changes upon adsorption corresponds to a relaxation process leading to macroscopically observable effects such as a reduced amount of adsorbed protein, an altered secondary structure, and an increased resistance to elution [29, 30, 32, 97]. Experimental techniques that directly provide information about changes in the secondary structure include ATR-IR [108, 111, 112] and CD spectroscopy [53, 91, 104, 113] whereas the elution properties are amenable through adsorption and desorption kinetics. Significant conformational changes of a protein are usually slow processes since often a whole cascade of angular rotations takes place [114-117]. Many proteins initially bind loosely on the surface and only increase their surface affinity afterwards in time by structural changes. As a result, desorption kinetics of a protein layer strongly depend on the time that has been passed since the adsorption event. That means, a freshly established protein layer is hardly resistant to elution whereas after a certain time period (scaling between a few minutes and several hours) proteins can even be found to be irreversibly attached to the surface. In this way it has been proven that the protein Immunoglobulin G (IgG) undergoes conformational changes on a variety of model surfaces [118]. By using a simple kinetic model the transition rates between the initial and the apparent equilibrium state could be quantified.

It can be expected that conformational and/or orientational changes upon adsorption affects the protein's biological function. On the one hand, some proteins or peptides exhibit their function only after adsorption [30, 92]. By contrast, adsorption can also lead to the irreversible alteration of proteins that do not refold into their native structure after desorption. It is assumed that such events potentially inactivate certain species such as receptors or enzymes [53, 119]. Further, surface adsorption can stabilize the structure of proteins and hence improve their resistance to denaturation as compared to dissolved proteins [120-123]. Also it was shown experimentally that the activity of enzymes after surface adsorption can be indifferent [124] or reduced [125] compared to dissolved enzymes if the orientation of the active sites is directed toward the solution or toward the surface, respectively.

4 The ensemble behavior of proteins at interfaces

4.1 Structure of the protein layer

Provided that a surface is sufficiently equilibrated in the presence of a protein solution a saturation coverage will establish. The protein layer structure at this point can be a densely or loosely packed monolayer or even a multilayer. Multilayers are found under specific conditions that promote protein aggregation or repress inter protein repulsion [41, 126]. Monolayers establish when protein-protein attractions are only weak or repulsive which is often the case for proteins bearing charges of equal sign [98, 127]. A general observation is that the packing density of monolayers depends on the strength of the electrostatic repulsions between surface-adsorbed proteins. If proteins bear a relatively high net charge (pH ≠ pI and low ionic strength conditions) they assemble into a loose layer whereas proteins that are net neutral (pH = pI or high ionic strength conditions) assemble in a more densely packed layer [39-41]. The highest possible monolayer density, i.e., a close-packed monolayer, implies the formation of two-dimensional surface aggregates [4, 37, 128]. Interestingly, the surface density has also been found to be dependent on the bulk protein concentrations even in the case of irreversible adsorption [98, 129-131]. The following explanation for such a behavior was suggested by Ramsden [37]. At low bulk protein concentrations the surface coverage increases slowly and conformational as well as orientational changes that are accompanied with an augmentation of the protein's footprint can take place. If, by contrast, the bulk concentration is high and the surface rapidly fills up such structural changes are hindered due to a lack of available surface leading to a higher saturation level. Alternatively, it is suggested that a surface crystallization of proteins takes place once a certain minimum 'supersaturation' is exceeded [130].

In the absence of significant protein-protein interactions except short range repulsions proteins populate the surface in a pure randomized arrangement which is described by the *random sequential adsorption (RSA)* theory. According to this framework proteins do only adsorb to the surface if they approach an area which does not overlap with any other pre-adsorbed protein. Otherwise, if a protein were to hit an adsorbed protein on its way toward the surface it is rejected back into the bulk volume. This ends up in a very inefficiently packed protein layer leaving undefined gaps between adsorbed proteins which are not large enough to accommodate another protein. The probability by which an incoming protein finds an

available adsorption site decreases faster with increasing surface coverage as is the case in the simple Langmuir adsorption theory. So far, an exact description for the 'probability function' or more commonly termed the *available surface function* $\Phi(\theta)$ (where θ is the surface coverage) exists only for the one-dimensional problem which is also known as 'random parking' [132]. Concerning the more relevant two-dimensional problem an approximate function was proposed by Schaaf and Talbot (1989) [133] which satisfactorily matched the results of a Monte-Carlo simulation.

$$\Phi^{RSA}(\theta) = \frac{\left(1-\frac{\theta}{\theta_j}\right)^3}{1-0.812\cdot\left(\frac{\theta}{\theta_j}\right)+0.2336\cdot\left(\frac{\theta}{\theta_j}\right)^2+0.0845\cdot\left(\frac{\theta}{\theta_j}\right)^3} \qquad (1)$$

Using hard discs as model particles a simulation of the RSA mechanism yielded a saturation coverage or 'jamming limit' of θ_j = 54.7% implying a minute degree of surface order [134]. Considering that the RSA theory allows an adsorption of particles at any free position it is certainly the more realistic model compared to the Langmuir adsorption theory which restricts the adsorption to discrete binding sites. Up to date no better formalism for the two-dimensional reference RSA probability function was proposed. Experimental evidence that proteins under certain conditions follow the RSA mechanism was found through analyzing adsorption kinetics [44, 132, 135]. However, the RSA model was also suggested to be insufficient as additional effects may contribute to the adsorption process. Following the first treatments of the RSA problem, formalisms that allow a generalization were developed including processes like bulk diffusion, conformational and orientational changes, protein desorption, multilayer formation and even gravitational effects in the case of large polymer particles [135-137].

4.2 Lateral interactions

Lateral interactions refer to the possibility that proteins not only interact with the sorbent surface but also with one another. Unless the buffer pH is chosen to match their isoelectric point, proteins of the same species usually bear a net charge of equal sign that causes long-

range inter protein repulsions. This explains why at pH values below and above the pI the final packing densities of protein layers are usually smaller than at pH = pI [39-41]. Su et al. reported a five times higher saturation coverage when BSA adsorption at the silica-water interface was conducted at pH = pI as compared to the adsorption at pH 3 [98, 99]. Thus, if the buffer conditions are chosen such that proteins bear a net charge the resulting protein monolayer is most likely a rather lose layer. However, an increase of the protein packing density is observed when the electrostatic repulsions between like-charged proteins is shielded by the screening effect of dissolved ions. Since at high ionic strength conditions the Debye length decreases a closer distance between adsorbed charged proteins is possible [42]. To this end it is efficient to assign adsorbing proteins an effective size or interaction radius that is typically larger then their real size [138].

During the course of adsorption beginning at an empty surface the mean protein-protein distance averaged over all neighboring adsorbed proteins continuously decreases. Therefore, some effects based on lateral repulsions only enter the scene once a certain coverage has been exceeded. One example is the so called ‘catalysis of desorption’ by adsorbed neighbors primarily observed by Kurrat et al. which accounts for rising desorption rates at higher surface coverages [139]. Further, even though mechanistically somewhat different, it has been shown that proteins approaching the surface may hit other pre-adsorbed proteins which, as a result, are released from the surface [10, 140]. This could be understood as ‘catalysis of desorption’ by adsorbing proteins. In the case of protein mixtures it was concluded from theoretical considerations that larger proteins tend to repel smaller ones from the sorbent surface [45].

4.3 Cooperative effects

Apart from an enhanced desorption the inverse, namely the enhanced adsorption of proteins mediated by already adsorbed proteins, is a frequently discussed phenomenon. Experimentally, this concept is manifested by sigmoidal adsorption isotherms or by increasing adsorption rates as a result of increasing surface coverages [130, 141-144]. That means, somehow simplistically, that a protein diffusing in close proximity to the surface is more likely to adsorb if there are already pre-adsorbed proteins. Therefore, the concept of positive cooperativity is nowadays widely used for this effect. A first complete definition of the terms positive cooperative adsorption, negative cooperative adsorption, and apparent non-

cooperative protein adsorption was achieved by Chatelier and Minton [145]. In this notation positive cooperative adsorption refers to adsorption isotherms that are steeper in relation to the reference Langmuir isotherm whereas negative cooperative adsorption leads to broadened adsorption isotherms [145, 146]. This naturally implies a definition for protein adsorption kinetics. Adsorption rates that are generally higher than expected from the classical Langmuir adsorption theory result from positive cooperativity, adsorption rates which are below the expected Langmuir theory result from negative cooperativity [147].According to this framework the above discussed *random sequential adsorption* model that accounts for the size exclusion caused by purely randomized surface packing of particles is equivalent to negative cooperativity. Apparent non-cooperative adsorption is encountered when adsorption isotherms and kinetics are in agreement with the Langmuir-adsorption model. However, Minton emphasizes that a Langmuir-like adsorption behavior can be erroneously interpreted as the lack of cooperative effects. In fact such pseudo-Langmuir-type adsorption isotherms or kinetics most probably conceal a balance between positive and negative cooperativity [146, 147]. Mechanistically, the attractive forces that lead to (positive) cooperative adsorption have been suggested to be tightly connected to surface aggregation mechanisms [145-148]. Indeed, self-association of surface-bound proteins has been found experimentally mainly through scanning force microscopy [67, 128, 149, 150]. However, this technique is invasive and potential influences of the scanning process to the protein distribution on the surface need to be considered during data interpretation.

In a recent study by Rabe et al. the ideas of cooperative adsorption were tested experimentally with the proteins BSA and Fibrinogen [44]. Comprehensive experimental data sets have proven that protein aggregation on the surface is not an indispensable explanation for cooperative protein adsorption events. It was considered that the complex electrostatic field in the circumference of adsorbed proteins induces a kind of electrostatic self-assembly which in turn enhances the protein uptake rate [151-153]. Thus, instead of a strict formation of protein clusters in which monomers are in contact with each other, the preferred adsorption of approaching monomers in the close vicinity (but without a direct contact) of pre-adsorbed proteins was suggested. The positive cooperative effect is explained by a guiding mechanism through which proteins are at the same time attracted toward the surface in vertical direction and repelled from the neighboring protein in horizontal direction caused by a patchy charge distribution within the protein (Fig. 2). As long as the sorbent surface is empty or contains at least some empty regions proteins also adsorb via the non-cooperative adsorption pathway. The adsorption kinetics that follow from such an overlap of cooperative and non-cooperative

adsorption turned out to excellently describe adsorption kinetics measured with different experimental techniques [10, 101, 130, 142-144, 153]. A characteristic feature of cooperative adsorption kinetics are an almost linear or even a convex shape before the saturation level is reached [151-153].

Clearly, mechanistic aspects of cooperative adsorption like the surface clustering or the guiding mechanism are still under debate. Although the observable cooperative adsorption kinetics are similar there are widely differing concepts how to mathematically express the coverage dependence of the adsorption rate constant including square-root [154], linear [10], or exponential [155] functions.

4.4 Overshooting adsorption kinetics

The expected shape of protein adsorption kinetics is a monotonically increasing curve that after a sufficiently long time period reaches saturation. In this final equilibrium stage the number of adsorbing proteins equals the number of desorbing proteins. Alternatively, in the case of pure irreversible adsorption, proteins can not adsorb to the surface any more as all binding sites are occupied. A rather unexpected phenomenon is the observation of an overshoot during the adsorption kinetics which refers to a situation where the adsorption kinetics pass a local or global maximum before the saturation is reached (Fig. 3, down). So far, general insights which experimental conditions promote this effect and which proteins do and do not show this behavior are lacking. Definitely, an experimental artifact can be excluded as quite a few research groups using differing techniques have observed overshooting protein adsorption kinetics [93, 101, 102, 156-158].

