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Albumin binding and insertion into PS-*b*-PEO monolayers at air-water interface

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Abstract

Interaction of human serum albumin with poly(styrene)-*b*-poly(ethylene oxide) (PS-*b*-PEO) monolayer at air/solution interface was studied by measuring surface pressure. The density of PEO chains in the monolayer was controlled using Langmuir trough barriers. The thickness of PS-*b*-PEO monolayer prior to and after albumin adsorption was computed from *in situ* surface plasmon resonance (SPR) measurements. Depending on the initial PEO surface density the surface pressure kinetics of albumin insertion displayed two different regimes: below the PEO “pancake-brush” transition albumin binding was initially very rapid and itself induced the “pancake-brush” transition in the monolayer, and above the “pancake-brush” transition where some albumin penetration into the free PS-*b*-PEO monolayer still occurred into the PEO “brush”. In the case of SPR-immobilized monolayer, more than 0.1 PEO chain/nm² was required to inhibit albumin or ferritin adsorption. A half-reduction of albumin adsorption required approx. threefold higher PEO surface density than the half-reduction of ferritin adsorption.

Keywords

albumin; protein adsorption; Langmuir monolayer; monolayer insertion; poly(styrene)-*b*-poly(ethylene oxide); poly(ethylene oxide); PEO brush

1. INTRODUCTION

Biomaterial surface-induced thrombosis, one of the major problems in clinical applications of materials in contact with circulating blood, begins with the adsorption of plasma proteins [1-3]. Coating biomaterial surface with hydrophilic polymer chains usually reduces such adsorption. Among various hydrophilic polymers, poly(ethylene oxide) (PEO) has been extensively studied as biocompatible material suitable for creating protein adsorption “resistant” surface coatings [2,4]. A recent review addresses many theoretical and practical aspects of tethered chains, including PEO [5]. A densely grafted PEO surface layer results in so-called “brush” configuration. Both the “brush” thickness and the surface density of PEO chains in the “brush” are known to affect the extent of protein adsorption [6-13]. Halperin outlined the limits of inhibiting adsorption of different size proteins by grafted PEO chains: densely grafted, short PEO “brush” is efficient in preventing adsorption of small proteins but may allow adsorption of large proteins to the “brush” surface, while a lower surface density PEO “brush” made out of longer PEO chains is an effective barrier for large proteins while

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possibly allowing smaller proteins to penetrate into the “brush” and bind to the underlying surface [9]. McPherson et al. reached similar conclusions using numerical self-consistent field computation [10]. Clearly, the control over the surface density of grafted PEO is essential in designing protein adsorption resistant coatings. There are different ways to create a PEO surface layer, for example:

- by adsorption of molecules containing PEO chain(s), including surfactants and block copolymers,
- by adsorption and subsequent chemical reaction of PEO molecules that contain one or more reactive chemical groups,
- by interfacial segregation of PEO-containing block copolymers between two bulk polymer phases followed by a selective solvent removal of the bulk phase in which PEO chains segregated, or
- by a variety of *in situ* polymerization methods.

Combination of water-insoluble, PEO-containing molecules and the Langmuir trough technique is very useful in creating dense PEO brushes: water-insoluble, PEO-containing molecules residing on the air/solution interface can be compressed by Langmuir trough barriers to control the surface PEO density. One of such water-insoluble PEO-containing molecule is a block copolymer, poly(styrene)-*b*-poly(ethylene oxide) (PS-*b*-PEO). Upon compression the trough barriers force the PEO chain of PS-*b*-PEO to form a “brush”. The surface density of such “brush” is dependent on the length of PEO chain and the size of PS block; for long enough PEO chains and small enough PS blocks, each PEO chain will be in the “brush” configuration stretching its neighbors into the subphase [11]. Such compressed PS-PEO monolayer can be transferred to a suitable substrate [12,13] for protein adsorption experiments or used *in situ*. The compressed PS-PEO monolayers transferred to hydrophobic silica are unstable; the exposure to air or to the poor PEO solvents results in the PS-PEO surface hemi-micelle formation and monolayer restructuring [14]. The aim of the present study was to investigate how proteins penetrate into and/or bind onto the PEO “brush”; hence, the compressed PS-PEO monolayers were used *in situ* on the surface of the Langmuir trough. Human serum albumin (HSA) and ferritin (FER) were used to assess the efficacy of PEO adsorption inhibition as a function of PEO surface density. The HSA insertion was monitored by measuring monolayer surface pressure as a function of time. Increase of the amount of protein adsorbed onto the PS-*b*-PEO monolayer was measured in parallel using an *in situ* surface plasmon resonance (SPR) technique.

