Time Dependence of Lysozyme Adsorption on End-Grafted Polymer Layers of Variable Grafting Density and Length

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**Supporting Information

ABSTRACT: A combined experimental and theoretical approach establishes the long-lived nature of protein adsorption on surfaces coated with chemically grafted macromolecules. Specifically, we monitor the time dependence of adsorption of lysozyme on surfaces comprising polymer assemblies made of poly(2-hydroxyethyl methacrylate) brushes grafted onto flat silica surfaces such that they produce patterns featuring orthogonal and gradual variation of the chain length (N) and grafting density (σ). We show that in the kinetically controlled regime, the amount of adsorbed protein scales universally with the product σN, while at equilibrium the amount of adsorbed protein is governed solely by σ. Surprisingly, for moderate concentrations of protein in solution, adsorption takes more than 72 h to reach an equilibrium, or steady state. Our experimental findings are corroborated with predictions using molecular theory that provides further insight into the protein adsorption phenomenon. The theory predicts that the universal behavior observed experimentally should be applicable to polymers in poor and theta solvents and to a limited extent also to good solvent conditions. Our combined experimental and theoretical findings reveal that protein adsorption is a long-lived phenomenon, much longer than generally assumed. Our studies confirm the previously predicted important differences in behavior for the kinetic versus thermodynamic control of protein adsorption.

■ INTRODUCTION

The adsorption of proteins from solution onto solid surfaces has attracted much attention in the last two decades due to its scientific importance and applications in many areas.1–4 For instance, buildup of bacterial films on the surfaces of heat exchangers results in reduced heat transfer efficiency of the exchanger. A similar problem afflicts the marine industry as well, where biomass fouling on the surfaces of naval vessels, i.e., the deposition of unwanted biofilm on the surfaces of objects in contact with aqueous solution, is triggered by protein adsorption. The economical loss associated with biofouling is estimated to run into millions of dollars annually. In the food industry, adsorption of proteins on food processing equipment causes adverse biological consequences.5 Performance of many surgically inserted materials, i.e., implants, catheters, or pacemakers, inside the human body depends critically on the adhesion of proteins from bodily fluids onto the surfaces of these materials.6–8 If protein adhesion is not checked in time, uncontrolled adsorption of proteins at the device surface can result in encapsulation and ultimately deterioration of the implant.9 Many blood-contacting medical devices meet similar fate due to surface-induced thrombosis.10 Thus, prevention of nonspecific protein adsorption is the primary focus in the design of implants and biomedical devices. In contrast to the aforementioned examples, there exist areas where protein adsorption on man-made surfaces is required. For instance, the development of artificial organs via tissue engineering requires that surfaces are “pre-treated” with proteins, which subsequently serve as functional bedding for cell growth.11,12 Successful biological development of a tissue-engineered product thus calls for precise tuning of the protein and cell adhesion to the scaffolds around which the organ is constructed. These two opposite examples illustrate why tailoring the adsorption of proteins at surfaces and interfaces remains one of the most extensively researched topics.13–17