In a number of colloid and polymer adsorption studies overshoots and even oscillations of adsorption kinetics are reported and mechanistically explained by the so-called *time delay model* [159-161]. According to this model an overshoot during the adsorption occurs when the surface is temporarily oversaturated and equilibration is reached through a net desorption of polymers despite a further supply of polymer solution. This means, adsorption begins when desorption from the surface is not allowed. After a certain time delay, however, desorption starts due to conformational rearrangements which may cause the overshoot provided the surface is fairly covered and consequently oversaturated. Considering that proteins are in fact biopolymers consisting of up to several thousands of amino acids as monomeric units it can be inferred that protein adsorption kinetics could also include

overshoots. However, the properties and shapes of these overshoots differ broadly which has led to a variety of concepts seeking to explain this peculiar behavior (Fig. 3). The most prominent work on this issue was conducted by Vroman et al. [162-164] who investigated the adsorption of proteins from blood plasma to a solid interface. It turned out that the protein Fibrinogen rapidly adsorbs to the surface but after a short time passes through a coverage maximum and finally covers the surface in smaller amounts at the equilibrium state than in the intermediate state. Experiments with differing protein compositions revealed that this behavior is actually a displacement effect due to which Fibrinogen is replaced by other proteins of higher surface affinity, predominantly by the protein High molecular weight kininogen (HMWK) (Fig. 3 upper row). Numerous subsequent studies confirmed this mechanism which was hence concluded to be of general validity [45, 165, 166]. In recognition to his initial studies this effect is now called 'Vroman effect' [167, 168]. Interestingly, displacement events are not necessarily restricted to the adsorption from protein mixtures. Elofsson et al. observed an overshoot during the adsorption of β-Lactoglobulin which was attributed to an initial adsorption of metastable octamers that were subsequently replaced by the more stable monomers and dimers [156].

However, studies on Lysozyme (Lys) in its monomeric form have revealed that other mechanisms than described by the Vroman effect must also be taken into consideration. In two separate studies, Daly et al. [101] and Wertz et al. [93] found overshooting adsorption kinetics when fluorescently labeled Lys was adsorbed at neutral pH (7.4) on hydrophilic or hydrophobic surfaces, respectively. On the hydrophilic surface (investigated by Daly et al.) the adsorption from relatively small bulk protein concentrations ranging from ~0.07 μM to ~0.7 μM resulted in an overshoot whose peak width turned out to be the broader the lower the bulk concentration was. The authors argued that an orientational rearrangement from an initial end-on to a final side-on orientation takes place which is accelerated by a high flux of incoming proteins. As a result of the orientational change the fluorescent label fluorescein isothiocyanate (FITC) is moved closer to the negatively charged surface where the pH is practically increased leading to a preferred protonation of the fluorophore. Since FITC is a pH-dependent fluorophore its protonation in turn reduces the fluorescence emission intensity [169]. In this sense, the overshoot is believed to be only a signal loss caused by the characteristics of the experimental method and hence does not reflect a reduction of the surface coverage (Fig. 3 second row) [101]. On the hydrophobic surface, by contrast, Wertz et al. observed the overshoot only at bulk concentration as high as ~700 μM or higher [93]. The overshoot in this case was described as a displacement of the more loosely bound proteins in

the end-on orientation by the more strongly bound proteins in the side-on orientation. When the adsorbed proteins change their orientation they require 1.5 times more space on the surface than before such that the equilibrium coverage is lower than the intermediate maximum (Fig. 3 third row). The authors concluded that overshoots occur when the adsorption rate is high compared to the transition rate of the orientational change. No dependence of the peak shape on the bulk concentration was considered.

The comparison of these two works highlights the complexity of protein adsorption phenomena. Both research groups work under almost identical experimental conditions including the same technique, the same fluorophore (FITC), and the same buffer pH (7.4). The most important difference is the used sorbent surface which is either net negatively charged and hydrophilic [101] or neutral and hydrophobic [93]. In agreement with each other they both consider the overshoot of the adsorption kinetics to be a consequence of an orientational rearrangement of surface adsorbed Lys molecules from the reversibly bound end-on to the tightly bound side-on orientation. From this point on, however, the overshoot is explained in completely different ways, namely by a loss of fluorescence intensity, on the one hand, and by a displacement of the species which requires less surface area, on the other hand. Unfortunately, desorption experiments through rinsing the surface with protein free buffer at different stages before and after the overshoot are lacking in both studies. Therefore, the assumed difference of the binding affinities of the end-on and side-on oriented proteins is not supported experimentally.

Based on comprehensive experimental investigations Rabe et al. have suggested a consistent explanation of the overshooting effect that combines the idea of orientational or conformational rearrangements with some aspects of the time delay model [102]. The authors studied the adsorption kinetics of the model protein β-Lactoglobulin (β-Lg) on a hydrophilic glass surface using fluorescence detection. It turned out that in the beginning all proteins bind in an irreversible manner to the surface as no desorption can be observed upon buffer rinse. However, once a certain coverage level has been exceeded in the course of adsorption a sudden alteration of the binding behavior from irreversible to reversible takes place. In contrast to some previous explanations that suggested the formation of a first irreversible protein layer on which a second reversible layer is built [152, 170, 171], here it was proven that all proteins, including the new ones and those which were already adsorbed before on the surface, are affected by this affinity alteration. The key experiment for this finding consists of recording protein adsorption kinetics of first fluorescently labeled (i.e. visible) and second unlabeled (i.e. invisible) proteins. The experiment is designed such that the critical coverage

is exceeded during the adsorption of the unlabeled proteins. In the case of β-Lg it was found that the fluorescently labeled proteins which had been already adsorbed in the beginning desorbed from the surface such that a two layer model could be excluded (Fig. 4). The critical coverage was found to scale around 20% of the saturation coverage and to be independent of time and bulk concentration. To this end the concept of an affinity change of adsorbed polymers from irreversible to reversible was adapted from the *time delay model* whereas the idea of a 'time delay' could not be supported. Proteins remained in their irreversible state for any arbitrary time period until the critical coverage was reached. To explain the sudden affinity change mechanistically, the proposed concept of an orientational and/or conformational change of adsorbed proteins mediated by the increasing importance of lateral protein-protein interactions during the adsorption was recalled (see Fig. 1) [93, 101, 153]. The initial state of a protein becomes obviously disfavored when more and more proteins adsorb in the vicinity. As a consequence, the proteins undergo a transition into a new state in which the sum of lateral protein-protein and protein-surface interactions is optimized which apparently involves a weakening of the surface affinity. The effect of suddenly rising desorption rates upon reaching a certain minimum coverage was reported before [103]. Additionally, many experimentally observed overshooting adsorption kinetics are consistent with the proposed concepts [101, 156-158].

4.5 Protein aggregation

A highly important aspect connected with protein adsorption is the aggregation or clustering of proteins into oligomers of a few monomers or into clusters of up to several hundreds of protein monomers. This process can accommodate protein adsorption at solid interfaces and influence the adsorption kinetics as well as the resulting layer structure. Aggregation or clustering can be vital for the control of signal transduction pathways [172-176] or can enable protein machines to exhibit complex cellular functions [177, 178]. Conversely, precursor protein aggregates or amyloid structures have been associated with over 20 human diseases including Alzheimer's disease, Parkinson's disease, and Type II diabetes [179, 180]. These diseases are linked to either a direct cytotoxic effect of protein clusters or to the inhibition of biological function when monomers are aggregated.

The term 'protein cluster' can refer to different entities. One meaning which is particularily wide spread in the protein adsorption community assigns protein clusters to two-

dimensional assemblies of aggregated protein monomers on a surface. The formation mechanisms of these protein clusters may include either the diffusion of surface bound molecules towards precursor aggregates or the direct adsorption of bulk proteins adjacent to other surface bound proteins or protein aggregates [146, 147]. This implies that these protein clusters evolve directly on the surface mediated by strong protein-protein interactions [150, 181]. In a comprehensive set of theoretical investigations on the formation mechanisms and kinetics of such protein clusters, Minton suggests mathematical formalisms to model the thermodynamics and kinetics resulting from cluster formation on the surface. According to this framework protein monomers in the bulk solution preferably aggregate to surface clusters compared to an adsorption at empty surface sites. Moreover the affinity to existing protein clusters is assumed to depend on the cluster size: the larger the cluster the stronger the affinity [146, 147]. As a natural consequence the observable adsorption rate increases with the growing coverage during the adsorption because the mean cluster size increases. To this end Minton inherently coupled the formation of protein clusters to the term (positive) cooperative adsorption and pointed out which characteristics of the adsorption kinetics and isotherms can be associated with two-dimensional protein cluster formation on the surface. Herrig et al. indeed found experimental evidence for this connection between protein clusters and cooperativity by comparing the adsorption kinetics of the protein ezrin on supported lipid bilayers with scanning force microscopy images that clearly show cluster formation (Fig. 5) [128]. Considering that the observation of increasing adsorption kinetics alone can also be misinterpreted [44], scanning force microscopy (SFM) is undoubtedly the method of choice to experimentally support the idea of two-dimensional cluster formation due to its atomic scale resolution. Numerous further SFM studies in which protein adsorption was directly measured in solution, i.e., without a preceding drying step, could reveal the existence of two-dimensional protein surface clusters [149, 182-186]. However, the often proposed cluster growth mechanism through a surface diffusion or a direct attachment of protein monomers can not be observed directly but can only indirectly concluded from the increase of the cluster size. Care must be in particular taken in interpreting time series of scan images as tip induced protein displacements can occur when the same surface section is repeatedly scanned. An alternative mechanism that can lead to two-dimensional clusters consist of the formation of larger protein assemblies in the solution which subsequently deposit onto the surface where they spread and flatten [187]. Moreover, there are also a few experimental studies in which the growth of protein clusters was not restricted to the surface but could also proceed orthogonally to the surface, i.e., into three dimensions [126, 188].

Another meaning of the term protein cluster is the assembly of proteins on the surface in a highly regularly ordered manner which is referred to as two-dimensional surface crystal. By means of atomic force measurements the protein annexin A5 was found to form such surface crystals on supported phospholipid bilayers [65-67, 189]. Unlike disordered surface clusters two dimensional surface crystals are generally believed to serve as nuclei for the growth of large three-dimensional crystals [190, 191].

So far it has been pointed out that protein clustering or aggregation can be a surface induced process. However, protein clusters have also been reported as stable, ordered or amorphous aggregates that grow in a protein solution [192]. Their appearance ranges from linear, fibril-like to spherical with diameters of up to a few hundreds of nanometers [193, 194]. Many different protein species were found to form such soluble clusters as proven by a broad spectrum of different analytical techniques including circular dichroism [195], static and dynamic light scattering [192, 196], electron microscopy [193, 197], size exclusion chromatography [173], and fluorescence methods [198]. The generally accepted formation mechanism of this kind of protein cluster includes a nucleation step resulting in a seed of one or a few aggregated monomers followed by the cluster growth through monomer addition [195, 199]. Universal detailed mechanistic models for protein cluster formation, growth, and adsorption are currently under debate [200, 201]. It is, for instance, known that BSA [202] as well as other proteins [203, 204] can form aggregates in solution; however, the fate of these particles after adsorption to a surface has only recently been investigated by Rabe et al. [187]. Using the technique of Förster resonance energy transfer it was shown that protein clusters can deposit from the solution onto the surface and subsequently start to spread (Fig. 6). The spreading rate is strongly influenced by the surface chemistry: fast spreading on a hydrophobic surface, slow spreading on a hydrophilic surface. An investigation of the interplay between protein clusters and a monolayer of pre-adsorbed proteins revealed that the cluster spreading mechanism is connected to the ability of protein molecules to move on the surface (Fig. 7). A high surface mobility correlates with a high spreading rate whereas a low mobility slows down the spreading process. As a consequence the deposition of protein clusters on the surface can be suppressed by a dense protein monolayer provided that it displays a low surface mobility of adsorbed proteins. In this case protein clusters diffusing in the bulk solution can not disrupt the layer of adsorbed proteins and hence a deposition on the surface does not take place. Such a scenario was found when BSA was adsorbed on a hydrophilic surface at pH 3. On a hydrophobic surface, by contrast, even a dense monolayer

of BSA can not hinder the deposition and subsequent spreading of protein clusters. This finding is particularly interesting considering that BSA is traditionally used as a blocking agent to prevent unspecific binding of analyte molecules to surfaces. A possible source of error in the read out of receptor-ligand reactions arises when target proteins form clusters in solution and deposit somewhere on the sensor surface even though a layer of BSA was previously adsorbed. Such unspecific binding/deposition events lead to overestimates or even false responses which is a significant problem in the field of assay technology.