2. MATERIALS AND METHODS

Poly(styrene)-*b*-poly(ethylene oxide) (PS-*b*-PEO, Polymer Source Inc.) block copolymer was used as received. Some properties of the copolymer are given in Table 1. Solution of PS-*b*-PEO copolymer was prepared in spectroscopic grade chloroform at a concentration of 1 g/L. The solutions were stirred overnight to ensure complete dissolution.

Human serum albumin (fraction V, fatty acid free, ICN Immunobiologicals) and horse spleen ferritin (Sigma) were used as received. Protein solutions were prepared in phosphate buffer saline (PBS) solution of pH 7.4 at a concentration of 1 g/L. Some properties of HSA and FER are listed in Table 2. PS-*b*-PEO monolayer was generated by depositing PS-*b*-PEO solution in chloroform onto the water surface of a Langmuir trough (KSV 5000 System 3, KSV Instruments) and allowing chloroform to evaporate for 15 minutes. Pressure-area (π -*A*) isotherms of PS-*b*-PEO monolayers were measured using poly(oxymethylene) moving barriers and Pt Wilhelmy plate. Solvent quality for the PEO block was varied, from good (H₂O) to θ -condition (at 0.45 M K₂SO₄), by changing the concentration of K₂SO₄ in the subphase.

Protein-monolayer experiments were performed using a smaller area Langmuir trough (KSV Minitrough, KSV Instruments) and PBS as a subphase. π -A isotherms of PS-*b*-PEO on water and PBS subphases were nearly identical. Before protein insertion experiment a desired volume of PS-*b*-PEO solution in chloroform was deposited to the air/PBS interface and the chloroform was allowed to evaporate for 15 minutes. Monolayer was then slowly (at 2 mm/min) compressed to a preset barrier position. Once the barriers reached that position, monolayer was allowed to equilibrate for another 15 minutes and the resulting surface pressure was recorded. By depositing different amounts of PS-*b*-PEO copolymer and using identical preset barrier position, it was thus possible to control the PEO surface density in the PS-*b*-PEO monolayer while keeping the total monolayer area constant in each protein-monolayer experiments.

A syringe with a long needle was used to inject 5 mL of protein solution into the subphase (total volume 100 mL) so that the final protein concentration was 0.05 g/L. Small stir-bar was used to facilitate mixing of protein solution with the subphase. Changes of the surface pressure, π , were monitored concurrently with the SPR measurements (see below) by using Wilhelmy plate in the trough area adjacent to the SPR sensor (Figure 1). Simultaneous acquisition of surface pressure and SPR signals was carried out for approx. 2 hours after protein injection. An integrated optics SPR sensor (SPREETA, Texas Instruments) was used to quantitatively measure protein binding to the PS-*b*-PEO monolayer (Fig 1). The sensing gold surface of the SPR probe was first hydrophobized by immersion in 1 mM octadecanethiol (ODT) solution in ethanol for 10 min. This surface modification resulted in a water contact angle of 107°. The SPR sensor was then pre-initialized in air, calibrated in double-distilled water (refractive index, $n = 1.33$) and a baseline SPR reading was obtained using the PBS solution already in the trough. The SPR sensor was then positioned above the air/PBS interface before deposition of the PS-*b*-PEO monolayer. Once the monolayer was equilibrated, the sensor was slowly lowered into the contact with the monolayer and to a depth of ca. 2 mm using a micromanipulator. This procedure was equivalent to a horizontal monolayer transfer to a solid substrate but without ever removing substrate (i.e. the sensor) with the transferred monolayer from the trough. Upon the contact of the SPR sensor with the monolayer a step increase of the SPR signal was recorded and a new SPR baseline was established for the protein adsorption experiments. This baseline signal was monitored over a period of 10 min to ensure the integrity of the monolayer. Protein binding kinetics were recorded for each protein at different PEO surface densities as a change in refractive index units in time, Δ RIU vs. time. The measured Δ RIU was used to calculate the effective thickness of adsorbed protein layer, d , as previously described by Jung et al. [17]. A pristine gold surface and an ODT-modified gold surface in contact with a clean air/PBS interface were used as negative controls for protein-PS-*b*-PEO monolayer experiments.

