Specific and Non-Specific Adsorption of Proteins at Solid Interfaces

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ABSTRACT

Two studies in the field of protein adsorption are presented. First, we comprehensively investigated the non-specific adsorption mechanism of β-Lactoglobulin on a solid interface by monitoring adsorption kinetics under varying conditions. The experimental observations are translated into mathematical concepts resulting in a complete and accurate mechanistic model which is exploited to calculate long term kinetics and an adsorption isotherm. Second, we propose a new and very simple method to create highly porous functionalized surface coatings with specific protein adsorption characteristics. Such surfaces are considered to have a high potential in biosensor applications.

Keywords: Protein adsorption, Protein enrichment, SAF, Kinetics, Biosensors

1 INTRODUCTION

Protein adsorption is an active area of research promoted by a wide interest in fields like biology, medicine, pharmacology, chemical analysis or food processing. For a deeper understanding of many natural processes it is essential to explore the mechanisms behind protein adsorption on a molecular level. In the field of analytical sciences, on the other side, materials allowing for controlling the process of protein adsorption are required. Finally, in the case of biomedical implants protein adsorption must be either hindered to prevent blood coagulation or promoted to allow tissue growth.

To date there is a broad spectrum of experimental methods to approach the field of protein adsorption studies. In our lab a specific fluorescence detection method was developed that enables the highly sensitive and selective detection of surface generated fluorescence. This so called supercritical angle fluorescence (SAF) biosensor was used in this work to perform real-time measurements of the adsorption of proteins on various transparent surfaces [1, 2]. DY-647 labeled β-Lactoglobulin and Lysozyme served as model proteins during the study of specific and non-specific protein adsorption.

2 NON-SPECIFIC ADSORPTION

The adsorption behavior of β-Lactoglobulin in citrate buffer (pH 3) on hydrophilic glass was investigated in a large range of protein bulk concentrations from 1.0×10^{-8} M up to 1.5×10^{-6} M. In the high concentration range it was found that the reversibility of the non-specific binding in the presence of protein-free buffer was slowly altered in time. Rinsing after distinct adsorption times revealed that the reversibly bound fraction of all adsorbed molecules depleted the longer the adsorption process proceeded. In agreement with a former study this can be explained by a slow relaxation of reversibly adsorbed β-Lactoglobulin molecules to an irreversible state [3].

If rinsing is conducted with an equally concentrated solution of unlabeled proteins, potential interactions between adsorbed molecules and molecules which are in close proximity to the surface can be studied. In this way it was observed that incoming proteins enhance the desorption of adsorbed proteins.

Figure 1 shows the adsorption kinetics of β-Lactoglobulin at different bulk protein concentrations. As can be noticed for concentrations above 0.125×10^{-6} M (Figure 1, curves a, b) the adsorption curves tend to reach a saturation limit of approximately 110 counts×ms^{-1}.

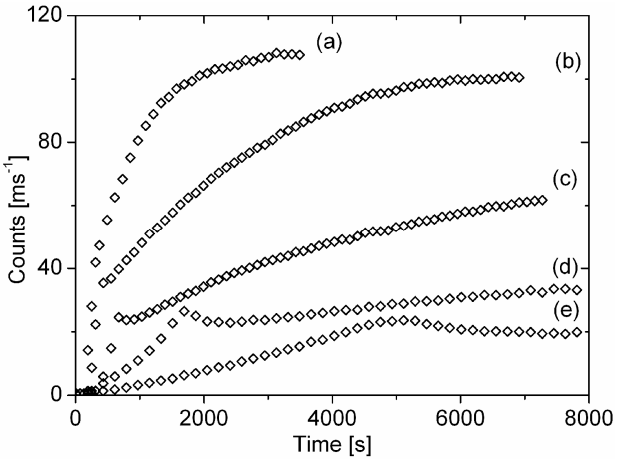

Figure 1: Measured adsorption kinetics of β-Lactoglobulin at different bulk concentrations:
(a) 1.5×10^{-6} M, (b) 0.5×10^{-6} M, (c) 0.125×10^{-6} M,
(d) 4.0×10^{-8} M, (e) 1.0×10^{-8} M