5 Mathematical models for protein adsorption

In the field of protein adsorption studies the primary objective is to understand the behavior of proteins in close proximity to or deposited onto the surface. This includes their behavior as individual species and as a component in an ensemble. Given the considerable albeit not unlimited technical opportunities to date, large amounts of experimental data are available. However, techniques allowing a direct observation of the undisturbed adsorption of proteins in molecular dimensions are still far from being mature. Thus, experimental data typically contain macroscopic information resulting from the individual behaviors of one or several proteins. At this point the design of a model that mathematically describes the experimental data is an efficient way to unravel or confirm mechanistic details of the adsorption process. A model always opens the opportunity to 'play' with different ideas or to test different sets of parameters which in the end helps to argue what is possible and what is not. However, models are typically restricted to the experimental limits in which their hypotheses can be tested and generalization to other systems must be done with care. There are two main directions for developing mathematical model description in the field of protein adsorption. Kinetic models on the one hand describe the events and phenomena during the course of adsorption or desorption. They typically start with an empty surface and model the adsorption kinetics until surface saturation is reached. Often the desorption process upon rinsing the surface with protein buffer is also included. On the other hand there are thermodynamic models that account for the energetic aspects involved in protein adsorption and predict the final equilibrium state. However, before the most relevant models are outlined the reader shall be reminded to what is the proper meaning of the word model: A model is a theoretical construct that serves as a simplified substitute of a real system. The degree of simplification depends on the specific scientific question in mind, and researchers asking different questions will come

up with all legitimacy with distinct model designs. In this sense the validation of a model is not that it is 'true' or 'false' but that it generates verifiable hypotheses in the context in which it was developed.

5.1 Kinetic models

As the mechanisms behind protein adsorption events strongly affect the adsorption kinetics, the majority of models developed in this field are 'kinetic models' which are usually expressed through rate equations. In general it is rather uncomplicated to construct a kinetic model by using terms that represent the mathematical translation of adsorption phenomena such as structural rearrangements, lateral interactions, cooperative effects, or overshootings. The easiest way is to start with a reference model, for instance the Langmuir adsorption model, which is successively modified or extended.

$$\frac{\mathrm{d}\theta}{\mathrm{d}t} = k^{on} \cdot c_s \cdot \left(1 - \frac{\theta}{\theta_{max}}\right) - k^{off} \cdot \theta \tag{2}$$

In Eq. (2) θ refers to the protein coverage, θ_{max} is the maximum coverage level at which no more binding site is available, k^{on} and k^{off} are, respectively, the on-rate and off-rate constants and c_s is the protein concentration directly above the surface. The adsorption of proteins from the bulk solution causes a depletion of the surface concentration c_s which in turn leads to a protein transport from the bulk solution to the region above the surface. As a consequence, the surface concentration varies during the adsorption process which is often taken into consideration in the model design [10, 93, 151, 155, 205]. Typically protein adsorption measurements are conducted under flowing conditions such that a continuous supply of analyte is maintained. In this case, a straight-forward possibility to implement the transport of proteins towards the surface was proposed by Corsel et al. who argued that the surface concentration changes very slowly in time throughout the adsorption and is hence approximately constant ($\mathrm{d}c_s / \mathrm{d}t \approx 0$) [205]. Consequently, the protein flux to the surface equals the protein adsorption rate leading to the following expression of the surface concentration which has been implemented in some works [10, 93, 205].

$$c_s(t) = c_b - \frac{1}{k^{transport}} \cdot \frac{\mathrm{d}\theta}{\mathrm{d}t} \tag{3}$$

The transport rate constant $k^{transport}$ has to be calculated according to some further models accounting for the used measuring cell system. However, a number of researchers judges the influence of transport to the surface less important to the resulting adsorption kinetics and leaves the surface concentration as a constant that is equal or at least directly proportional to the bulk concentration [101, 141, 206].

The term $\left(1-\theta/\theta_{max}\right)$ in Eq. (2) which accounts for the continuous reduction of available surface sites is often replaced by the available surface function $\Phi(\theta)$ as defined by Eq. (1) [132, 135, 152]. In this way a more realistic depletion of surface sites considering the random sequential adsorption of proteins is achieved.

Lateral interactions between surface bound proteins are implemented into kinetic models by expressing the corresponding rate constants as functions of the surface coverage [10, 154, 155].

$$k^{on,off,trans} = f(\theta) \tag{4}$$

This concept was in particular useful to include cooperative effects. By defining the on-rate constant being proportional to the surface coverage, increasing adsorption kinetics expressing the acceleration of the adsorption rate due to pre-adsorbed proteins were described [44].

$$k^{on} = \alpha \cdot \theta \tag{5}$$

In most published models proteins can adopt different states on the surface, for instance compact and expanded, monomer and dimer or end-on and site-on. If their adsorption characteristics differ from one another, each state needs to be expressed with a specific rate equation. Transition or exchange mechanisms between them require a coupling of these equations resulting in a rate equation system. The following coupled rate equations demonstrate a two-states system where the subscripts 1 and 2 refer to the respective state.

$$\frac{\mathrm{d}\theta_{state\_1}}{\mathrm{d}t} = k_1^{on} \cdot c_1 \cdot \Phi(\theta) - k_1^{off} \cdot \theta_1 - k_{1\to 2}^{trans} \cdot \theta_1 + k_{2\to 1}^{trans} \cdot \theta_2 \tag{6 a}$$

$$\frac{\mathrm{d}\theta_{state\_2}}{\mathrm{d}t} = k_2^{on} \cdot c_2 \cdot \Phi(\theta) - k_2^{off} \cdot \theta_2 - k_{2\to 1}^{trans} \cdot \theta_2 + k_{1\to 2}^{trans} \cdot \theta_1 \tag{6 b}$$

$$\frac{d\theta_{tot}}{dt} = \frac{d\theta_1}{dt} + \frac{d\theta_2}{dt} \qquad (6\ c)$$

It is, however, highly desirable to keep the number of different species and likewise the number of adjustable parameters at a minimum as a higher complexity of the model degrades its validity. The idea behind this is a rather general heuristic principle in science often referred to as *Occam's razor*. Models comprising two [101, 106, 115], three [10, 102], or even six [207] different species are common.

A simplified and schematic overview of some of the most important kinetic models proposed during the past decades is presented in Fig. 8. As already mentioned the most basic and therefore the most often applied reference model is the *Langmuir* adsorption model that accounts for the adsorption and desorption of particles at distinct surface sites (Fig. 8 A). Only because of its simple mathematical format (see Eq. 2) this model is still applied today although there is general accordance in the community that this formalism is inadequate to accurately describe protein adsorption kinetics.

One of the major weaknesses of the Langmuir model was overcome by the development of the *random sequential adsorption (RSA)* model which accounts for the much more realistic adsorption of proteins from the bulk solution to random binding sites (Fig. 8 B) [133, 136, 208]. In contrast to the *Langmuir* model the protein distribution resulting at the saturation level is highly inefficient and it was determined that only up to 55% of the total surface area is actually covered. The RSA model was first developed for irreversible adsorption and later extended to include other effects such desorption, diffusion, structural rearrangements etc. [135-137]

To account for the frequent experimental observation that adsorbed proteins are apparently only partially removable upon rinsing the surface with protein free buffer, McGuire et al.[106, 209] proposed a *two states* model in which proteins can adsorb in a reversible or in an irreversible state on the surface (Fig. 8 C). Two conceivable adsorption mechanisms were proposed: The first one is a *transition* pathway through which all proteins must initially adsorb in the reversible state and can subsequently undergo a transition into the irreversible state. The second one is a parallel adsorption of the protein either into the reversible or directly into the irreversible state.

An extension of the *transition* model was proposed by Szöllősi et al. by introducing an undefined number of different states of adsorbed proteins which exhibit increasing footprints and as a consequence increasing surface affinities (Fig. 8 D) [210]. The transition between 'neighboring states' on the surface is allowed which overcomes the limitation that only two

distinct conformations of adsorbed proteins are possible. Although it is very likely that several conformations of adsorbed proteins do exist, current experimental methods are by far too inaccurate to distinguish such subtle details.

A model that explicitly implements lateral interactions between adsorbed proteins was developed by Wahlgren and Elofsson to describe the adsorption kinetics of β-Lactoglobulin A and B to a methylated silica surface (Fig. 8 E) [10]. The model includes the adsorption of two types of monomers with differing resistance to desorption and the adsorption of dimers that can displace pre-adsorbed monomers from the surface. Apart from this *monomer/dimer exchange* mechanism, adsorbed dimers are also suggested to laterally interact with adsorbed monomers of type 1 such as to induce their transition into monomer type 2 which is then stronger adhered to the surface. Therefore the rate equations are designed such that the transition rate between monomer type 1 and monomer type 2 is linear dependent on the surface concentration of adsorbed dimers.

Wertz and Santore presented two simple models termed *displacement* and *rollover* model that can both explain the overshooting effect frequently occurring in protein adsorption kinetics (Fig. 8 F) [93]. The displacement model is in principle analogous to the *Vroman* effect except that only one protein species is involved. This protein rapidly but rather weakly adsorbs to the surface in its end-on orientation and slowly but tightly adsorbs in its side-on orientation. As a consequence the surface is initially, i.e., in the non-equilibrium state, mainly covered with proteins in the end-on orientation. In the long term, however, the side-on oriented proteins will dominate on the surface as their surface affinity is higher and thus their desorption rate is slower. Since the side-on orientation of a protein such as Lysozyme may occupy 1.5 times more space on the surface there is the possibility that the surface coverage passes a maximum during adsorption which is the known overshoot. In contrast to the displacement model the rollover model allows the transition of the end-on oriented protein into its side-on orientation. A direct adsorption into the side-on orientation, however, is not possible. Both models predict overshooting adsorption kinetics provided the parameters are properly set. Only the shape of the kinetic curves described by the two models or by a combination of them differs slightly. After comparing the model predictions with experimental data obtained for Lysozyme adsorption to a hydrophilic surface the authors concluded that the *rollover* model fitted best to their system.