Long-term stability of the PS-*b*-PEO monolayer in contact with the SPR ODT-Au surface was tested in separate experiments: a stable SPR signal was recorded for periods over 5 hours. After each protein adsorption experiment, the SPR sensor was cleaned using oxygen plasma (Plasmod, Tegal Corp.). The sensor response was unaffected by repeated plasma cleaning and ODT surface modification procedures.

3. RESULTS

Pressure – area isotherms of PS-*b*-PEO

π -A isotherms of PS-*b*-PEO monolayer measured on four different subphases are shown in Figure 2. The conformation of PEO chains at each subphase condition can be deduced from the π -A isotherm. At low surface pressures ($\pi < 5$ mN/m) PEO chains are known to be adsorbed to the air-water interface in so-called “pancake” configuration [11,19,20]. Poly(styrene) block of the copolymer exists in a collapsed state because both water (or K₂SO₄ subphases) is a non-solvent for PS. Fig 2 shows that increasing K₂SO₄ concentration makes the solvent quality for

PEO incrementally worse, which, in turn, results in more PEO segments attached to the interface and in higher surface pressures [19,20].

Upon compression of the monolayer each PS-*b*-PEO molecule becomes confined to a smaller area/molecule, A , and the π - A isotherm starts to level off (at $\pi = 8 - 12$ mN/m, depending on the K_2SO_4 concentration). This isotherm region, where π rises only slowly with decreasing A , is known as a “pancake-brush” transition [20]. The onset of the “pancake-brush” transition corresponds to an interface filled with adsorbed PEO chains. At such a condition, any decrease in A leads to the desorption of attached PEO segments and their submersion into the subphase, thus converting the “pancake” configuration into a “brush.” Both the steric repulsion between neighboring PEO chains and the dislike between PS and PEO segments contribute to this transition [20]. Further reduction in A results in a high density region where the ultimate area/molecule becomes limited by the size and the compressibility of the PS block. The change from the “pancake-brush” transition into a high density region is characterized by a steep rise in π . The π - A isotherms recorded on different subphases all displayed very similar slope, $d\pi/dA$, in the high density regime (which is omitted from Fig 2 for clarity). Table 3 compares the A values at $\pi = 25$ mN/m for water and three different K_2SO_4 subphases. The average area/molecule, $\langle A \rangle$, was 8.34 ± 0.25 nm², a value comparable to the cross-section area of the PS block calculated from the respective radius of gyration, R_{gPS} (see Table 1). It is the same average 8.34 ± 0.25 nm² area per molecule that is, at high surface pressures, available for each tethered PEO chain. When compared with the area of PEO chain calculated from the R_{gPEO} , (184.81 nm², see Table 1) one concludes that the PEO chains at $\pi = 25$ mN/m are strongly stretched in the “brush” configuration.

Thickness of PS-*b*-PEO monolayer

Initial contact of SPR sensor with PS-*b*-PEO monolayer caused the SPR-measured refractive index to increase (insert, Figure 3). The first refractive index step was due to the change from water to PBS and the second step was due to the sensor contact with the PS-*b*-PEO monolayer on PBS. The latter refractive index changes can be used to estimate the thickness of PS-*b*-PEO monolayers. The key parameter required for such an analysis is the refractive index of PEO “brush” at various PEO surface densities. Since the effective refractive index of PEO “brush” is expected to vary with the amount of water contained in the “brush,” it has to be calculated using some geometric model. Figure 3 shows the thickness of PS-*b*-PEO monolayer computed from the refractive index changes using an approximate model for inhomogeneous monolayer as proposed by Sauer et al. [21].* The calculated thickness of PS-*b*-PEO monolayer was lower than what the scaling law [22,23] predicts for the thickness of grafted PEO, especially at low PEO surface densities. The difference was expected because the scaling law-based prediction does not take into account adsorption of PEO segments at the interface and neglects the thickness contribution made by the PS block.

Monitoring HSA insertion into the PS-*b*-PEO monolayer

Prior to each protein-monolayer experiment, the PS-*b*-PEO monolayer was pre-equilibrated at a desired initial surface pressure, π_{in} . These pre-equilibrium surface pressures were selected to cover the π - A isotherm, from low to high density region. The surface pressure changes recorded for the interaction of HSA with each PS-*b*-PEO monolayer are shown in Figure 4. The time, $t = 0$ was defined as the time of protein injection into the subphase. In the control experiment ($\pi_{in(t=0)} = 0$, Fig 4A) HSA adsorption took place at a pristine air/PBS interface: after a brief time lag the surface pressure rapidly increased and, in agreement with the previous