Although a great deal of progress has been made in understanding the interfacial behavior of proteins, the issue is...
The concept of utilizing grafted polymer layers to prevent protein adsorption has been inspired by extensive past work on colloidal stabilization. The basic idea is that in a good solvent environment, polymers end-grafted to the surface present a steric barrier that prevents the proteins from reaching the surface. The seminal theoretical work of Jeon et al.,\textsuperscript{1} based on the Alexander–de Gennes model,\textsuperscript{2,3} was applied to predict the ability of polymer brushes to prevent protein adsorption. This model assumes that the polymer layer is not deformed upon protein adsorption since it calculates the free energy of interactions between a surface with grafted polymers and the protein, which is modeled as an infinite surface. This simple and insightful approach is valid only when (1) the polymeric grafts are long and (2) the distance between the grafting points on the surface is much smaller than the protein size. While this approach is not quantitatively applicable to many experimental cases, it provides general comprehension that long chain lengths and high surface densities of the polymer will be more efficient in preventing protein adsorption than shorter and/or more sparsely spaced grafts. In 1997, a molecular theory\textsuperscript{4} was introduced, in which the conformations and interactions of the proteins and the polymer molecules were accounted for explicitly. Application of this model revealed that the deformation of the polymer layer is one of the most important components to be considered in determining the equilibrium amount of adsorbed proteins. This molecular approach has been applied successfully to a variety of experimental systems (vide infra).\textsuperscript{5,6} In particular, the model has been employed to determine how different molecular architectures modify the ability of the grafted layer to prevent protein adsorption including the possibility of protein denaturation upon adsorption.\textsuperscript{7,8} The coupling of polymer molecular architecture with deformation of the polymer layer upon protein adsorption is important, as it offers insight into the competition between polymer deformation and protein–surface interactions. Very relevant to the work presented here, the theory predicts that different design rules exist for kinetically and thermodynamically governed regimes of protein adsorption. In particular, while the molecular weight of polymeric grafts plays no role in the thermodynamics of adsorption (once the layer thickness is comparable to the size of the protein), the time scale for adsorption (kinetics) depends in a dramatic way on the polymer chain length under all solvent conditions.\textsuperscript{9,10} Halperin generalized the work of Jeon et al., by presenting a systematic analytical approach that treats both large and small proteins, based on classical theory of polymer brushes. This work addresses both the equilibrium and kinetic aspects of protein adsorption on surfaces coated with polymeric grafts. It presents scaling predictions for the role of both the molecular weight and surface grafting density for protein adsorption separating into different modes of adsorption, namely, on top of the grafted layer and on the surface itself. It is important to emphasize that the analytical approach does not include the role that polymer deformation upon protein adsorption plays. More recently, Halperin and co-workers also looked at the role of the temperature of thermoresponsive brushes on protein adsorption.\textsuperscript{11} They further studied the possibility that proteins adsorb due to attractive protein–polymer interactions.\textsuperscript{12} Van Tassel and Belfort provided alternative solutions to the kinetics of protein adsorption for surfaces without grafted polymers.\textsuperscript{13,14} In all of this work, the treatment is based on quantifying the free energy contributions associated with the adsorption of proteins in different modes, hence, providing the balance of...
interactions between an unperturbed polymer brush and the proteins, i.e., without including the response of the brush to the presence of the proteins.

Previous systematic experimental studies have shown that surface grafting density ($\sigma$) and molecular weight ($M$) (or, equivalently, the number of monomer repeat units, $N = M/M_0$, where $M_0$ is the molecular weight of a repeat unit) of the grafted macromolecules are key parameters governing protein adsorption. Apart from a few notable cases where the effect of $M$ or $\sigma$ was not monotinous, empirical observations indicate that protein adsorption decreases with increasing $M$ and/or $\sigma$ of the anchored chains.\textsuperscript{46} For the equilibrium case, once the polymer layer thickness reaches the same order of magnitude as the protein size, the adsorption isotherm becomes independent of polymer molecular weight;\textsuperscript{35,36,46} and only the grafting density of the polymer on the surface remains the relevant parameter. A molecular theory (vide supra) was used to interpret experimental observations pertaining to adsorption isotherms measured for Lys and fibrinogen on surfaces decorated with short and long chain length PEGs.\textsuperscript{38} The predictions were found to be in excellent agreement with the experimental observations. The kinetic behavior, however, is predicted to be governed by both the chain length and surface grafting density. The longer the chain length and the higher the surface grafting density, the longer the adsorption process takes. The time scales are predicted to vary from seconds to years, depending on the molecular weight and surface grafting density of the polymers on the surface. Recent work on surfaces with grafted polypeptide chains has shown that at short times protein adsorption is inhibited for all cases studied.\textsuperscript{47} However, the molecular theory predicts that for the shortest chain length the observed experimental observations are not in equilibrium, and long time protein adsorption should be expected. This has been confirmed indirectly by long-term studies of cell adhesion.\textsuperscript{47}

The findings that similar protein adsorption behavior is observed for both PEG as well as peptoids provides support to the idea that it is not the detailed chemical structure of the grafts that determines their ability to prevent protein adsorption, but rather some other general property, such as their solubility and flexibility. These experimental observations and the ability of the molecular theory to quantitatively predict the adsorption isotherms for several different proteins on surfaces with PEG and peptoids provide support to the predictions of the theory that have not yet been checked experimentally. Here we are particularly interested in the predictions of the different role that surface grafting density, molecular weight, and molecular interactions play in the kinetic and thermodynamic control of protein adsorption. For example, it has been predicted that the presence of polymer–surface interactions can modify the rate of adsorption as well as the partition of different adsorbed conformers on the surface. As we mentioned above for the equilibrium adsorption, the polymer chain length plays no role once the thickness of the layer is similar to, or larger than the size of the protein. However, the predictions for the kinetic behavior are rather different, particularly because the molecular weight plays a large role in determining the kinetics of adsorption, leading to changes of orders of magnitude in the initial adsorption time at a fixed surface density.