A comprehensive model that implements elements from practically all models presented above was developed by Rabe et al. based on experimental data obtained from studying the adsorption of β-Lactoglobulin on a hydrophilic glass surface [102]. The model contains three

different adsorbed states, an irreversible, initial state, a reversible intermediate state, and a final irreversible state (Fig. 8 G). In the beginning of the adsorption process, as long as the surface is empty or only sparsely covered, bulk proteins are solely allowed to adsorb on the surface via the initial state. The transition between the initial and the intermediate state is a result of lateral protein-protein interactions and takes place once a critical surface coverage has been exceeded. According to experimental observations this transition proceeds rapidly suggesting an orientational change rather than a (multistep) conformational rearrangement. Proteins in the intermediate state undergo a further transition into the final relaxed state. This transition is much slower and therefore most likely involves some conformational rearrangements. The coverage dependent first transition implies that large numbers of surface adsorbed proteins abruptly change their surface affinity from irreversible to reversible which is the key element of the overshooting effect in this model. Due to the second slow transition the surface coverage can still increase after the overshoot which is in line with the experimental observations.

A somewhat special model has been proposed by Minton to describe the growth of two-dimensional protein clusters on the surface (Fig. 8 H) [147]. In this model each surface bound i-mer, that means monomer, dimer, trimer, etc., has a specific tendency to attract a further incoming protein that increases the cluster by one unit. Proteins are either allowed to adsorb as individual species on the surface which can then diffuse and aggregate to a pre-existing cluster or to directly deposit at the edge of a two-dimensional surface cluster via a *piggyback* pathway. In either pathway the predicted adsorption kinetics exhibit an S-shaped curve with an increasing adsorption rate in the beginning, meaning positive cooperative adsorption, followed by a decreasing adsorption rate when the available surface becomes the limiting factor. Unfortunately, the mathematical format of this model is far from being simple as each cluster species (i-mer) is described with one specific equation. As a consequence the number of coupled rate equations is expanded to infinity, at least in theory. In practice the formalism has to be cut at an upper limit of cluster species to allow the computation of adsorption kinetics with reasonable effort. In any case, this pioneering work of Minton for the first time treated cooperative effects during protein adsorption with a comprehensive mathematical framework. Later, Rabe et al. implemented the key ideas of Minton into the new and very simple *tracking* model to account for cooperative protein adsorption kinetics (Fig. 8 I) [44]. Instead of restricting cooperative adsorption to the growth of tight two-dimensional surface clusters it was suggested that bulk proteins approaching pre-adsorbed proteins on the surface are attracted vertically to the surface due to the overlap of the electrostatic forces of the

protein and the surface. In lateral direction to the surface, however, the distribution of the charges of the pre-adsorbed protein and of the adsorbing protein leads to repulsion. The sum of all electrostatic interactions leads to a tracking or guiding of adsorbing proteins to the vicinity of other pre-adsorbed proteins. This mechanism necessarily leads to the formation of a patchy surface that contains regions of higher and regions of lower protein density. Adsorption kinetics are described with only two rate equations, one for the adsorption of proteins in the vicinity of pre-adsorbed proteins, one for the adsorption in an empty surface region. Depending on the strength of the cooperative forces, the curve shape of adsorption kinetics can vary between S-shaped, quasi linear, and exponential-like, which in many cases fits much better to experimental data than the Minton model [10, 101, 130, 141-143, 153].

5.2 Equilibrium models

Apart from kinetic models there are also models in which the focus is put on thermodynamics such that they can be classified as 'equilibrium models'. The primary objective of these approaches is to predict the equilibrium state of an adsorption event based on some selected input parameters such as pH, ionic strength, temperature, surface chemistry, protein composition, etc. A key element of equilibrium models is a suitable expression of the free energy of a given system. In a simplified view, this includes enthalpic contributions from protein-protein and protein-surface interactions as well as the entropic contributions from the respective protein adsorption state and the surrounding solvation shell. Finding the free energy minimum among a selected choice of systems gives access to adsorption isotherms or other specific information that are experimentally testable for instance about cooperative and surface clustering effects [146, 211].

Another strategy is to develop an equilibrium model for a specific adsorption mechanism which is then used to calculate adsorption kinetics. This was done by Szölösi et al. who proposed the multistep conformational change upon protein adsorption already discussed above (Fig. 8, D) [210]. Using arbitrary parameters for the internal energies of the particular states which differ in their footprint and for the energy barriers between them, MC simulations were performed that gave rise to calculated adsorption kinetics. In this way the authors predicted exchange effects between adsorbing and pre-adsorbed proteins as well as the overshooting effect. Szleifer et al. proposed expressions for calculating the free energy of systems containing proteins of different size that are either neutral or arbitrarily charged [45,

212-214]. The equilibrium surface compositions and densities as a function of bulk composition and ionic strength of the buffer solution were obtained. Again, dynamic simulations were performed that gave rise to the adsorption kinetics resulting from mixtures of large and small proteins under varying input parameters and varying surfaces.

6 Experimental approaches

The experiment in the field of protein adsorption is indispensible. Adsorption phenomena are observations made during experimental investigations which are interpreted in terms of adsorption mechanisms and eventually translated into mathematical concepts that are preferably combined into a complete model. This model can be used to formulate further hypotheses which again have to be tested through the experiment. Thus, a cyclic procedure that puts the experiment before and after the formulation or refinement of the model is the best way to achieve a reliant and consistent description of the adsorption process.

Of course, there are always constrains resulting from technical and physical principles, such as a limited sensitivity or selectivity, temporal or spatial resolution limits, interferences of the measurement process to the adsorption behavior, and many more. Hence, the used technique has to be chosen according to the category of asked questions, and a combination of complementary techniques is the key to acquire information from different perspectives. Subjects on which experimental investigations focus include adsorption kinetics and isotherms, the protein layer thickness and density, the secondary and tertiary structure of adsorbed proteins, protein-surface and protein-protein interactions, etc. Additionally, the effect of the adsorption of proteins on their biological behavior is a further field of interest. In the following, an overview of the most important experimental techniques and their fields of application is given.

6.1 Label-free techniques

There are several widely applied optical techniques that allow for the detection or analysis of unlabeled proteins adsorbed to an interface. Ideally suited for recording adsorption kinetics and isotherms are ellipsometry (ELM), surface plasmon resonance (SPR), and optical waveguide lightmode spectroscopy (OWLS). Ellipsometry is based on the change of the

polarization state of light after reflection from a surface and provides information about the mass of the adsorbed protein layer. ELM was used by a considerable number of researchers to measure adsorption kinetics [106, 143, 155, 156, 215, 216]. The technique requires planar, reflecting substrates, preferably quartz, silicon, or silica, and a sufficiently strong change of the refractive index upon protein adsorption.

Surface Plasmon resonance makes use of the light excitation of surface plasmons in thin metal layers. The resonance frequency and the angle of reflection minimum depend on the boundary conditions of the interface. Thus, by measuring these parameters the layer thickness, the adsorbed amount, and density fluctuations can be determined which also allows to record protein adsorption kinetics [92, 217]. However, SPR necessarily requires substrates coated with a metal layer which limits its versatility.

Optical waveguide lightmode spectroscopy (OWLS) is based on coupling a light beam into a waveguide through a specific incident angle. This angle is very sensitive to the refractive index change occurring upon protein adsorption. The adsorbed mass of a protein layer is amenable through the use of appropriate models. OWLS requires planar, optically transparent substrates such as quartz or $Si(Ti)O_2$ but no metal layer coating. Probably due to its high sensitivity and easy handling this technique has been used for numerous studies of protein adsorption kinetics [103, 142, 151, 152, 218, 219].

Techniques that specifically focus on the secondary structure of adsorbed proteins such as attenuated total internal reflectance – infrared spectroscopy (ATR-IR) and circular dichroism (CD) spectroscopy are valuable tools to study conformational changes. By measuring ATR-IR frequency and amplitude shifts of the amide I and amide II band of surface bound proteins, changes of the secondary structure in time were revealed [108, 111]. CD spectroscopy exploits the interaction of circular polarized light in the near UV range with the secondary structure of proteins. In contrast to most other techniques presented here, CD spectra are measured in the solution. Therefore proteins are adsorbed to colloidal silica particles in solution which do not interfere with the resulting spectra. Several studies report the use of this technique for direct observations of structural rearrangements of adsorbed proteins [53, 91, 104, 113].

In recent years the opportunities of detecting the intrinsic fluorescence of proteins that do not contain an extra fluorescent marker has been explored. This approach relies on detecting the fluorescence of the amino acid tryptophan (excitation max.: ~280 nm; emission max. ~360 nm) in tryptophan-rich biomolecules [101, 220-223]. The primary advantage of this method is

that any influence that a fluorescent marker may have on the properties or the behavior of the biomolecule of interest can be excluded.

In contrast to optical methods the working principle of the quartz crystal microbalance (QCM) technique is a change of the oscillating frequency of the substrate upon mass load. In protein adsorption studies the mass includes also the water coupled to or trapped within the layer which is an important difference to optical techniques like ELM, SPR, and OWLS that actually measure the adsorbed dry mass. To this end the QCM is inappropriate to determine the absolute mass whereas it is sensitive for water rich and extended layers. The technique was used to record protein adsorption and desorption kinetics, mostly in combination with other techniques [67, 128]. Modern QCM instruments are extended to allow energy dissipation measurements within the adlayer upon off-switching the driving voltage (QCM-D). Slow and fast dissipation thereby refer to rigid and flexible layers, respectively. Höök et al., for instance, made use of the combination of frequency shift and energy dissipation measurements to reveal a biphasic adsorption of the protein hemoglobin [41, 224]. First, a monolayer is formed that reaches strong adhesion and rigidity due to conformational relaxations. On the top of this layer a second protein layer establishes which constitutes more flexible proteins that were suggested to stay in their native state. Concerning the required substrate the QCM technique is quite flexible since the electrodes embedding the piezoelectric crystal can be coated with practically any desired thin film whereas optical properties like transparency or reflectivity do not need to be considered.

One of the most powerful techniques to measure the layer thickness of adsorbed protein films is neutron reflectometry. A collimated neutron beam at wavelengths ranging between 0.1 nm and 1.0 nm is exposed to and reflected from a protein covered surface. The reflectivity, i.e., the intensity of the reflected beam, is recorded as a function of the momentum transfer which depends on the chosen wavelength and the angle of incidence. In comparison to UV or visible light the wavelength of the neutron beam is considerably smaller, up to three orders in magnitude. Thus, film thickness information with sub nanometer resolution can be measured. Data analysis is performed via appropriate models in which values for the film thickness and the composition-dependent scattering length densities are fitted to the measured reflectivity profiles. In a whole series of publications Lu et al. [94-97] and Su et al. [98-100] could provide detailed information about the preferred orientations and the mass of surface adsorbed protein layers using neutron reflectometry. As an example, even minute differences in the layer thickness between side-on (30 Å) and end-on orientation (35 Å) of surface adsorbed Lysozyme were noticed precisely [100]. However, the technique is

very cost-intensive as it requires a neutron source which noticeably limits the number of instruments around the world.