*In this analysis, the effective monolayer refractive index, n_m , was an weighted average of two polymers (n_{PS} , n_{PEO}) and water (n_w) refractive indices. n_{PS} and n_{PEO} were each calculated from Lorentz-Lorenz equation by assuming that the molar refractivity of their respective constituents were additive [for more details see ref. 14].

report [24], within 6000 s smoothly reached a steady-state value of 15.5 mN/m. In the presence of PS-*b*-PEO monolayers, the surface pressure changes were dependent on the surface pressure of the monolayer at the time of protein injection. Well below the “pancake-brush” transition, for $\pi_{\text{in}} < 6$ mN/m, after the initial time lag the surface pressure kinetics displayed a two-step behavior: a fast initial rise of surface pressure, a kink at approx. 10 - 11 mN/m, and a second surface pressure increase that ultimately reached a steady-state at 15 - 16 mN/m (Fig 4A, full lines). When the equilibrium surface pressure was at the onset of the “pancake-brush” transition, at $\pi_{\text{in}} = 9.7$ or 9.9 mN/m, the surface pressure kinetics displayed an S-shaped form; a slower increase of surface pressure followed by an inflection point and leveling off at 18 mN/m (Fig 4B). As the starting surface pressure was made slightly higher, at $\pi_{\text{in}} = 10.0$ or 10.5 mN/m, the S-shaped kinetics reached lower steady-state surface pressures (Fig 4B). At even higher starting surface pressures ($\pi_{\text{in}} = 11.7$ and 12.7 mN/m, Fig 4A dashed lines) there was still a noticeable increase in surface pressure following a longer induction period, indicating that some HSA penetration into the PS-*b*-PEO monolayer might have taken place.

Binding of HSA to PS-*b*-PEO monolayers

The SPR experiments were run consecutively with the surface pressure measurements. The SPR sensor surface was brought in contact with the pre-equilibrated monolayer and HSA binding was initiated by protein injection into the subphase. Figure 5 shows the HSA binding kinetics for each pre-equilibrium surface pressure and controls, gold and ODT-modified gold surfaces (insert, Fig 5). In the case of ODTAu surface (negative control for PS-*b*-PEO) the adsorbed amount of HSA, Γ_{HSA} , calculated from steady-state value of ΔRIU , was $\Gamma_{\text{HSA}} = 1.53 \cdot 10^{-7}$ g/cm².

Unlike the surface pressure kinetics, the protein adsorption kinetics fell in two distinct groups: one below and other above $\pi_{\text{in}} = 11$ mN/m. For $\pi_{\text{in}} < 9.7$ mN/m HSA adsorption was generally lower than the adsorption measured for the control ODT-gold surface. In the “pancake-brush” transition region, the adsorption kinetics attained ever lower steady-state levels and for $\pi_{\text{in}} > 12$ mN/m, the adsorption was effectively reduced to the level barely measurable with SPR.

Adsorption of FER to PS-*b*-PEO monolayers as a function of PEO surface density

Adsorption of ferritin followed the identical protocol as in the HSA adsorption experiments; the PS-*b*-PEO monolayer was pre-equilibrated at the desired initial surface pressure before protein injection. Figure 6 shows the SPR adsorption kinetics of FER onto control surfaces and surfaces with PS-*b*-PEO monolayers. Unlike the HSA adsorption, the increase of the pre-equilibrium surface pressure caused a continuous reduction of both initial adsorption rates and the long time adsorption levels. In addition, FER adsorption did not seem to attain the steady-state condition and was still found increasing, even after 6000 s of adsorption time, except at higher π_{in} .

It is useful to relate protein adsorption to the PEO surface density, σ_{PEO} , which is equal to A^{-1} (units: number of PEO chains/area) rather than to π_{in} . In the all SPR experiments the PEO density was assumed not to change with protein adsorption as PS-*b*-PEO monolayer was effectively transferred to ODT-gold surface. The protein layer thickness, d , as calculated from each ΔRIU measurement [17], is shown as a function of PEO surface density, σ_{PEO} , in Figure 7. Values at zero PEO surface density are from the adsorption onto the ODT-Au control surface. At low PEO surface density d_{HSA} varied between 0.82 – 1.2 nm. Above the onset of the “pancake-brush” transition ($\sigma_{\text{PEO}} > 0.019$ chain/nm²) the thickness of HSA layer monotonously decreased with increasing PEO surface density. When σ_{PEO} was 0.1 chain/nm² or higher, the inhibition of HSA adsorption was the most effective. These results suggest that a critical PEO surface density, at approx. 0.1 PEO chain/nm², is required for a full inhibition of albumin adsorption.