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One of the most striking observations during this study is the overshooting behavior of β-Lactoglobulin in the early adsorption process which is clearly seen at concentrations below 0.125×10^{-6} M (Figure 1, curves c, d, e). The overshoot appears as a "kink" in the adsorption kinetics at high protein bulk concentrations whereas a clear peak shape is encountered at low bulk concentrations. It is also noticed that the overshoot occurs at roughly equal surface coverage levels of approximately 30±5 counts×ms^{-1}. Consequently, the adsorption time before the overshoot is the longer the lower the bulk protein concentration is.

To give a mechanistic interpretation of the observable kinetics the complete adsorption process can be divided into three stages. In the beginning the free surface is rapidly populated by adsorbing proteins in a non-exponential-like manner. In fact, a closer look into the curvatures of the adsorption kinetics in the low concentration range even reveals that the initial rate increases with a growing surface coverage. Once the coverage reaches a critical density, a maximum in the adsorption kinetics is observed followed by a temporal decrease of the total coverage. Interestingly, the peak widths of the overshoots are broad at low bulk concentrations and narrow for higher concentrations. Thereafter, the surface density passes a local minimum and continues to grow in an exponential-like manner until the equilibrium coverage is reached.

The key process which explains the overshooting behavior during the adsorption is an abrupt transition of adsorbed proteins from an initial irreversible state to a reversible state. By means of rinsing experiments it was recognized that this transition takes place when the growing surface coverage exceeds a certain critical surface density. As this transition proceeds very fast, the surface is temporarily populated with an excess of reversibly bound proteins which desorb from the surface, leading to the observed decrease. Once the fraction of reversibly adsorbed proteins is depleted to a quasi equilibrium amount, the adsorption kinetics (i.e. the sum of adsorption and desorption rate) passes a minimum. As discussed before, there is also a slow relaxation of the reversible state to a strongly bound final state which eventually causes the adsorption kinetics to grow again but with a significantly reduced rate. Hence, in the final stage the surface is mainly populated with the relaxed state of the protein.

Based on all discussed experimental observations a mathematical model was developed that accurately describes the adsorption and desorption behavior of β-Lactoglobulin in the given conditions. It basically includes three different states of adsorbed proteins, referred to as initial (init), reversible (rev), and irreversible (irr) state. The abrupt transition of the initial into the reversible state is easiest described by defining specific processes for the adsorption at low ($\theta\leq\theta_{crit}$) and high ($\theta>\theta_{crit}$) surface densities. The following rate equation system is used to express the observed behavior.

$$\frac{\mathrm{d}\theta_{init}}{\mathrm{d}t} = k^{on}\cdot c\cdot\Phi - k^{trans}_{init\_irr}\cdot\theta_{init}, \qquad \theta\leq\theta_{crit} \tag{1a}$$

$$\frac{\mathrm{d}\theta_{rev}}{\mathrm{d}t} = 0, \qquad \theta\leq\theta_{crit} \tag{2a}$$

$$\frac{\mathrm{d}\theta_{init}}{\mathrm{d}t} = -k^{trans}_{init\_rev}\cdot\theta_{init} - k^{trans}_{init\_irr}\cdot\theta_{init}, \qquad \theta>\theta_{crit} \tag{1b}$$

$$\frac{\mathrm{d}\theta_{rev}}{\mathrm{d}t} = k^{on}\cdot c\cdot\Phi + k^{trans}_{init\_rev}\cdot\theta_{init} - k^{trans}_{rev\_irr}\cdot\theta_{rev} - k^{off}_{rev}\cdot\theta_{rev}, \qquad \theta>\theta_{crit} \tag{2b}$$

$$\frac{\mathrm{d}\theta_{irr}}{\mathrm{d}t} = k^{trans}_{init\_irr}\cdot\theta_{init} + k^{trans}_{rev\_irr}\cdot\theta_{rev} - k^{off}_{irr}\cdot\theta_{irr} \tag{3}$$

$$\theta = \theta_{init} + \theta_{rev} + \theta_{irr} \tag{4}$$

$$\Phi = 1 - \frac{\theta}{\theta_{max}} \tag{5}$$

Some of the observable phenomena, such as the increasing adsorption rate in the beginning, lateral repulsions between adsorbed proteins in the high concentration range, and the concentration-dependent peak width are not satisfactorily described by these equations yet. For this reason coverage dependent rate constants for the on-rate, the off-rate, and the transition rates were implemented.