Whilst neutron reflectometry has a strong resolution in direction normal to the surface it lacks a powerful resolution in lateral directions. At this point the surface imaging techniques atomic force microscopy (AFM), scanning tunneling microscopy (STM) and electron microscopy (EM) with a lateral resolution down to the atomic level are of great value. In AFM a sharp tip positioned at the end of a cantilever scans over the surface either in contact mode or in tapping mode. At close distances surface-tip interactions increase the force acting on the cantilever in the case of contact mode or reduce the amplitude of the oscillating cantilever in the case of tapping mode. A feed-back loop moves the tip up and down to keep the strength of these interactions at a constant level which directly yields height information. In the field of protein adsorption AFM is often applied to image the distribution of proteins within a layer with a special regard to surface aggregation [149, 150, 182-186, 225]. Even protein adsorption kinetics, albeit at very poor temporal resolution, and conformational changes of individual proteins were measured [226, 227]. The limiting factor of many AFM studies is that imaging is often performed after drying the surface which potentially affects the folding state of the individual proteins or the structure of the protein layer. Otherwise, if in-situ measurements are performed, tip-induced movement of adsorbed proteins is a potential source of error. In this context Lea et al. even reports on a 'molecular blooming effect' when the cantilever tip sweeps proteins across the surface leading to corrugations in the protein layer [228]. However, in addition to surface imaging AFM is a valuable tool to measure force-distance curves that give rise to the strength of protein-surface interactions. A single protein is immobilized at the end of the AFM tip and brought into contact with the surface. By slowly retracting the tip adhesive forces between protein and surface can be quantified. This technique was used to quantify protein-surface and protein-protein interactions [56, 58] as well as to probe the stability of surface tethered proteins [70]. Due to force-distance measurements AFM has directly revealed that most proteins adhere stronger to hydrophobic than to hydrophilic surfaces and stronger to charged than to uncharged surfaces [68, 69]. An alternative method for measuring protein-surface interaction is the so called surface force apparatus (SFA). Using the SFA Belfort et al. measured the intermolecular forces between and within adsorbed protein layers [229, 230] as well as between proteins and polymer films [59, 61].

As a comparable technique to AFM, scanning tunneling microscopy (STM) is based on a constant tunneling current between tip and surface to obtain topographic information. This

naturally requires an electrically conductive layer which is reached by coating the sample with a thin layer of metal or graphite. As a consequence, STM has minor importance in the field of protein adsorption. Publications reporting the use of this technique are often proof-of-principle studies [141, 231, 232]. A similar problem is encountered in EM where imaging is achieved through an electron beam. Again, a conductive layer on top of the sample has to be established which hinders in-situ imaging. Nevertheless, EM was applied to study protein aggregation on surfaces [193, 233, 234].

6.2 Fluorescence detection techniques

Over the past decades techniques based on fluorescence detection have become highly appreciated tools in life sciences. In particular the astonishing sensitivity down to the single molecule, the versatility, and the easy handling are strengths that lead to the wide use of fluorescence methods in the field of protein adsorption. Instruments can be divided into 'sensing devices' focusing on a fixed position and as 'imaging devices' visualizing objects on a given surface area. In order to study protein adsorption events, a selective detection of proteins bound to the surface is essential whereas proteins which diffuse in the solution close to the surface need to be precluded from detection. Comparable to the working principle of OWLS, total internal reflection fluorescence (TIRF) detection exploits the evanescent field upon total reflection to exclusively excite fluorophores in close proximity (~100 - 200 nm) to the surface. Thus the excitation light hits the substrate at an incident angle above the critical angle and the fluorescence emission is collected perpendicularly to the surface. Measurements can be performed in aqueous media on transparent substrates which renders the technique to a low cost and versatile tool. TIRF sensing has been frequently applied to record protein adsorption kinetics with a high sensitivity [93, 101, 126, 141, 227]. TIRF imaging was, for instance, applied to observe enzymatic reactions of single surface immobilized proteins or to visualize the mobility of myosin on actin [235-237].

An alternative method to TIRF is supercritical angle fluorescence (SAF) detection which exploits the effect that fluorophores positioned at the glass/water interface emit a considerable fraction of the fluorescence light into the substrate above the critical angle. The optical set-up is inverse to that of TIRF with an excitation beam illuminating the sample perpendicularly and a collection of fluorescence light emitted into the supercritical angle of the substrate using a parabolic mirror objective. The SAF technology itself (2000) [238, 239], a SAF sensing

instrument (2003) [240], and also a SAF imaging instrument (2007)[241, 242] were recently developed. The strength of the SAF technique to study protein adsorption phenomena was demonstrated in some recent publications [44, 102, 118, 187] [243].

Apart from protein detection and imaging there are two further important techniques related to fluorescence methods, namely fluorescence correlation spectroscopy (FCS) and Förster resonance energy transfer (FRET). In FCS fluorescence fluctuations are monitored as a function of time and statistically evaluated. The resulting autocorrelation function describes the probability to find a molecule within the detection volume a certain time after a molecule was detected. A curve fit of appropriate models to the autocorrelation function can provide physical parameters such as the diffusion coefficient of particles, aggregation states or molecular interactions. In terms of instrumentation FCS is routinely combined with confocal or TIRF set-ups and, more recently, also with the SAF optics [244]. In relation to protein adsorption the applicability of FCS to reveal the aggregation of proteins in the solution or on the surface has been demonstrated [245, 246].

FRET is the non-radiative transfer of the excited state energy from a donor fluorophore to an acceptor fluorophore via dipole-dipole interactions. This process requires an overlap of the donor emission spectrum with the acceptor absorption spectrum, a favorable relative orientation of the two involved transition dipole moments, and a donor-acceptor distance in the range of the Förster radius (3 ~ 7 nm). Since the energy transfer efficiency is inversely proportional to the sixth power of the spacing between donor and acceptor fluorophore it can be used as a 'spectroscopic ruler' which is highly sensitive to small distance changes. The FRET technique has been successfully applied in the solution to map protein folding processes at the single molecule level or to understand the relation between protein unfolding and aggregation [247, 248]. Further, FRET imaging of surface adsorbed proteins was performed to study protein-protein interactions and aggregation [129, 249, 250]. The combination of FRET with SAF imaging to investigate the spreading of surface adsorbed protein clusters in time has also been demonstrated [187].

A limiting factor of fluorescence imaging methods as well as of optical methods in general is that only those details can be separately detected that are about one half of the wavelength apart from each other. However, even this paradigm formulated by Ernst Abbé is being overcome by some remarkable recent developments in fluorescence microscopy. Already in the 1980s near-field scanning optical microscopy (NSOM) was developed by combining the scanning facilities of AFM with near-field optics. The method is based on a sharp tip scanning in close distance across the sample which provides a resolution of down to

20 nm [251, 252]. One of the most prominent recent developments is stimulated emission depletion (STED) microscopy which is based on the de-excitation of fluorophores around the center of an excitation beam [253-255]. Further promising approaches that have been proven to enhance the optical resolution down to the order of only a few tens of nanometers are saturated pattern excitation microscopy (SPEM) [256], photoactivated localization microscopy (PALM) [257], and stochastic optical reconstruction microscopy (STORM) [258]. Recently, Winterflood et al. have succeeded to further develop the SAF technique such as to allow for a spatial resolution in z-direction in the nanometer range. At the present this so called 3D-SAF-microscopy technique is amongst the highest in spatial resolution [243]. The application of such high resolution imaging techniques will certainly have a strong impact on the research in the field of protein adsorption in the nearer future.

Another decisive drawback connected with fluorescence methods is the need of a label attached to the analyte molecule unless this molecule is a fluorophore itself. Comfortably, a large number of fluorescent dyes with coupling groups for a covalent attachment to biomolecules is commercially available. However, the potential interference of the additional tag to the experimental result needs to be clarified prior to any scientific conclusion [253]. Given the fact that size and mass of common fluorescent dyes are comparable with those of small biomolecules, it is essential to question whether the adsorption behavior and in particular the surface affinity of the dye-labeled analyte molecules is identical to that of the native analyte molecules. Several studies report that dye-labeled proteins may exhibit a stronger adsorption affinity to the selected surfaces than unmodified proteins under identical conditions [259-261]. A second issue concerns the emission intensity of fluorescent dyes under varying local conditions. In the case of fluorescein isothiocyanate (FITC) it has been reported that the fluorescence intensity can decrease when the fluorophore approaches a negatively charged surface as a result of the re-orientation of the protein. This alteration of the local electrostatic potential is assumed to favor the protonation of the carboxyl residues of fluorescein which in turn reduces the emission intensity [101, 169]. An intensity decrease of fluorophores can also be the result of photodestruction processes caused by repeated excitation/emission cycles [262]. When protein adsorption kinetics are recorded with fluorescence methods, it has to be shown that the adsorption curves are not significantly influenced by the attached fluorophore. An efficient experiment to probe this issue consists of diluting fluorescently labeled proteins with unlabeled, native proteins at different ratios. If the total concentration is kept equal the resulting kinetic curves should exactly reflect the applied ratio of unlabeled to labeled proteins. As an example, Fig. 9 A presents pairs of adsorption

kinetics of a sample whose content of dye-conjugated proteins was set to differ by a factor of two. On examining these pairs (lower and upper adsorption curves in Fig. 9 A), it is noticed that their fluorescent intensities also differ by a factor of two. In particular, the saturation levels are always reached after the same adsorption time independent of the content of labeled proteins. Only in the case of a preferential adsorption of dye-conjugated proteins one would expect a factor smaller than two as well as different adsorption times until saturation. A second experiment that serves to exclude the inverse problem namely that labeled proteins adsorb much slower or weaker than unlabeled ones is shown in Fig. 9 B. Two different adsorption kinetics were recorded, a reference kinetic curve containing labeled proteins during the whole experiment (upper curve) and a second kinetic curve consisting of the adsorption of unlabeled, native proteins for the first 800 s and the adsorption of labeled proteins at the same concentration thereafter. It is evident that both curves exhibit a characteristic feature, in this case an overshoot, at exactly the same point in time. This is only explainable if unlabeled proteins adsorb at more or less the same rate as labeled proteins. If such a behavior as demonstrated in Fig. 9 A and B can be shown, any influence of the dye on the adsorption behavior of the protein can be excluded.

7 Computational approaches

An increasingly important access to molecular-scale studies of protein adsorption events has been opened by computational methods [263-266]. The adsorption of one or several proteins to a selected surface is simulated with differing degrees of exactness based on physical laws. Although, at the present, computer simulations are far from being suitable to replace experimental work they provide a growing amount of useful information about some details of adsorption mechanisms. Without the technical and physical constraints ruling experimental methods, computational approaches to protein adsorption practically allow to ‘see’ the movement of every protein or even of its atoms. Limitation, however, results from the computational costs which rise rapidly with increasing structural detail and precision.

Starting at the highest level of precision, quantum mechanical (QM) simulations were performed on single amino acids or small peptides adsorbing on a solid surface to draw fundamental conclusions about the adsorbate state or binding mechanisms and energies at the atomic level [267-274]. It was found, for instance, that in contact to the surface the zwitterionic form of the adsorbed amino acid is preferred over the neutral form [269, 272].

Concerning the binding situation it was shown that the amino function of amino acids does not bind directly but via water molecules to silica-like surfaces [267, 268]. Due to the high computational expanses of QM methods their use in the field of protein adsorption is restricted to extremely small systems, usually consisting of only one or a few amino acids interacting with a restricted and artificial model surface area and a limited number of solvent molecules. If trajectories of protein adsorption processes are simulated they are usually limited to a few picoseconds which is by far too short to reach equilibrium [267, 268]. The advantage is, of course, that no empirical parameters are needed and a high level of accuracy can be expected.