The thickness of FER adsorbed layer (recorded at 6000 s of the adsorption time) as a function of PEO surface density is also shown in Fig 7. On the control ODT-gold surface, $\Gamma_{\text{FER}} = 5.7 \cdot 10^{-7} \text{ g/cm}^2$ which was equivalent to an effective FER layer thickness, $d_{\text{FER}} = 4.3 \text{ nm}$. A significant decrease (68%) in the adsorbed FER amount was noticed at the onset of the “pancake-brush” transition region ($\pi_{\text{in}} = 9.7 \text{ mN/m}$, $\sigma_{\text{PEO}} = 0.019 \text{ chain/nm}^2$) where d_{FER} dropped to 1.4 nm. In the “pancake-brush” transition region FER adsorption decreased less drastically with σ_{PEO} . At $\sigma_{\text{PEO}} = 0.097 \text{ chain/nm}^2$ it was lower by 96% compared to the ODT-gold control. Overall, the effective inhibition of FER adsorption appeared to follow a biphasic behavior: a significant, two-third decrease at the onset of the “pancake-brush” transition followed by a monotonous reduction of adsorption until the full inhibition was achieved at high PEO densities.

4. DISCUSSION

Control over the surface density of tethered PEO chains is essential to the design of protein inert coatings. In many practical surface immobilization situations such control is difficult, if at all possible. In a laboratory one can accomplish such a control by using water-insoluble, PEO-containing molecules on the surface of Langmuir trough. A few protein adsorption studies were carried out using *in situ* or transferred PEO monolayers [5,12,13,25-28]. Because the compressed PS-PEO monolayers were found unstable in air when transferred to hydrophobic substrates [14], this study utilized Langmuir trough to control the surface density of PEO chains while measuring protein adsorption *in situ* and *in real time* using a combination of surface pressure and SPR techniques. Comparison of surface pressure and SPR results (Figs 4,6,7) requires a brief review of the differences between these two techniques.

- Surface pressure technique measures the change of air-solution interfacial tension. Any change of interfacial chemical composition, such as positive adsorption of protein amino acid segments *to*, or negative adsorption (i.e. desorption) of PEO segments *from* the interface, will contribute to the measured π . It is also possible that the adsorbed protein molecule unfolds at the interface resulting in more adsorbed protein's segments, thus causing a surface pressure change at a constant number of adsorbed protein molecules. As a consequence, the surface pressure increase upon protein adsorption is not proportional to the number of adsorbed protein molecules.
- SPR measures protein adsorption onto PS-*b*-PEO monolayer which is effectively transferred to the sensor surface by horizontal deposition but kept in the same subphase. In doing so SPR senses the increase of refractive index due to the protein mass increase within a 200-300 nm thick interfacial region, i.e. insertion *into* and adsorption *onto* the monolayer.

The surface pressure increase upon HSA adsorption to the pristine air-buffer solution interface (i.e. with no PS-*b*-PEO) was very rapid after the initial lag time of approx. 300 s (Fig 4A). It is known that a certain amount of protein has to be present at the interface before an increase in surface pressure is observed [29-31]. The time lag increased with the increasing the initial surface pressure; at higher π_{in} values it was approximately 1000 s before any increase of surface pressure was observed. At $\pi_{\text{in}} = 1.6 \text{ mN/m}$, HSA adsorption showed a two-step kinetics: a fast rise of π which occurred within a few minutes and a kink at $\pi = 9.5 \text{ mN/m}$ followed by a second increase. The same features were found when π_{in} was 5.3 mN/m (Fig 4A). In both cases the kink coincided with the surface pressure region of the “pancake-brush” transition (see Fig 2); an indication that a further desorption of PEO segments from the interface is caused by the adsorbing protein molecules. Question is whether this transition is primarily caused by adsorption of new protein molecules, or by structural rearrangement (i.e., by unfolding) of already adsorbed protein molecules. The SPR-measured adsorption of HSA at the time of the kink was slightly below the adsorption steady state (Fig 5). After the kink appeared the surface