$$k^{on} = k^{on}_0 + c_1\cdot\frac{\theta_{init}}{\theta_{max}} \tag{6}$$

$$k^{off}_{rev} = k^{off}_{rev,0} + c_2\cdot\frac{\theta_{rev}}{\theta_{max}} \tag{7}$$

$$k^{trans}_{init\_rev} = c_3\cdot\frac{\theta_{rev}}{\theta_{max}} \tag{8}$$

$$k^{trans}_{rev\_irr} = c_4\cdot\frac{\theta_{rev}}{\theta_{max}} \tag{9}$$

The numerical solution of the rate equations (1)-(3) plus the equations accounting for the coverage dependencies yields the respective fraction of the initial, reversible and irreversible state of the total amount of adsorbed β-Lactoglobulin as a function of time. All parameters were determined by fitting the model to the experimental data with the least-squares fitting method and are summarized in [4]. Using these parameters the adsorption kinetics of 10 different protein bulk concentrations between 1.0×10^{-8} M and 1.5×10^{-6} M were calculated (Figure 2). Comparing these curves with the measurements presented in figure 1

highlights that the proposed model is a valid description of the experimental data. In addition, the equilibrium coverages of any desired bulk concentration can be obtained easily allowing for the calculation of the adsorption isotherm as shown in figure 3. Interestingly, the isotherm predicts a local maximum at a coverage level of the critical surface density at a bulk concentration of 4.91×10^{-10} M. The abrupt decrease of the equilibrium coverage above this concentration is a direct consequence of the transition between initial and reversible state. Especially in the low concentration range the experimental determination of the equilibrium coverage may not always be practical as it takes up to several days until the equilibrium is reached.

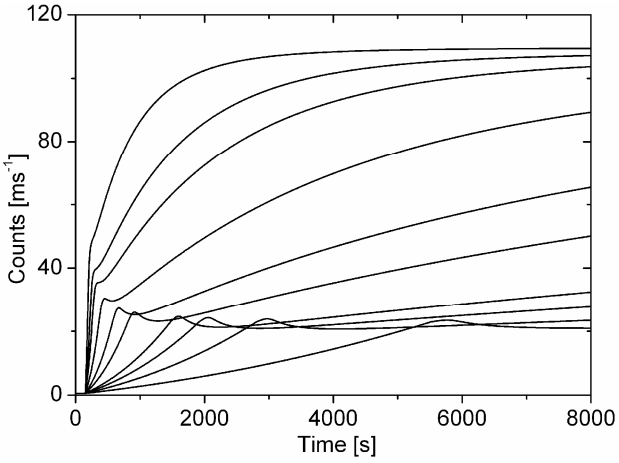

Figure 2: Calculated adsorption kinetics of β-Lactoglobulin at different bulk concentrations. From up to down: 1.5×10^{-6} M, 0.75×10^{-6} M, 0.5×10^{-6} M, 0.25×10^{-6} M, 0.125×10^{-6} M, 8.0×10^{-8} M, 4.0×10^{-8} M, 3.0×10^{-8} M, 2.0×10^{-8} M, 1.0×10^{-8} M.