The question that arises is whether the dependence of the kinetics and thermodynamics of protein adsorption on properties of surface-grafted polymer are universal. The answer to this question is that the type of polymer–solvent interaction is the one that determines the type of universal behavior. As the protein molecule has to penetrate through the grafted polymers, the process of protein adsorption on surfaces with bound polymers depends strongly on the structure of the polymer layer and its deformation upon protein adsorption. Water represents a good solvent for PEG, and the experimental and theoretical findings described above and elsewhere hold. Given that the grafted polymer used in this paper poly(2-hydroxyethyl methacrylate) (PHEMA) is known to only marginally be soluble in water, we need to consider theta and poor solvents. Chains in contact with a poor (and to a somewhat lesser degree theta) solvent form a compact layer, and it is therefore not surprising that the kinetics of adsorption depends on the total amount of polymer segments, i.e., the initial time-dependent adsorption is a universal function of $\sigma N$. The equilibrium amount of protein adsorbed will depend on how the finite amount of adsorbed proteins deforms the polymer layer. As such, this will depend mostly on the surface grafting density of the grafted macromolecule and, to a much lesser degree, on its length.

In order to thoroughly probe the effect of $M$ and $\sigma$ on protein adsorption and address the role of adsorption kinetics, one needs to have the capability to vary $M$ and $\sigma$ systematically and concurrently in a single experiment. Over the past few years, protein adsorption has been studied on samples comprising systematic variation of $M$, $\sigma$, and other material properties, such as surface topography. Detailed account of those studies can be found in recent reviews.\textsuperscript{48–52} Here we demonstrate that combinatorial orthogonal\textsuperscript{53} polymeric substrates comprising tethered PHEMA brushes with simultaneous gradual variation of $M$\textsuperscript{54} and $\sigma$\textsuperscript{55} that facilitate systematic, reliable, and unambiguous platform, enabling comprehensive exploration of the broad parameter space governing protein adsorption. By combining the experimental studies employing these complex polymeric substrates with molecular theory, we demonstrate unambiguously for the first time that protein adsorption is a long-lived process; as suggested indirectly by the cell adhesion studies,\textsuperscript{47} i.e., it takes many days for the protein to reach its equilibrium state. This time is much longer than what has commonly been believed and employed in literature, and it thus questions the validity of using short-time protein adsorption as a measure of nonfouling in the design of surface modifiers.

\section{MATERIALS AND METHODS}

\textbf{Molecular Theory.} The basic idea of the molecular theory is to look at each conformation of the molecules, whether polymer chain or protein, and determine the probability of that conformation given fixed values of the experimentally controllable variables, i.e., temperature, bulk protein density, and polymer grafting density. The distribution of molecular species and conformers is determined by minimizing a free energy functional, written in terms of the probability of the different conformers and the position-dependent density of protein and solvent. The free energy accounts explicitly for all the intermolecular and surface interactions. The free energy functional for a surface of total area $A$ with $N_p$ polymers grafted to the surface, i.e., surface grafting density $\sigma = N_p/A$, in contact with a solution of proteins whose bulk chemical potential is $\mu_{prot}$ is given by