To simulate larger systems and longer time scales all-atom empirical force field methods are applied including molecular dynamics (MD) simulations, monte carlo (MC) simulations, and energy optimization methods. The critical point with these methods is the choice of a valid force field which is always designed for a specific problem. Naturally, the more accurate the implemented forces are the higher are the computational costs. Compromises are typically made by treating solvent molecules implicitly via an effective dielectric medium or by using force fields that have no extra term for H-bonds [275-279]. In quite a number of recent studies empirical all-atoms force field methods were successfully applied to determine the preferred orientations of surface bound proteins [275-278, 280-286], to visualize conformational rearrangements upon adsorption [276-278, 287], and to explore effects on the solvation shell [277, 288, 289]. By using a combination of MC and MD simulations to study the adsorption of the proteins IgG and cytochrome c (Cyt-c), Zhou et al. were able to show that there is a surface charge-driven mechanism of protein orientation [281-283]. In particular they simulated Cyt-c adsorption on a self assembled monolayer terminating with carboxylic functionalities at varying dissociation degrees, i.e. at different charge densities [283]. In a first step the protein was kept rigid and placed a few nanometers above the surface in a random orientation. By using MC simulations one million configurations were sampled to obtain a preliminary optimized orientation. Subsequently, the resulting configuration was used as a starting point for an MD simulation over a period of 1 ns. In this way the authors found the preferred orientation of Cyt-c with its dipole moment oriented orthogonally to the surface at high surface charge densities and a slightly tilted orientation at lower charge densities (Fig. 10). A similar investigation was conducted by Hagiwara et al. to study the adsorption mechanism of the protein β-Lactoglobulin on a positively charged model surface [275]. The authors showed that after an initial surface adsorption through van der Waals interactions some acidic residues near the surface dissociate and hence strengthen the protein-surface

binding through additional electrostatic interactions. A drawback of MD over MC simulations is that the simulated time period is often too short to ensure a sufficient sampling of the configurational phase space as the system can easily be trapped in one of the numerous local low-energy wells [264, 278, 288]. Therefore, the combination of an MC simulation to find a rough optimum with a subsequent MD simulation to perform a kind of fine adjustment is the preferred approach. However, still at the present these methods are limited to systems comprising mostly no more than one protein and surface areas that are a bit larger than the size of the protein. Given that most simulated time periods amount to only a few nanoseconds one can easily infer that simulations using all-atom force field methods are out of question for realistic systems comprising several proteins and time scales of at least seconds.

A promising step forward toward larger systems and more realistic time scales are simulations based on coarse-grained models in which structural information is maintained in a strongly simplified manner [290]. Skepö et al. studied the influence of electrostatic and short-range interactions on the adsorption of the salivary proteins proline rich protein 1 (PRP 1) and statherin on charged and uncharged surfaces [291-293]. The proteins were represented by a flexible chain of beads connected by harmonic bonds. Depending on the nature of the amino acid that is represented, those beads bear, respectively, a positive, negative, or no charge (Fig. 11). The different conformations and orientations of these proteins adsorbed to positively, negatively, or uncharged surfaces were examined. In particular the adsorption of net negatively charged proteins to negative surfaces could be illustratively visualized with these simulations. In the work presented by Carlsson et al. the protein Lysozyme was represented by a hard sphere with embedded positive and negative charges (Fig. 12) [294]. Using an ensemble of 64 proteins information about preferred surface orientations and about the effect of varying pH, ionic strength, and surface charge on the adsorbed amount was obtained. Thus, at this level of simplification real ensemble effects like the structure of the adsorbed protein layer can be studied. Additionally, the coarse graining approach opens the opportunity for the comparison of simulation results with experimental data. In practice, however, correlating simulation results with experimental observations often requires even ‘coarser’ graining.

The general strategy to computationally explore protein adsorption phenomena with large protein ensembles over reasonable periods is to abandon any detail of structural information. This is done by defining proteins as single particles that may be spherical, disk shaped, hexagonal, or rectangular, with a specific charge or binding site located somewhere within or at the edge of the particle [138, 295-300]. The adsorption process is then simulated with a Metropolis Monte-Carlo simulation according to appropriate algorithms. To evaluate the

Metropolis criterion after each MC step, the potential energy has to be calculated using valid pair-potential models. This is certainly the most critical point in this method as a unique mathematical description of the potential energy terms arising for a protein close to a solid surface possibly surrounded by further adsorbed proteins is simply non-existent. Most models are again based on crude approximations such as a uniform, coverage-independent protein-surface potential which may be inappropriate for some specific problems. An exact description of the potential energy near the surface would be a non-trivial high dimensional function considering the short- and long range interactions between the adsorbing protein and all pre-adsorbed proteins plus the surface at least in a local environment. In other words, the loss of structural information hinders an accurate evaluation of the energy difference resulting after each MC step. One solution is to propose arbitrary interaction energies for the adsorption of a protein, its transition into another state, or its aggregation with other pre-adsorbed proteins [186, 297, 298, 300]. Another possibility consists of replacing approximate models with empirical parameters [149, 186, 298, 299]. Calculating the Boltzmann factor from the energy gain/loss of an MC step serves to determine the probability that this step is accepted. In many simplified simulation studies an MC step corresponds to a microscopic event such as the adsorption, desorption, diffusion, or aggregation of a single protein. From a macroscopic perspective, average values of the probability of such an event are simply reflected by the respective adsorption, desorption, diffusion, or aggregation rate constant. It is therefore efficient to make use of these experimentally accessible parameters to circumvent the difficulty of constructing an approximate model for calculating the total energy difference before and after the MC step. This strategy was applied by Rabe et al. to address the phenomenon of cooperative protein adsorption from a microscopic point of view [301]. The MC algorithm was designed to differentiate between a protein that adsorbs at the surface in an empty area and a protein that adsorbs in the vicinity of other pre-adsorbed proteins (Fig. 13). Basically, the simulation reflects the tracking or guiding mechanism through which proteins diffusing freely in the bulk close to the sorbent surface can be tracked to an available binding in the vicinity of one or a several pre-adsorbed proteins. The attraction is due to the complex electrostatic field resulting from the overlapping fields of the surface charges and of the charges of the pre-adsorbed proteins. With the help of a convolution of the protein covered surface with the point spread function of a laser scanning microscope and by determining the amount of adsorbed proteins in time the simulation gives direct access to simulated microscopy images (Fig. 13, middle row) and simulated adsorption kinetics (Fig. 13, lower row). Thus, the macroscopic response to varying microscopic parameters, in particular the

length of the maximum allowed tracking distance, was obtained by this simulation. Because of an excellent agreement of the simulation results with experimental data the proposed mechanism underlying cooperative protein adsorption was justified. Additionally, the maximum tracking distance for some experimental systems was determined to range up to 35 nm depending on the protein and the buffer conditions.

8 Concluding Remarks

Investigating protein adsorption phenomena on solid surfaces is a complex and also fascinating endeavor. Its scientific relevance is manifested in various problems encountered in research areas such as designing biocompatible materials, tracing biological events that trigger or prevent diseases, improving analytical devices, or control fouling processes to name a few. Ever since the 1970s, when Leo Vroman had observed exchange mechanisms between adsorbed plasma proteins, researchers are aware of the great complexity inherent to protein adsorption events [162, 164]. Although continuous effort in this field has been advancing our understanding considerably over the past decades we are far from a complete and unique view on how do proteins behave upon contact with solid interfaces. Many phenomena, such as the overshooting effect, cooperative adsorption, or protein aggregation, are still controversially discussed in the scientific community. From a practical point of view this can be understood considering that even minute changes in the experimental conditions have a tremendous impact on the outcome. Thus researchers working on similar questions may come to opposing answers simply because of a differing experimental detail. It is exactly the vast number of those details, such as the buffer composition (including pH, ionic strength, etc.), the temperature, the sorbent surface, the protein itself, and even the measuring technique, which renders the field of protein adsorption to a highly complicated problem. An ongoing effort to increase the accuracy and reliability of experimental work is needed as a prerequisite of a systematic and step by step approach to understand protein adsorption phenomena. This naturally includes advances in the development of measuring techniques which will offer better opportunities concerning sensitivity, resolution, and most importantly, reliability. In choosing the experimental technique to study protein adsorption events researchers so far have to make a decision between techniques that either offer high resolution for the price of being an invasive technique, or non-invasive techniques with limited resolution that are based on attaching a label to the analyte molecule, or label-free detection techniques whose sensitivity is rather weak. Fortunately there are promising recent advances in breaking the

Abbé limit with a new generation of high-resolution fluorescence microscopes. It can be assumed that there will be soon solutions that combine the advantages of different techniques like non-invasiveness, high sensitivity, and high spatial resolution.

Experimental data are the basis for mathematical models that help to test and to refine mechanistic hypotheses. Once the validity of a model has been proven it can be used to describe and predict protein adsorption phenomena. This will be of major relevance for both, a controlled manipulation of protein adsorption to implants, analytical devices, or container walls, and for deciphering biological and biochemical processes in living cells. The vast number of differing models of protein adsorption suggests that there is an urgent need for more clearness and unification. Conciseness but also accuracy and general applicability are the essential prerequisites and so far only a few models discussed in the field of protein adsorption can attend to all those requirements.

Future work must also include investigations on the influence of adsorption phenomena on the protein's functionality. Many effects such as the transitions between different adsorption states necessarily induce conformational re-orientations which certainly affect the biochemical activity of the individual proteins. Moreover, there is still little information on how important ensemble effects like overshootings, cooperativity, or aggregation are for the control of biological processes. These questions imply a considerable increase in experimental complexity as suitable model systems have to be designed that are in accordance with real biological systems. Additionally, the investigation of the protein's functionality in-situ adds a new dimension to the techniqual requirements.

In principle, computational approaches to the field of protein adsorption are promising as there are independent of experimental constraints. There is no doubt that the contributions arising from this side will rise considerably in the nearer future. However, the limiting point is that the computational expanses rise in a very unfavorable relation with the system size in particular when a high level of accuracy is demanded. As a consequence, successful strategies of future research will rely on a clever hyphenation of computational and experimental methods to elucidate the molecular rules behind protein adsorption phenomena from different perspectives.

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Figures

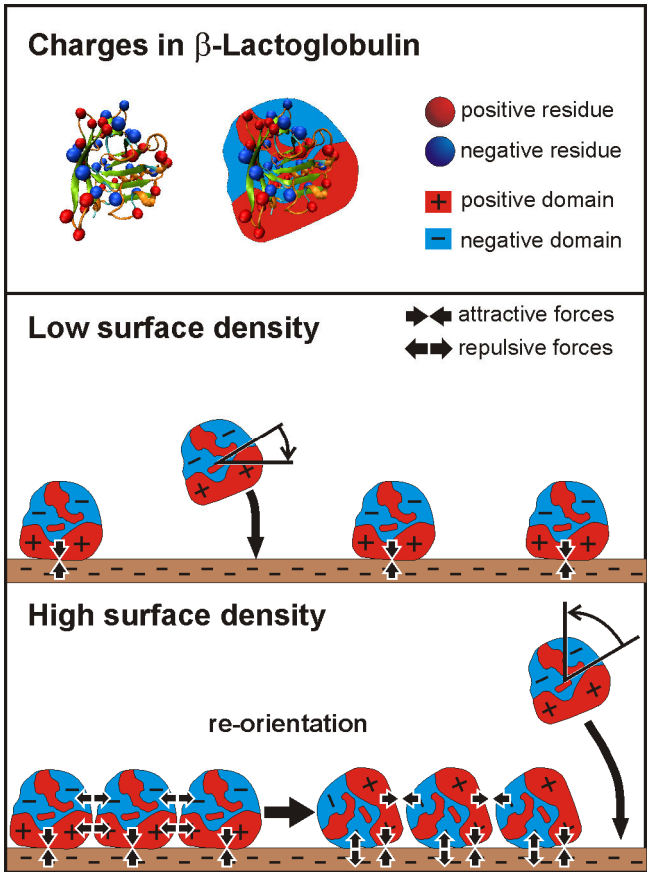

Fig. 1. Schematic representation of orientational changes of surface adsorbed proteins. Top: A cartoon representation of β-Lactoglobulin depicts the distribution of positively (red spheres) and negatively (blue spheres) charged amino acids. After strong simplification the protein is represented as globular entity consisting of positive and negative domains. Middle: At low surface densities the protein orientation is solely determined by surface-protein interactions. Bottom: At high surface densities increasing protein-protein interactions can trigger orientational changes leading to a decrease of protein-surface interactions.

