Adsorption of Poly(ethylene glycol)-Modified Lysozyme to Silica

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Covalent grafting of poly(ethylene glycol) (PEG) to pharmaceutical proteins, "PEGylation", is becoming more commonplace due to improved therapeutic efficacy. As these conjugates encounter interfaces in manufacture, purification, and end use and adsorption to these interfaces may alter achievable production yields and in vivo efficacies, it is important to understand how PEGylation affects protein adsorption mechanisms. To this end, we have studied the adsorption of unmodified and PEGylated chicken egg lysozyme to silica, using optical reflectometry, total internal reflection fluorescence (TIRF) spectroscopy, and atomic force microscopy (AFM) under varying conditions of ionic strength and extent of PEG modification. PEGylation of lysozyme changes the shape of the adsorption isotherm and alters the preferred orientation of lysozyme on the surface. There is an abrupt transition in the isotherm from low to high surface excess concentrations that correlates with a change in orientation of mono-PEGylated conjugates lying with the long axis parallel to the silica surface to an orientation with the long axis oriented perpendicular to the surface. No sharp transition is observed in the adsorption isotherm for di-PEGylated lysozyme within the range of concentrations examined. The net effect of PEGylation is to decrease the number of protein molecules per unit area relative to the adsorption of unmodified lysozyme, even under conditions where the surface is densely packed with conjugates. This is due to the area sterically excluded by the PEG grafts. The other major effect of PEGylation is to make conjugate adsorption significantly less irreversible than unmodified lysozyme adsorption.

Introduction

Covalent attachment of poly(ethylene glycol) (PEG) chains to therapeutic proteins, "PEGylation", is growing in application due to increased renal clearance time, decreased proteolytic degradation, and decreased immune response relative to the unmodified forms.[1,2] The PEGylated forms of adenosine deaminase and asparaginase are now approved for human use by the U.S. Food and Drug Administration,[2,3] and other PEG-modified proteins are being developed for possible therapeutic use, including PEG epidermal growth factor[4] and PEGylated single-chain Fv proteins.[5]

The numerous scenarios in which PEGylated proteins encounter solid/liquid interfaces during their manufacture, storage, and end use motivate this study of the effect of PEGylation on protein adsorption behavior. When PEGylated proteins are prepared for therapeutic applications, it is necessary that the distribution of modified forms be reproducible. Over-PEGylation can decrease bioactivity, and under-PEGylation may allow a molecule to be cleared from the circulatory system too rapidly. A reproducible population of modified forms could be achieved by controlling the PEGylation reaction and/or by fractionation of the PEGylated product. Chromatography is a common technique for therapeutic protein fractionation, and most chromatographic methods depend on adsorption behavior.

Furthermore, PEG-modified proteins may also encounter solid/liquid interfaces during drug delivery. Protein loss to deposition and surface-induced conformational changes at these interfaces is manifested as lost bioavailability. Tzannis and co-workers observed ∼90% loss of the biological activity of interleukin 2 after 24 h of continuous delivery via commercial silicone rubber catheter tubing.[6] Additionally, insulin activity is reduced upon delivery from micropumps, due to surface-induced aggregation.[7] In delivery technologies, where dosage is critical, unpredictable protein−surface interactions can have a significant impact on the amount of biofunctional drug administered to a patient. The extent of adsorption, the adsorption kinetics, and the layer structural evolution upon the adsorption of protein will dictate the yield of biochemically functional protein and will all likely be affected by PEGylation.