\begin{equation}
\mu_{prot} = \mu_{prot}' - T \ln \left( \frac{N_p}{A} \right) - \frac{V_p}{A}
\end{equation}
where the first term in eq 1 represents the conformational entropy of the grafted polymers, with $P(\alpha)$ representing the probability of finding a polymer in conformation $\alpha$. The second term (first integral) represents the translational entropy of the proteins followed by the bare protein surface interaction, $U_p(z)$, and the last term in the same integral accounts for the fact that the surface is considered to be in equilibrium with a bulk solution with protein at chemical potential $\mu_{prot}(z)$ is the position-dependent density of proteins, and $v_w$ is the volume of a water molecule, and it is used as the volume unit. Thus, $\mu_{prot}(0)$ is the amount of adsorbed protein. The third term in eq 1 is the $z$-dependent translation (mixing) entropy of the solvent with $\rho_w(z)$ being the density of water at $z$. The following term represents the attractions between the polymer segments where $\chi$ measures the strength of the attraction, and $\phi_i(z)$ is the volume fraction of polymer segments at $z$. The last term represents the intermolecular repulsive interactions, with $\phi(z)$ being the volume fraction of species $i$, and $\pi(z)$ is the intermolecular repulsive field, which is determined by imposing packing constraints at each distance $z$ to account for the intermolecular excluded volume interactions. The free energy is minimized for given values of the polymer surface grafting density.

The details of the minimization and the numerical procedure have been explicitly presented in several publications.$^{35,37,39}$ Here we just mention that as input to the theory we include a very large set (5 $\times$ 10$^6$) of independently generated conformations for each polymer molecule; the protein and polymer conformations are generated as discussed in detail elsewhere.$^{35,37,39}$ The other input is the polymer surface grafting density. The calculations are then repeated for different solvent qualities, as a function of surface grafting density for each polymer chain length.

Experimental Section. The substrates for the study were prepared by following the procedures outlined earlier.$^{36,57}$ Specifically, silicon wafers (Silicon Valley Microelectronics, Ltd.) were cut into pieces of desired sizes (3.5 $\times$ 3.5 cm$^2$), washed with methanol, and cleaned in a ultraviolet/ozone chamber for 15 min, which generated a high density of surface-bound silanol groups. A molecular gradient of n-octytrichlorosilane (OTS) was formed along one edge (X-direction) of the specimens by using organosilane vapor diffusion technique pioneered by Chaudhury and Whitesides. The specimens were then immersed in toluene solution of 11-(2-bromo-2-methyl) propionloxy)-undecyl trichlorosilane (BMPUS) and maintained at $-10$ °C for ~12 h. PHHEMA brushes were prepared by surface-initiated atom transfer radical polymerization (ATRP) of HEMA monomer by following the recipes outlined in refs 56 and 57. Specifically, degassed polymerization solution (HEMA/solvent/ligand/activator/deactivator) was transferred to the reaction chamber built in a custom-made polymerization apparatus, and the BMPUS-coated wafer was immersed slowly (~0.33 mm/min) into the polymerization solution along the Y-direction (i.e., with the BMPUS gradient oriented horizontally). This method resulted in an orthogonal variation of the PHHEMA $\phi(X$-direction) and $M(Y$-direction). After polymerization, the specimens were washed and sonicated thoroughly with deionized (DI) water and blow-dried with nitrogen gas. The dry thickness of the polymer layer is comparable to or larger than the size of the protein, the equilibrium adsorption is determined exclusively by the number of segments in the region where the protein adsorbs and how these parts of the layer are deformed. As shown in detail in refs 35 and 39, this is independent of molecular weight for Lys in the range of chain length shown in Figure 1a. The equilibrium theory was used in the past to interpret experimental observations pertaining to adsorption isotherms measured for Lys and fibrinogen on surfaces decorated with short$^{38}$ and long$^{37}$ chain length PEGs. The predictions were found to be in excellent agreement with the experimental observation, supporting the validity of the

![Figure 1](image-url)
results presented here. As we will show below, the experimental observations seem to suggest a different dependence on surface grafting density. Namely, that the adsorption seems to be a universal function of the total number of polymer segments on the surface, i.e., of the product $\sigma N$. As can be seen from the equilibrium protein adsorption calculations in Figure 1b, the equilibrium adsorption is not universal in that variable, and therefore it suggests that it may be that the kinetics of adsorption are responsible for the universal behavior in the product $\sigma N$.