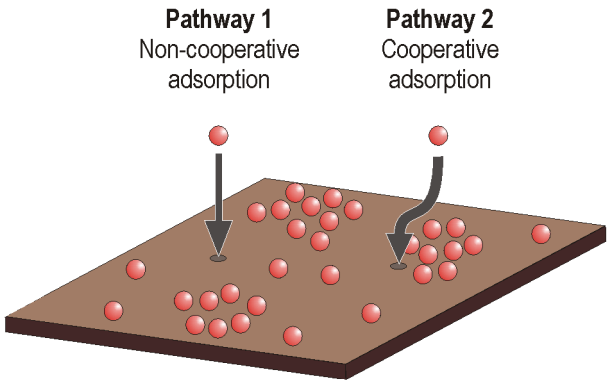

Fig. 2. Schematic representation of the non-cooperative (pathway 1) and the cooperative (pathway 2) protein adsorption mechanism. During cooperative adsorption proteins are vertically tracked toward the surface and at the same time horizontally repelled by neighboring proteins.

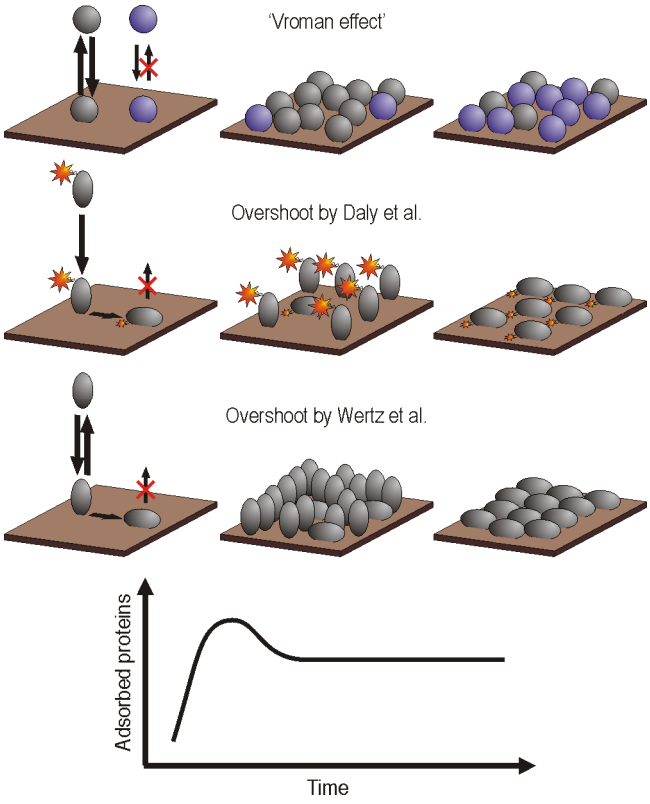

Fig. 3. Three different explanations for overshooting adsorption kinetics: The Vroman effect (upper row) describes the competitive adsorption of a fast adsorbing species of relative low surface affinity and a slowly adsorbing species of high surface affinity. The explanation by Daly et al. (middle row) is based on a change of the protein's orientation after adsorption which results in a decrease of fluorescence emission intensity of the dye [101]. Wertz et al. (lower row) argue that the initial end-on orientation allows more species to adsorb on the surface as the final and energetically preferred side-on orientation [93].

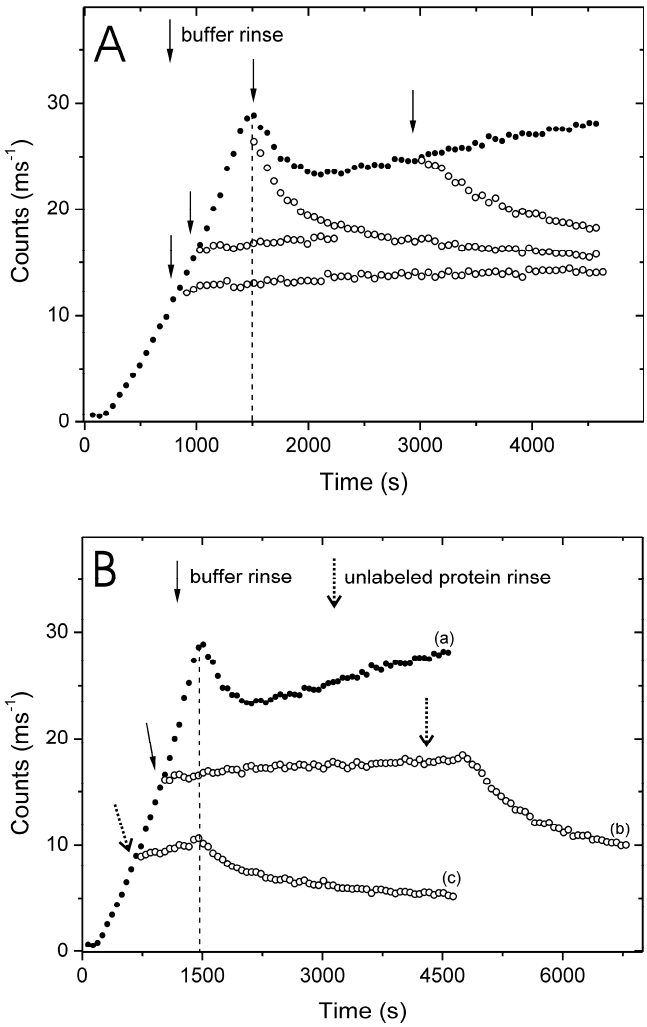

Fig. 4. Overshooting adsorption kinetics during the adsorption of β-Lg.
A) Reference adsorption kinetics (filled circles) and rinsing kinetics obtained from buffer rinse (open circles). Before the overshoot is exceeded proteins are irreversibly adsorbed; afterwards they are reversibly adsorbed.
B) Reference adsorption kinetics (filled circles, a) and rinsing kinetics obtained from rinsing with buffer and unlabeled proteins (open circles, b, c). Curve b shows the irreversible behavior of adsorbed proteins upon rinsing with buffer for ~1 h; only when an equally concentrated solution of unlabeled proteins is added, proteins become reversible. Curve c shows the behavior when the solution of labeled proteins is replaced with an equally concentrated solution of unlabeled proteins: adsorbed proteins abruptly become reversible once a critical surface density is exceeded.

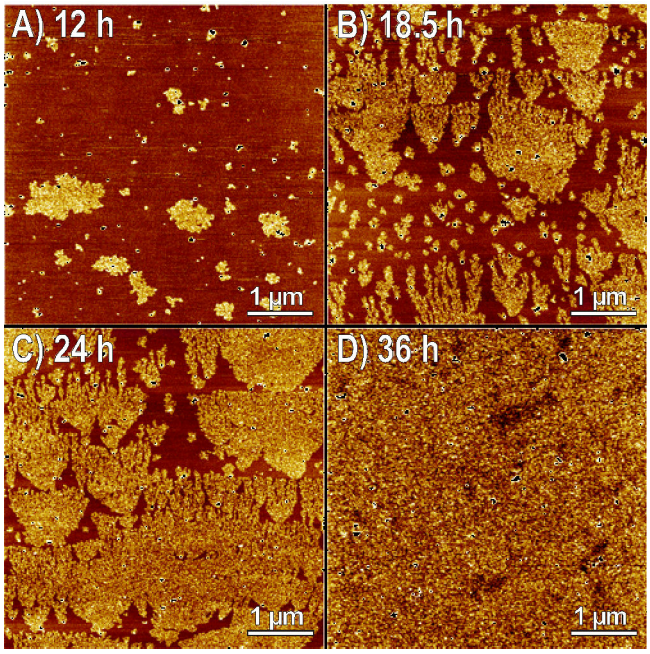

Fig. 5. Time-elapsed SFM images showing the topography (tapping mode) of a solid-supported membrane after the addition of ezrin. (A) 12 h after the addition of ezrin (c_{ezrin} = 0.35 µM) and (B) after 18.5 h, (C) 24 h, and (D) 36 h incubation time with ezrin (c_{ezrin} = 1.5 µM). Fig. reproduced from ref. [128] with permission.

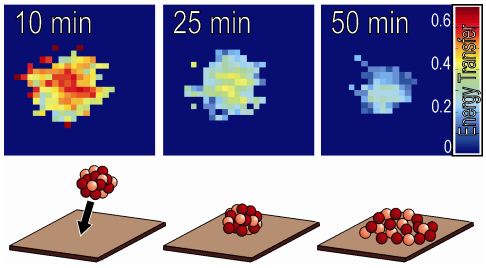

Fig. 6. FRET imaging reveals that protein clusters consisting of up to several hundreds of protein monomers can form in the solution and subsequently deposit onto an interface where they spread.

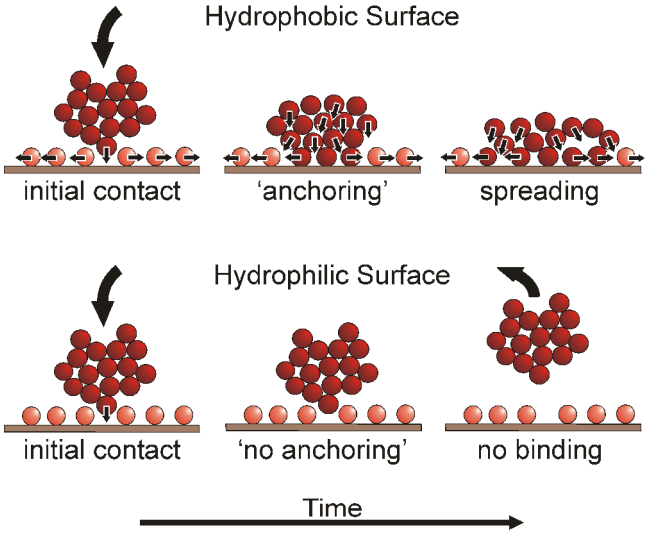

Fig. 7. Schematic illustration of the mechanism of the spreading process of a protein cluster in time on the hydrophobic and the hydrophilic surface.