pressure continued to rise, while the SPR-measured adsorption reached its steady-state. Hence, protein denaturation is likely to be the operative mechanism producing the kink. This scenario is illustrated in Figure 8. At large initial A there is enough available interface for the protein to penetrate and adsorb (Fig 8a). At progressively smaller initial A , surface-adsorbed PEO segments are confined to smaller available area by the adsorbing HSA molecules. The effect is akin to the reduction in the surface area available for PEO segments by the compression of trough barriers. HSA molecules occupy progressively more area at the interface, either by adsorption or denaturation, thereby forcing the PEO segments to desorb from the interface (Fig 8b). In this scenario, the second step in the surface pressure kinetics is related to the protein-mediated creation of PEO “brush”. Such PEO “brush” will then prevent (or slow down any further penetration and adsorption of HSA. The major uncertainty here is due to the differences in the nature of the two PS-PEO monolayers; one, sensed by surface pressure is free and mobile, while other, sensed by SPR, is captured by the horizontal transfer to the SPR sensor. Similar protein-mediated phase transitions have been observed in the case of protein insertions into lipid monolayers at air-water interfaces [32,33] and protein insertion into lipid monolayers containing lipid-PEO conjugates [34]. However, to the best of our knowledge, the “pancake-brush” transition effected by protein insertion into a block copolymer monolayer at air-solution interface has not yet been reported in the literature.

The HSA interactions with the PS-*b*-PEO monolayer in the “pancake-brush” transition showed a pronounced difference that strongly depended on the initial surface pressure, π_{in} (Fig 4B). When starting HSA binding at $\pi_{in} = 9.7$ or 9.9 mN/m, the surface pressure reached its steady state at approx. 18 mN/m after a 600 s time lag (diamonds and + symbols, Fig 4B). However, when the initial surface pressure was set at $\pi_{in} = 10.0$ mN/m the time lag was prolonged to approx. 1000 s and the surface pressure increased to only 15 mN/m. A slightly higher initial surface pressure at $\pi_{in} = 10.5$ mN/m resulted in a time lag of almost 2000 s and the steady state surface pressure only reached 14 mN/m (triangles, Fig 4B). Hence, the largest difference in surface pressure kinetics appears to be around $\pi_{in} = 10$ mN/m.

Interestingly, the surface pressure kinetics continued to show a small pressure increase even when the initial surface pressure was above 11.7 mN/m (dashed lines, Fig 4A), while almost no HSA adsorption was observed in parallel SPR measurement (Fig 5). This difference implies that, either PS-*b*-PEO monolayer at the air-PBS interface fluctuates in local surface density and rearranges itself to make way for insertion of some protein molecules, or that some unfolding of protein occurs even at such high surface pressures. The differences between the surface pressure and the SPR-measured adsorption kinetics are the consequence of the differences between the processes of protein insertion *into* and adsorption *onto* the monolayer that were initially kept at the same surface pressure. The monolayer immobilized by the SPR sensor surface is rigid in nature, its PS blocks are held at the SPR sensor surface with no or very little lateral mobility, while the same monolayer on the air-PBS interface is mobile and possibly contains a number of packing defects.

SPR was also utilized to determine the thickness of the PS-*b*-PEO monolayer (Fig 3). Calculated PS-*b*-PEO thickness reflects the change of PEO configuration along the π - A isotherm: for example, the increase of monolayer thickness below $A = 80$ nm² corresponds well to the “pancake-brush” transition region seen in Fig 2. Although the model used in calculating the monolayer thickness from SPR data is simplified, the use of SPR provides an interesting alternative to more sophisticated optical techniques and neutron reflectivity [5,11, 35]; it does not require monolayer transfer from subphase into another solvent and thus circumvents the problems related to the instability of transferred PS-*b*-PEO monolayers [14].

Both HSA and FER, used in this study as models for small and large proteins, are known to adsorb readily from dilute solutions to a variety of interfaces [35-39]. Saturation adsorption of

HSA and FER is approximately of 2.5×10^{-7} and 6.3×10^{-7} g/cm², respectively [18,37]. Corresponding thicknesses of the adsorbed HSA or FER layers are expected to approach each protein's molecular dimensions. However, when measured optically one has to account for water content within the adsorbed layer. Even in a well-ordered monolayer the actual protein packing efficiency rarely exceeds 2/3, so that the average protein layer thickness is typically found smaller than the size of the protein molecule [35]. Unlike the effective PS-*b*-PEO monolayer thickness discussed above, the adsorbed protein layer thickness was computed in this study by assuming a pure protein layer, i.e. with no water present within the layer. While this is physically unrealistic, it helped in comparing the HSA and FER adsorption at different PEO surface densities and with the data from the literature. Adsorbed amount of the protein was calculated from experimental SPR data at the steady-state level of the protein adsorption kinetics (or, at 6000 s for FER adsorption), and subsequently converted into the effective thickness of the protein layer [17]. The HSA layer thickness on control ODT-gold surface was 1.2 nm (Fig 7). This thickness was lower than 1.8 nm measured by SPR for an HSA monolayer on a similar substrate [17], but was similar to the results of HSA adsorption experiments carried out in a similar time frame [38,39].