The description of the kinetics of protein adsorption is based on the assumption that there is a separation of time scales between the fast local polymer rearrangement and solvent mobility and the slow protein. This translates into the assumption that the proteins move in an average potential that can be calculated readily using a local equilibrium approximation. Namely, the potential of mean-force is given by the minimal free energy of the system for the given frozen configuration of the proteins. The equations of motion can then be written in the form of a generalized diffusion approach. In the case that the barriers to protein adsorption are larger than the thermal energy, it has been shown that the kinetics of protein adsorption can be described by a transition state theory where the rate coefficients explicitly depend upon the molecular organization of the combined protein–polymer layer. The expression for the time-dependent adsorption of proteins is given by

$$\frac{d\rho_{ads}(t)}{dt} = k_{ads}(t)\rho_{pro}^{bulk} - k_{des}\rho_{ads}^{pro}(t)$$

where $\rho_{ads}^{pro}(t)$ is the number density of adsorbed proteins at time $t$, $\rho_{pro}^{bulk}$ is the bulk concentration of proteins, and $k_{ads}(t)$ and $k_{des}(t)$ are the rate coefficients for adsorption and desorption at time $t$, respectively. The coefficient of adsorption is given by

$$k_{ads}(t) = \frac{D}{\alpha(t)H(t)}e^{-\Delta U_{ads}(t)/k_BT}$$

where $D$ is the bulk diffusion coefficient of the protein, $H(t)$ is the wet thickness of the polymer layer at time $t$, $\alpha(t)$ is the width of the potential of mean-force at an energy $k_BT$ below the maximum, and $\Delta U_{ads}(t)$ is the height of the potential of mean-force barrier. All these quantities vary with time due to the changes in the potential of mean-force as proteins adsorb. Furthermore, $\alpha$, $H(t)$, and $\Delta U_{ads}(t)$ depend strongly on both $M$ and $\sigma$ of the polymer brush, since these properties determine the effective potential of interaction between the protein and the surface. The determination of the barrier for adsorption are determined using a free energy similar to that in eq 1 but in the absence of proteins; for details, see ref 40.

From the kinetic equations we can define a characteristic time of protein adsorption as $\tau = 1/k_{ads}(0)$, which provides a measure of the time needed for the adsorption process to take place. Clearly, $\tau$ is a function of $M$ and $\sigma$, as can be seen from eq 3. At times $t \leq \tau$ the amount of protein adsorbed is given by $\rho_{ads}^{pro}(t < \tau) \approx \rho_{bulk}^{pro}/\tau$. Since $\rho_{bulk}^{pro}$ is given by the solution conditions, the dependence of $\rho_{ads}^{pro}(t < \tau)$ on molecular weight and surface grafting density is the same as that of $\tau$. Therefore, if the predicted $\tau$ is a universal function of $N\sigma$, then the initial amount of proteins adsorbed depends only on the product $M\sigma$ (or $N\sigma$), which is what we observe experimentally, as demonstrated below.

The experimental systems presented below correspond to PHEMA, for which it is known that water is not a very good solvent (i.e., it is between the good and the theta solvent). Therefore, in Figure 2 we plot the predicted values of the initial adsorption time $\tau$ of Lys as a function of the product $\sigma N$ for good (Figure 2a), theta (Figure 2b), and poor (Figure 2c) solvent conditions. The data reveal that the time to reach equilibrium in protein adsorption depends strongly on the solvent quality. In good solvents, even the initial time of adsorption depends heavily on the degree of stretching of the polymer chains, which is a function of the grafting density, chain length, and the interactions between the polymer segments and the surface. For theta and poor solvents, polymer chains form a compact layer, and therefore it is not surprising that the kinetics of adsorption depends on the total amount of polymer segments, i.e., the initial time-dependent adsorption is a universal function of $\sigma N$. The equilibrium amount of adsorbed protein will depend on how the finite amount of adsorbed proteins deforms the polymer layer. As such, this will be governed primarily by surface grafting density of the grafted macromolecule and to a much lesser degree polymer length, as shown in Figure 1. As the protein has to penetrate through the grafted polymers, the process of protein adsorption on surfaces
with bound polymers depends strongly on the structure of the polymer layer. The orthogonal substrate comprising a gradual variation of the grafting density and molecular weight of PHEMA (cf. Figure 3a) was prepared and characterized as described in the Methods section. The amount of adsorbed protein on the gradient substrate was expected to decrease with increasing \( M \) (cf. Figure 3b) and increasing \( \sigma \) (cf. Figure 3c). In Figure 3d, we plot the dry thickness maps of PHEMA on five different orthogonal \( M-\sigma \) substrates (top panel, red color) and the corresponding dry thicknesses of Lys (bottom panel) in samples exposed to Lys solutions for 24, 53, 72, and 87 h. The amount of adsorbed protein is expected to decrease with increasing \( M \) of PHEMA (b) and \( \sigma \) of PHEMA (c). (d–g) Dry thickness profiles (in nanometers, measured by ellipsometry) of PHEMA (top panel) and the corresponding dry thicknesses of Lys (bottom panel) in samples exposed to Lys solutions for 24 (b), 53 (c), 72 (d), and 87 (e) h.