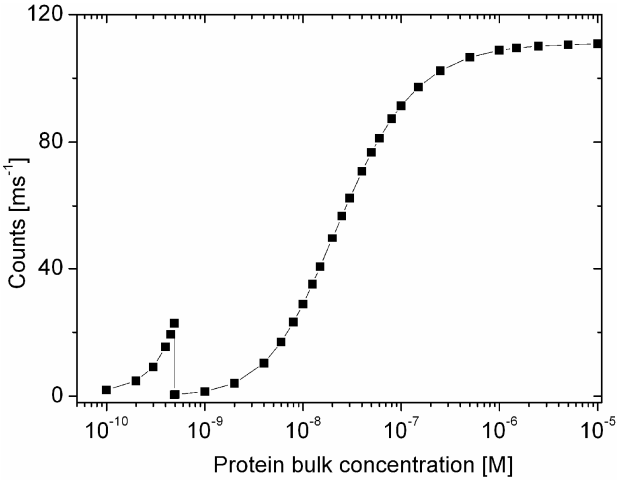

Figure 3: Calculated adsorption isotherm of β-Lactoglobulin. The final coverages were determined by simulating the kinetics of each concentration for 10^6 s.

In conclusion, we have introduced several concepts like different adsorption states, a critical surface density, or coverage dependent rate constants in the formalism of the kinetic model to account for the experimental observations. Using this model adsorption kinetics of different protein bulk concentrations and the adsorption isotherm can be readily calculated. Due to the comprehensive set of experimental data that was used to develop the proposed model we provided a mechanistic interpretation for certain adsorption phenomena like overshootings, lateral interactions, or increasing adsorption rates which are still poorly understood to date.

3 SPECIFIC ADSORPTION

The specific adsorption of proteins to solid interfaces is of special importance for biosensor applications in various fields of proteomics such as diagnostics, system biology or pharmaceutical research. In these systems highly efficient and selective enrichment methods are needed to improve their analytical performance.

In our group a simple and versatile coating technique was developed, by which a dense layer of silicone nanofilaments can be grown onto a variety of substrates [5, 6]. As shown in figure 4 the silicone nature of this coating results in a very high roughness.

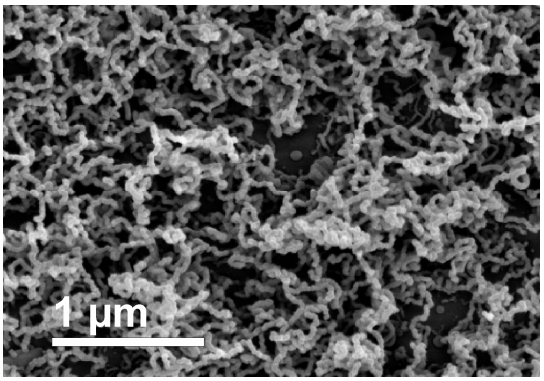

Figure 4: Electron microscopy image of the silicone nanofilaments grown on a coverslip.

By means of plasma treatment and the subsequent chemical modification of the activated sites we managed to manipulate the surface chemistry such as to create new functionalities on the silicone nanofilament coated substrates. It was possible to add carboxyl groups to the surface by using 2-(carbomethoxy)ethyltrichlorosilane (CETS) as reagent and cleaving the ester in alkaline buffer. Alternatively, amino groups were introduced to the surface by coating the activated template with APTES [7].

The potential of the resulting amino- and carboxyl-functionalized silicone nanofilament surfaces was explored by monitoring the adsorption kinetics of fluorescently labeled β-Lactoglobulin at pH 3 and pH 6 (citrate buffer, 50mM) in a continuous flow cell setup. The respective

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equilibrium coverages at the different pH levels are depicted in the left part of figure 5. A control experiment on a plasma activated, unmodified template has also been run.

β-Lactoglobulin is an amphoteric molecule with an isoelectric point (pI) of 5.2. At pH 3 it bears a net positive charge and at pH 6 a net negative charge. On the APTES and pretreated CETS modified surfaces, β-Lactoglobulin adsorption follows the behavior expected for charged proteins on cationic and anionic exchange resins: weak adsorption on the positively charged APTES surface at pH<pI, and strong adsorption at pH>pI. The negatively charged pretreated CETS surface shows the inverse behavior with strong adsorption at pH<pI, and weak adsorption at pH>pI.