A significant reduction in the effective thickness of HSA and FER layers was observed with the increase in the surface density of PEO chains (Fig 7). HSA adsorbed layer thickness was dramatically reduced (by nearly 85%) at $\sigma_{\text{PEO}} = 0.097$ chain/nm² and practically no adsorption occurred beyond $\pi_{\text{in}} > 12.7$ mN/m ($\sigma_{\text{PEO}} > 0.097$ chain/nm²). These findings are very similar to the results of BSA adsorption on PS-PEO brushes grafted to PS-covered silicon wafers [12,13,23]. Norde and Gage have used optical reflectometry to show that BSA adsorption on grafted PEO-PPO-PEO or PS-PEO brushes was minimal at $\sigma_{\text{PEO}} = 0.085$ chain/nm² (PEO-PPE-PEO, $M_{\text{PEOn}} = 5600$ Da), or at a PEO brush thickness of 9 nm (PS-PEO; $M_{\text{PEOn}} = 11000$ Da) [12]. Bosker et al. [13] and Currie et al. [25] observed similar results: the BSA adsorption steeply decreased between $0.1 > \sigma_{\text{PEO}} > 0.15$ chain/nm² for longer PEO chains ($M_{\text{PEOn}} = 20000$ - 34000 Da). The effect of low PEO surface density on HSA adsorption reduction was less dramatic. In this study the HSA adsorption monotonously decreased with increasing σ_{PEO} . In the case of PS-*b*-PEO monolayers transferred to PS-covered silicon wafers, it was found that bovine serum albumin (BSA) adsorption first *increased* with σ_{PEO} , then reached a maximum and finally decreased at higher σ_{PEO} [13,25]. This different behavior at low PEO surface density was likely due to the choice of the underlying substrates (ODT-Au vs. PS-film on silicon).

The adsorption of ferritin has been studied by others as well [18,40-41]. Caruso et al. studied ferritin adsorption to gold and found that ferritin formed a monolayer with a thickness of 4.5 nm [18], similar to the effective 4.3 nm thickness of ferritin monolayer on the control ODT-Au surface found in this study. The thickness of FER layer followed a biphasic behavior with increasing σ_{PEO} : a significant 2/3 decrease at the onset of the “pancake-brush” transition followed by a monotonous reduction until a full inhibition of FER adsorption was achieved by the PEO “brush.” This biphasic trend is somewhat similar to the adsorption reduction of another large protein, fibrinogen, by tethered PEO chains as observed by Efemerova et al. [26]. According to Halperin [9] protein penetration into the brush and adsorption to the underlying adsorbing surface is suppressed when the distance between the grafting points of PEO is smaller than the size of the protein. As the diameter of FER is larger than of HSA, it was expected that the amount of adsorbed FER would decrease at lower PEO chain surface density where such reduction of HSA adsorption was not observed (Fig 7). Efemerova et al. [26] also noticed that the adsorption inhibition of a small (HSA) and a large (fibrinogen) protein was different at same PEO grafting density. In the present study a half-reduction in albumin adsorption required approx. threefold higher PEO chain surface density than the half-reduction of ferritin adsorption. However, for an effective inhibition of albumin or ferritin adsorption, the PEO chains had to be in a “brush” configuration with PEO surface density, $\sigma_{\text{PEO}} \geq 0.1$ PEO chain/nm² (Fig 8c).

5. CONCLUSIONS

Inhibition of protein adsorption by PEO “brushes” which were created by compression of PS-*b*-PEO monolayer at the air-solution interface was studied using surface pressure and *in situ* surface plasmon resonance techniques. SPR data were also used to calculate the thickness of the PS-*b*-PEO monolayer. The monolayer thickness and the pressure-area isotherms of PS-*b*-PEO monolayer confirmed that at large area/molecule the PEO chains were adsorbed to the air/solution interface in a “pancake” configuration. Only after the monolayer was compressed to a small area/molecule, configuration of the PEO chains changed from a “pancake” to a “brush”. Maximum surface density of PEO chains in such “brush” was ultimately limited by the size of PS block.