Figure 3. (a) Schematic illustration of the formation of an orthogonal molecular-weight-grafting density \((M-\sigma)\) gradient of PHEMA on a flat substrate and the deposited protein on top of the \(M-\sigma\) PHEMA gradient. First, a molecular gradient of OTS is formed on a silicon wafer \((3.5 \times 3.5 \text{ cm}^2)\) by the vapor deposition method, and the empty spaces on the substrate are filled by BMPUS, an initiator for the ATRP. The BMPUS-decorated substrate is then placed vertically in the custom-designed polymerization chamber (the BMPUS gradient is positioned horizontally), and PHEMA chains are grown via ATRP by dipping the specimen into the solution containing HEMA + solvent (methanol/water) and an organic ligand. Lys was adsorbed onto the PHEMA \(M-\sigma\) specimen by immersing the PHEMA-containing substrates into Lys solutions (concentration 0.1 mg Lys/ml solution, pH = 10, phosphate buffered solution) for different times. The amount of adsorbed protein is expected to decrease with increasing \( M \) of PHEMA (b) and \( \sigma \) of PHEMA (c). (d–g) Dry thickness profiles (in nanometers, measured by ellipsometry) of PHEMA (top panel) and the corresponding dry thicknesses of Lys (bottom panel) in samples exposed to Lys solutions for 24 (b), 53 (c), 72 (d), and 87 (e) h. In the middle panel of Figure 4, we present dry thickness profiles of PHEMA and that of Lys; the SPM micrographs corresponding to the positions on the sample denoted by numbers are shown in the top panel of Figure 4. While the surface of bare PHEMA is very flat (see the rightmost SPM micrograph in the top panel in Figure 4), considerable roughening is observed on protein-coated specimens. The amount of Lys adsorbed decreases as one moves along the scanning direction on the specimen toward areas on the sample that possess higher \( M \) and higher \( \sigma \). The resultant root-mean-square roughness (rms) measured by SPM is plotted in the bottom panel of Figure 4 as a function of the position on the sample (expressed in terms of the \(X\)-coordinate of the position where SPM measurements were taken). The trend in the rms values matches nearly quantitatively with that of the dry Lys thickness measured by ellipsometry after 96 h of protein adsorption (see Supporting Information). In the same plot we
also denote the rms values collected from PHEMA−Lys specimens after 24 h of protein adsorption. While the trends in rms are identical, the absolute values are smaller than those observed on the sample exposed to Lys solution for 96 h. A close inspection of the SPM micrographs leads to a tentative description of Lys configuration on the surface; those trends are shown pictorially in the right portion of the bottom panel in Figure 4. At low M and low σ, Lys adsors on the PHEMA substrate readily and heavily. With increasing M and σ, the amount of Lys decreases dramatically. Small adsorption of proteins on surfaces takes place at 96 h. Ellipsometry cannot detect the proteins, yet SPM, being a local probe, can sense the protein’s presence. This is reasonable since the rms at shorter time at the same position on the substrate is negligible.