For proteins of different pI, adsorption on the modified silicone nanofilament coatings becomes specific. In the right part of figure 5 the adsorption of β-Lactoglobulin (pI 5.2) and Lysozyme (pI 11.5) on the APTES and pretreated CETS modified silicone nanofilament surface at a constant pH level of 6 is shown. On the positively charged APTES modified surface at pH 6, the negatively charged β-Lactoglobulin shows a fourfold increase of adsorption as opposed to the positively charged Lysozyme. On the negatively charged pretreated CETS surface the ratio is even 15:1 in favor of the positively charged Lysozyme. These results highlight that by choosing the appropriate surface functionality and the right conditions it is possible to enrich a specific class of proteins on the silicone nanofilament surface.

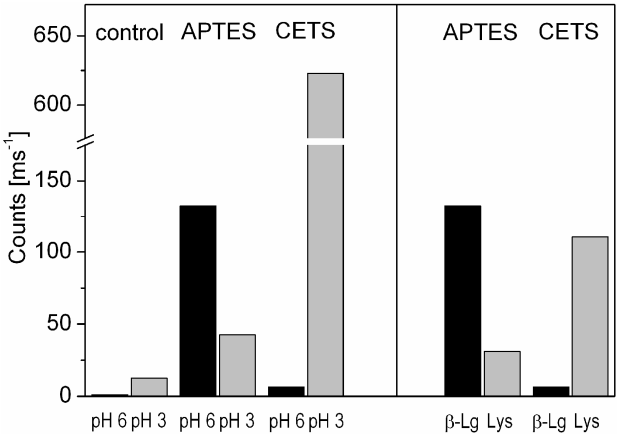

Figure 5: Left: Equilibrium coverage of β-Lactoglobulin adsorbed on modified silicone nanofilaments. Right: Equilibrium coverage of β-Lactoglobulin and Lysozyme on modified silicone nanofilaments at pH 6.

As can be concluded from the binding and elution kinetics the protein adsorption on the modified silicone nanofilament coatings is fully reversible. Figure 6 exemplarily shows the adsorption kinetics of β-Lactoglobulin on the pretreated CETS modified surface. When the protein solution at pH 3 is replaced by a protein-free buffer at pH 6 practically all surface bound molecules are released within a few minutes. Upon switching back to protein solution at pH 3, β-Lactoglobulin can be re-adsorbed again, following practically the same kinetics as before. This retention elution cycle can be repeated several times without alteration of the adsorption characteristics.

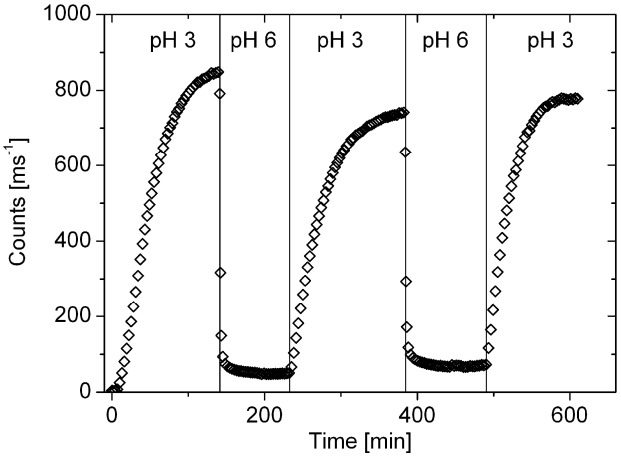

Figure 6: Adsorption and desorption kinetics of β-Lactoglobulin on a pretreated CETS modified surface.

In conclusion, we presented a simple and versatile process for the fabrication of surfaces with varying surface functions. The highly porous silicone nanofilament coating can be exploited to selectively and reversibly enrich proteins on a solid support. As there are no apparent constrains that hinder the immobilization of other active molecules the coatings open new opportunities for the development of efficient enzyme or receptor based biosensors. It is also noteworthy that we managed to create surface patterns in the sub mm range using the plasma activation process. This should in principle allow to transfer the proposed enrichment and separation methods to microchip format.

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