Interest in the adsorption of PEGylated proteins is piqued by the so-called "stealthy" characteristics of PEG. It is well-known that surfaces coated with PEG tend to be resistant to protein adsorption[8−10] and the protective effect of PEG modification on circulating proteins (as well as PEGylated liposomes) in vivo is attributed to strong steric and hydration repulsions between the PEGylated species and other proteins or cell surfaces.[11] Nevertheless, PEG itself is surface active on a variety of surfaces, and

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PEG modification of protein may not necessarily preclude or even decrease the extent of protein adsorption at the solid/liquid interface. Acid−base interactions between PEG ether groups and acidic groups on solid surfaces, such as the silanol groups on silica, make PEG surface active on many hydrophilic materials.[12,13] In addition, the somewhat hydrophobic character of PEG that causes it to have a lower critical solution temperature also makes PEG surface active on hydrophobic surfaces.[14] It is conceivable that the grafted PEG chain may have an adsorption affinity that is comparable to, or even stronger than, that of the protein. The variety of different possible driving forces for the adsorption of what amounts to a complex block copolymer, containing a protein "block" and one or more PEG blocks, makes for a complex adsorption energy landscape that is likely sensitive to the surface coverage and orientation of the PEG−protein conjugate.

Herein, we examine how the presence of PEG covalently grafted to a model protein, chicken egg lysozyme, affects the extent and reversibility of adsorption at the silica/water interface. We contrast the effects of covalently grafted PEG with the simple competitive adsorption of unmodified protein and PEG homopolymers. Chicken egg lysozyme is a commonly examined, relatively hard model protein with a low adiabatic compressibility that tends to resist surface-induced conformational changes on hydrophilic surfaces.[15,16] In addition to being a commonly used model substrate for protein adsorption studies, silica in various degrees of modification is also used as a chromatographic medium. We prepared our conjugates using 20 kDa PEG. Although early PEGylation strategies employed small PEG chains, on the order of a 5 kDa molecular weight or less, recent therapeutic applications focus on larger PEGs, including molecular weights of 20 and 40 kDa.[11] Increasing the molecular weight of the PEG moiety decreases the in vivo clearance rate of the conjugate.[17]

We recently reported experimental evidence that a two-stage, crowding-induced reorientation of unmodified lysozyme occurs during adsorption to the silica/water interface.[16,18] Consistent with a previously published model of electrostatic interactions within a lysozyme monolayer,[19] the reorientation optimizes both the lysozyme attraction to the oppositely charged silica surface and the lateral electrostatic repulsions among adsorbed protein molecules. The preferred orientation puts the active site toward the solution and the most positively charged amino acid patch (containing the N-terminus[20]) into contact with the negatively charged silica surface. This occurs when the adsorbed layer reaches a threshold surface coverage. The richness of this adsorption mechanism arises from spatially nonuniform intermolecular and surface forces. PEGylation imparts a new level of nonuniformity to the intermolecular forces and should be expected to significantly alter the adsorption mechanism.

Here, we report that PEG modification alters lysozyme adsorption isotherms, as well as the organization of the adsorbed layer at the silica/water interface. The complexity of this adsorption problem dictates the use of multiple independent experimental tools. We use optical reflectometry[21−25] to monitor the extent and kinetics of adsorption and desorption, atomic force microscopy (AFM) force measurements[22,26−28] to characterize the extension of adsorbed conjugates at different surface coverages, and total internal reflection fluorescence[29−33] (TIRF) to monitor the exchangeability of unmodified and PEG-modified proteins or PEG homopolymer. We combine these conventional TIRF exchange experiments with the pH-sensitive fluorophore TIRF approach[18] to monitor reorientation dynamics that may occur within the PEG-modified lysozyme layer.

Experimental Section

Materials. Chicken egg lysozyme (Sigma, cat no. L-6876, lot no. 65H7025); methoxy-poly(ethylene glycol)-propionaldehyde (mPEG-propionaldehyde), nominal molecular weight 20 kDa (Nektar Therapeutics, formerly Shearwater Corporation); and poly(ethylene glycol) homopolymer, nominal molecular weight 20 kDa (Sigma-Aldrich Company), were used without further purification. All adsorption experiments were conducted in triethanolamine hydrochloride (TEA; Sigma-Aldrich Company) buffers prepared with varying ionic strength. The matrix assisted laser desorption/ionization (MALDI) matrix used with undigested proteins was 3,5-dimethoxy-4-hydroxycinnamic acid (Fluka). For protein digestion, we used iodoacetate (Sigma-Aldrich Company), dithiothreitol (DTT; Sigma-Aldrich Company), sequencing-grade-modified porcine trypsin (Promega Corporation), and α-cyano-4-hydroxycinnamic acid (Fluka). Fluorescein isothiocyanate (FITC) was obtained from Molecular Probes. Size exclusion chromatography was conducted in phosphate buffer made with sodium phosphate monobasic and dibasic salts (Fisher Scientific) and potassium chloride (Sigma-Aldrich Company).