While SPM provides information about local arrangement of Lys molecules on the surface, it is limited in quantitatively assessing the amount of adsorbed protein over larger areas. In order to gain a quantitative understanding of how the adsorbed amount of Lys correlates with the density and length of the PHEMA grafts, in Figure 5 we plot the dry thickness of Lys as a function of the dry thickness of PHEMA for various times ranging from 24 to 96 h (see legend to Figure 5 for details). Note that the dry thickness of PHEMA (h) corresponds to the product of σ and M (i.e., M = ρpolN_A), where ρpol is polymer density and N_A is Avogadro’s constant. Close inspection of the data plotted in Figure 5 reveals that for all adsorption times, the adsorbed amount of Lys on the substrates decreases with increasing thickness (or σN) of PHEMA. Importantly, for adsorption periods of up to 72 h, the amounts of adsorbed Lys collected at various σ of PHEMA superimpose. In other words, for a given thickness (or σN) of grafted polymer, the amount of adsorbed proteins is independent of the actual value of σ. PHEMA thickness (~σN) is the factor determining protein adsorption. This is in contrast to the behavior observed for adsorption times longer than 72 h when data no longer fall on a

**Figure 4.** (top panel) SPM scans (10 × 10 μm², height range 75 nm) taken along various positions on the M−σ sample after adsorbing Lys for 96 h (see middle panel). The SPM image on the right depicts the topography of a bare PHEMA brush (10 × 10 μm², height range 25 nm) (height range 1 nm). (middle panel) Dry thickness (in nm) on PHEMA M−σ substrate (left) and dry thickness of Lys on the same sample after adsorbing Lys for 96 h. The positions of the SPM scans are denoted in the top panel. (bottom panel) rms collected at various positions on the Lys-covered samples after 24 (open symbols) and 96 (filled symbols) h of adsorption along the low M/σ to high M/σ diagonal on the specimen (expressed in terms of the X-coordinate of the position where SPM measurements were taken). The cartoons represent pictorially the proposed arrangement of Lys molecules on various positions on the sample corresponding to the first three SPM images shown in the upper panel.
master curve and the amount of adsorbed protein decreases with increasing $\sigma$ of PHEMA for a given thickness or $\sigma N$ (follow symbols corresponding to different $\sigma$ at constant thickness or $\sigma N$). In order to explain this behavior, we return to the theoretical predictions described in Figures 1 and 2 above.

PHEMA is not highly soluble in water; therefore the structure of the protein–polymer layers is rather different from those in the case of the most widely used polymer for minimizing fouling, i.e., PEG. The theoretical prediction, namely, that the amount of adsorbed protein is a unique function of $\sigma N$, is thus in excellent agreement with the experimental observation of amount of Lys adsorbed on PHEMA brushes scaling with $\sigma N$ for an adsorption period of $\leq 72$ h. The model also predicts that adsorption time of 72 h is shorter than the time needed to reach equilibrium. Lys adsorption reaches its thermodynamic equilibrium, but only at adsorption times of many tens of hours. Interestingly, the predictions show how relatively small changes in $\sigma N$ can lead to variations in the adsorption time ranging from days, to a month, to a year. Furthermore, the data in Figure 2 provide a convenient tool to design surface modifiers to control the time scales for initial adsorption of proteins on surfaces. While the calculations shown in Figure 2 have been carried out for Lys, similar results can be obtained for other proteins as well. For proteins larger than Lys, the time scale of adsorption on neutral polymer layers such as PHEMA is even larger due to the slow diffusion of larger proteins. Thus, the kinetic behavior observed in Figure 5a–c and explained theoretically in Figure 2 can be deemed universal for proteins adsorbing on grafted layers in theta or poor solvents.

To summarize, the initial time scale for adsorption, and thus the amount of protein adsorbed at short times, is a universal function of the total amount of polymer segments grafted to surfaces for compact polymer layers in poor and theta solvents. The initial time scale has to be measured in hours, and the time required for adsorption process to reach equilibrium is measured in days. However, the equilibrium amount of proteins adsorbed depends mostly on polymer grafting density on the surface with only a weak dependence on the chain length. The determination of the nonfouling capabilities of surface modifiers cannot be determined from short time adsorption experiments. The experimental observations presented in this article supported by molecular theory of protein adsorption on grafted polymer surfaces establish protein adsorption to be a long-lived interfacial phenomenon with profound consequences for many fundamental and industrial applications.

**ASSOCIATED CONTENT**

Supporting Information
Details pertaining to the characterization of PHEMA brush gradients and data pertaining to Lys adsorption from 1 mg/mL solutions onto PHEMA brush gradients. This material is available free of charge via the Internet at http://pubs.acs.org.

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