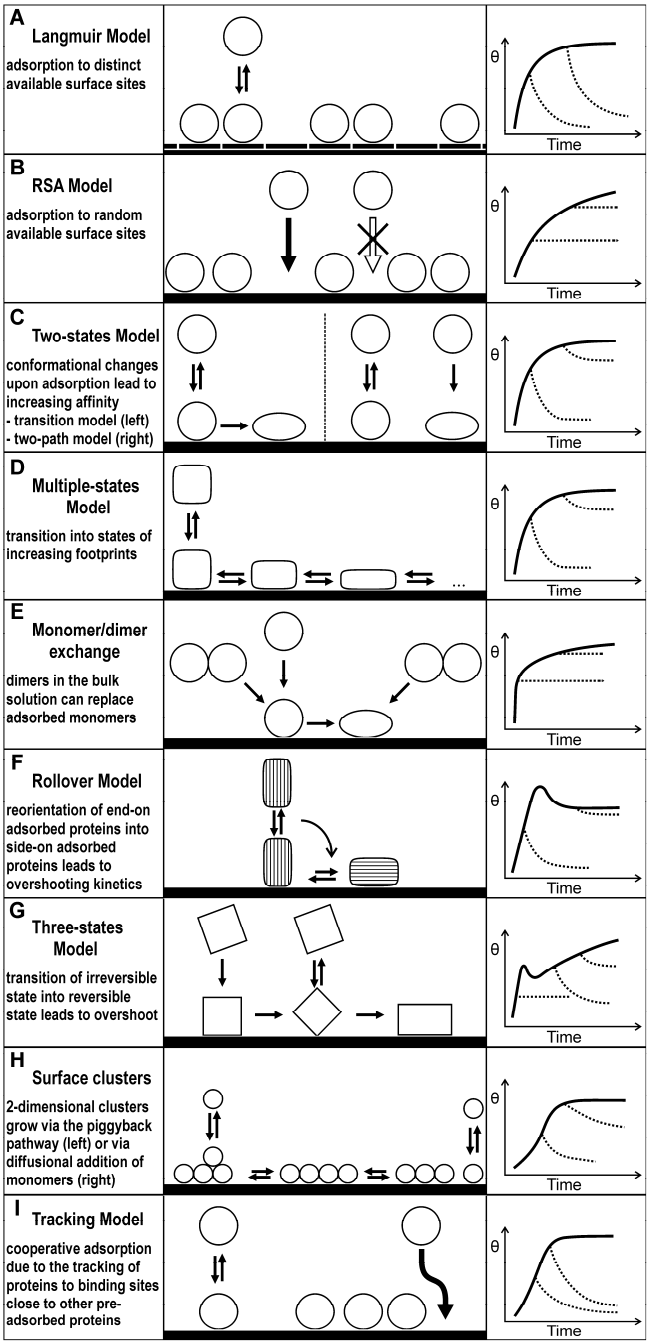

Fig. 8. Overview of kinetic models. A short scheme of each adsorption mechanism adapted from the respective references given in the text is presented in the middle column. The most important characteristics of the observable adsorption kinetics are depicted in the right column (solid line: adsorption curve; dashed line: desorption curve upon buffer rinsing).

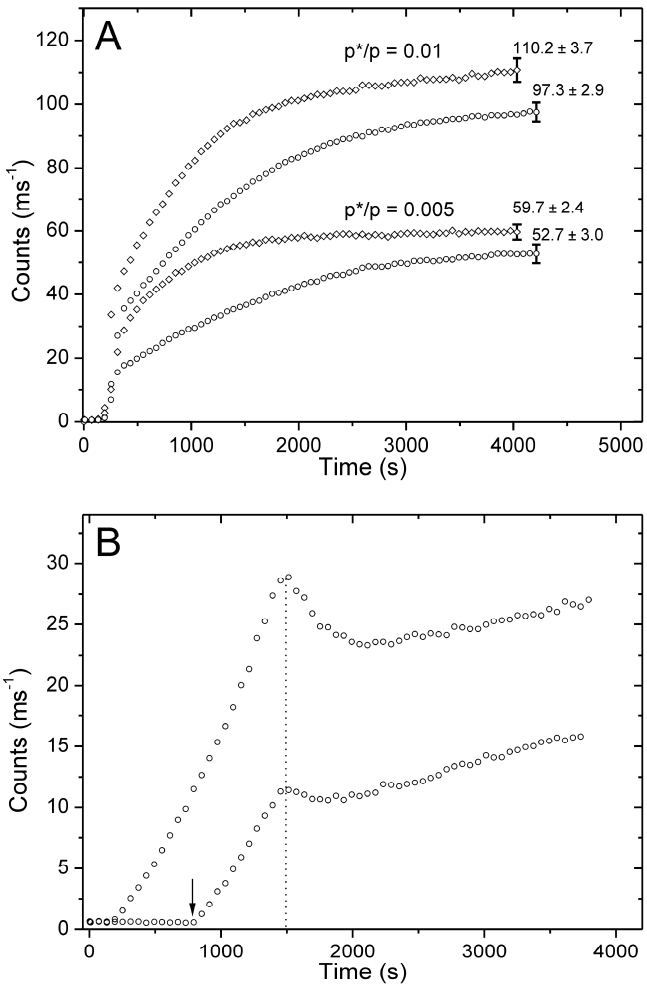

Fig. 9 A) Adsorption kinetics of fluorescently labeled β-Lg at bulk concentrations of 1.5 µM (◊) and 0.75 µM (○). The ratio of labeled to unlabeled proteins differs by a factor of two between the upper two curves and the lower two curves. As the final saturation levels also differ by a factor of two a preferential adsorption of labeled over unlabeled proteins can be excluded.

B) Lower curve: Adsorption of unlabeled β-Lg (50 nM) for 800s followed by the adsorption of fluorescently labeled β-Lg (arrow) at the same bulk concentration. Upper (reference) curve: Adsorption of fluorescently labeled β-Lg from the beginning. A characteristic feature, namely an overshoot, is observed at the same point in time in both experiments such that a hindered adsorption of labeled over unlabeled proteins can be excluded.

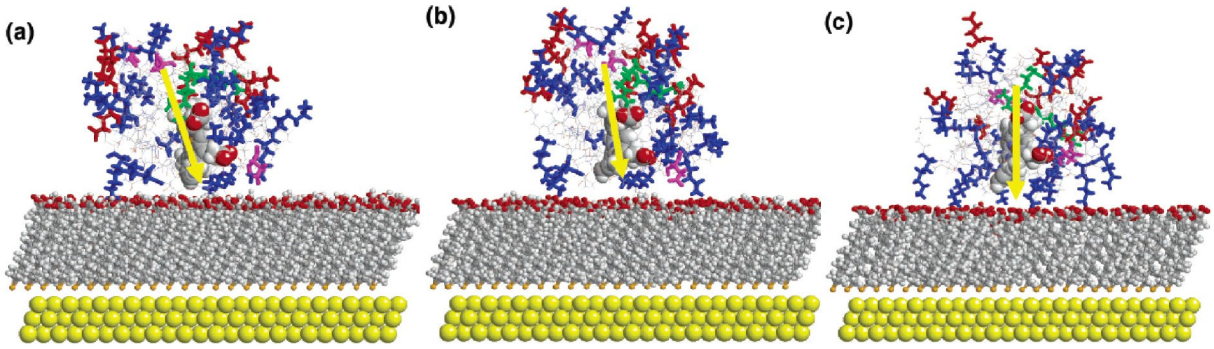

Fig. 10. Cyt-c configurations on carboxyl-terminated SAMs with increasing dissociation degrees of the terminal carboxyl groups, i.e. increasing surface charge densities: a) 5% b) 25% c) 50%. Reproduced from ref. [283] with permission.

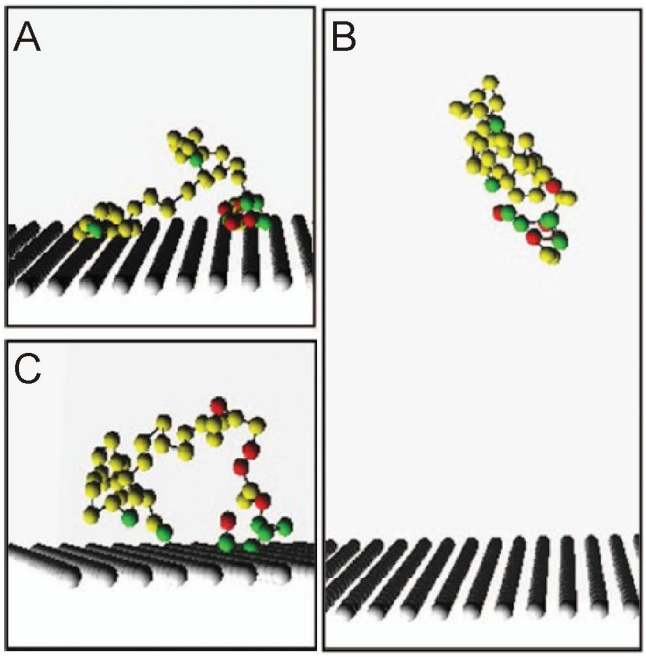

Fig. 11. Coarse graining to study protein adsorption I: Snapshots of the statherin model in different environments: (A) adsorbed to a pure hydrophobic surface; (B) adsorbing to a system with a hydrophilic negatively charged surface; (C) adsorbed to a hydrophilic positively charged surface. The simulated systems contain coil-like proteins with 44 amino acids represented as connected dots (red: positively charged amino acids; green: negatively charged amino acids; yellow: uncharged amino acids) Reproduced from ref. [291] with permission.

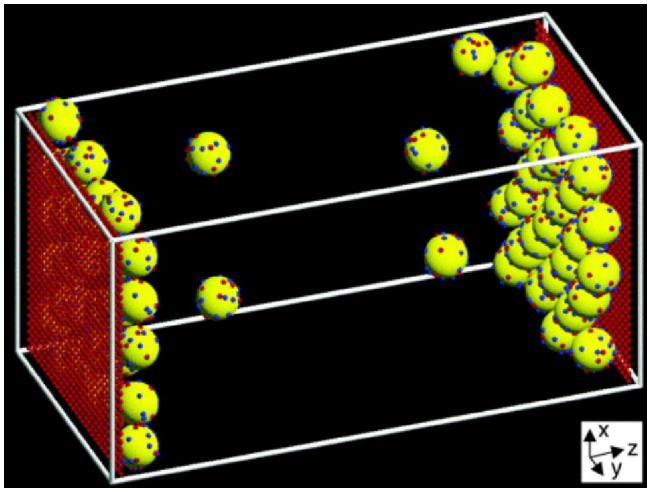

Fig. 12. Coarse graining to study protein adsorption II: Illustration of a simulation box with two positively charged surfaces and a total of 64 proteins modeled as hard spheres with positive and negative charges inside (red and blue dots). The snap shot was taken at the end of the simulation showing how proteins are arranged at the surface. Reproduced with permission from ref. [294]

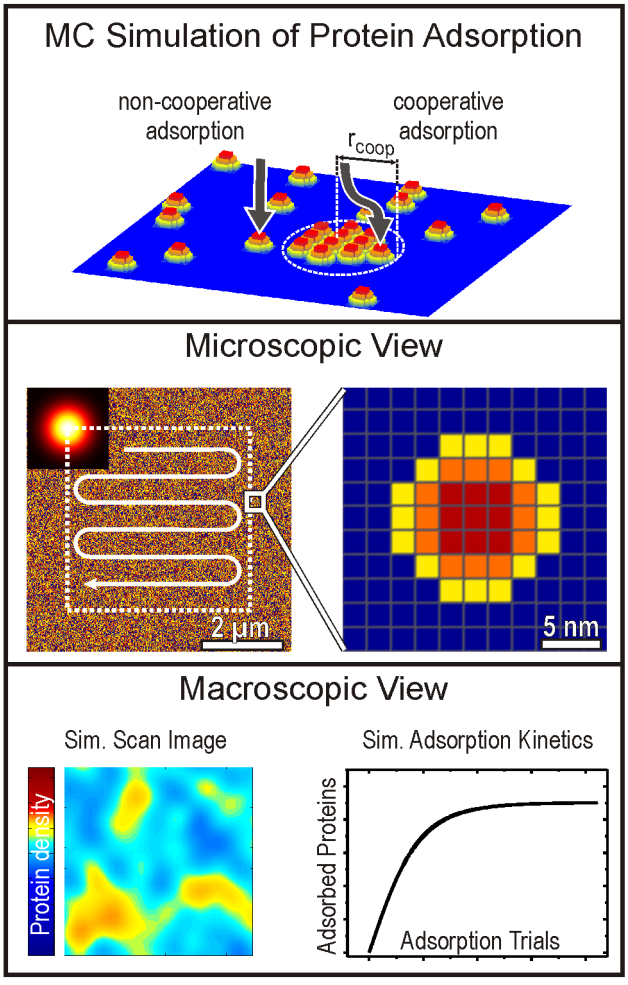

Fig. 13. Monte Carlo simulation to study cooperative effects during protein adsorption.