Surface pressure kinetics of HSA-monolayer interactions were sensitive to the initial surface pressure of the PS-*b*-PEO monolayer. Protein penetration into PS-*b*-PEO monolayer at low initial surface pressures itself induced a PEO “pancake-brush” transition. Some protein penetration into the mobile PS-*b*-PEO monolayer occurred even at the surface pressures above the “pancake-brush” transition. For an effective inhibition of albumin or ferritin adsorption the transferred PEO chains had to be in a “brush” configuration with PEO surface density, $\sigma_{\text{PEO}} \geq 0.1$ PEO chain/nm². At lower PEO surface densities the extent of protein adsorption was dependent on protein size: a half reduction of albumin adsorption required approx. threefold higher σ_{PEO} than the half reduction of ferritin adsorption.

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Figure 1. Schematics of surface pressure and SPR measurements performed in parallel on the surface of Langmuir trough.

Figure 2.
 π -A isotherms of PS-b-PEO diblock copolymer monolayers at air-water and air- K_2SO_4 solution interfaces. The concentration of K_2SO_4 in the subphase is shown in the legend. The parts of the isotherms recorded at high π are omitted for clarity.

Figure 3.

Effective thickness of PS-b-PEO monolayer shown as a function of area/molecule. The thickness was computed from the SPR-measured refractive index changes shown in the insert. Insert: the first refractive index step for water to PBS change and the second refractive index step was due to the contact of the sensor with PS-b-PEO monolayer kept at surface pressures as indicated in the legend.

Figure 4.

HSA interactions with PS-b-PEO monolayer measured as surface pressure kinetics. Each experiments started at different initial surface pressure of the PS-b-PEO monolayer ($t = 0$). Lower panel: initial surface pressures below and above the “pancake-brush” transition of PS-b-PEO monolayer. Upper panel: initial surface pressures within the “pancake-brush” transition of PS-b-PEO monolayer.

Figure 5. SPR adsorption kinetics of HSA to PS-b-PEO monolayers pre-equilibrated at different surface pressures (see legend) and transferred to SPR surface. The units on the y-axis refer to the change in refractive index next to the SPR sensing surface. The insert shows HSA adsorption kinetics to two control surfaces.

Figure 6. SPR adsorption kinetics of FER to PS-b-PEO monolayers pre-equilibrated at different surface pressures (see legend) and transferred to SPR surface. The units on the y-axis refer to the change in refractive index of the medium next to the SPR sensing surface.

Figure 7. Thickness of the adsorbed HSA and FER layers, calculated from the measured change in refractive index, shown as a function of PEO surface density.

Figure 8.

Scenario of HSA penetration into PS-b-PEO monolayer at different PEO surface density. (a) At low PEO surface density HSA molecules easily penetrate the monolayer and adsorb. (b) At higher PEO surface density HSA insertion of HSA molecules into PS-b-PEO monolayer further compresses PEO chains thus adding to the “pancake-brush” transition. (c) At high PEO surface density, transfer of the monolayer preserves the PEO brush which fully inhibits penetration and adsorption of HSA molecules.

Table 1

Molecular parameters for the PS and PEO blocks in PS-b-PEO block copolymer. N is the degree of polymerization. M_w is the molecular weight. Radius of gyration of PS, R_{gPS} , was computed as $R_{gPS} = (3 M_w / 4 \rho N_A)^{1/3}$ [15], where ρ is the density of PS (1.04 g/cm^3) and N_A is the Avogadro number. Radius of gyration for PEO, R_{gPEO} , (in nm) was calculated as $R_{gPEO} = 0.0215(M_w)^{0.583}$ [16].

block	M_w (Da)	N	R_g (nm)	area/molecule, from R_g (nm ²)
PS	12,200	117	1.69	8.97
PEO	23,900	543	7.67	184.81

Table 2

Properties of two proteins used in this study.

protein	M_w (Da)	shape and diameter (nm)	approx area/molecule ("footprint") (nm^2)	refractive index, n
HSA	68,000	"heart"-shaped, 7 (approx.)	40	1.51 [17]
FER	444,000	spherical, 12	113	1.45 [18]

Table 3

Area per PS-b-PEO molecule at 25 mN/m on different subphases.

Subphase	A, area/molecule at $\pi = 25$ mN/m (in nm ²)
H ₂ O	8.35
0.11 M K ₂ SO ₄	8.16
0.22 M K ₂ SO ₄	8.17
0.45 M K ₂ SO ₄	8.70