Adsorption Substrates. Optical reflectometry and TIRF experiments were conducted on surface-oxidized optical-grade silicon wafers (Virginia Semiconductor) and quartz substrates (Bioelectrospec), respectively. We cleaned all surfaces by a protocol that is described in detail elsewhere.[16]

The substrates for the in situ AFM force measurements consisted of fused silica slides (Esco Products) and ultrasharp contact silicon cantilevers (K-Tek) with a 10 nm tip radius of curvature. A cantilever with a spring constant of 0.08 N/m was used for all experiments. The cantilever and the silica slide were cleaned by exposure to an ozone-generating UV lamp (Jelight Company, Inc.) for 30 min. After assembly of the AFM fluid flow cell, a 10 mM NaOH solution was injected. The silica slide and tip were soaked in this solution for 20 min followed by a profuse water rinse. This base treatment step was included to match the last cleaning step of the TIRF and optical reflectometry substrates.

PEGylation of Chicken Egg Lysozyme. We reacted chicken egg lysozyme with mPEG-propionaldehyde (1:6 mole ratio) in

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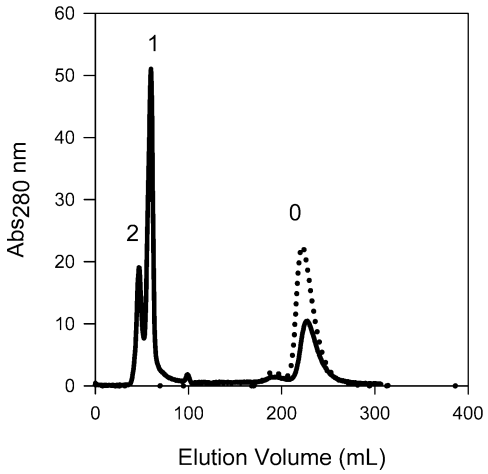

Figure 1. Size exclusion chromatography elution profile of unmodified lysozyme (dashed line) and PEG/lysozyme reaction mixture (solid line). The peak labels correspond to the number of PEG modifications on the lysozyme molecule.

pH 5.1, 100 mM sodium phosphate buffer that contained 20 mM sodium cyanoborohydride. The reaction was allowed to proceed for 17 h at 4 °C. These reaction conditions favor N-terminal modification of the protein.[34,35] PEGylation is not expected to alter the protein secondary structure.[36]

At the end of the reaction period, the PEGylated proteins, unreacted proteins, and unconjugated mPEG-propionaldehyde were separated via size exclusion chromatography on an Äkta Explorer (Amersham Pharmacia) chromatography system, as follows. Prior to sample injection, the Sephacryl S-300 column (1.6 cm inner diameter × 60 cm length) (Amersham Pharmacia) was equilibrated with pH 7.2, 10 mM phosphate buffer containing 150 mM KCl. We injected 2 mL of reaction mixture into the column at a flow rate of 0.5 mL/min and continued the separation at the same flow rate. Upon elution of the PEG/lysozyme reaction mixture from the column, we obtained the chromatogram, based on optical absorbance at 280 nm, shown in Figure 1. Peak 0 corresponds to the elution of unmodified lysozyme from the column, as is apparent from the overlap with the chromatogram for pure unmodified lysozyme. We collected peaks 1 and 2, sending ~5 mL of the material that eluted between the two peaks to waste so as to limit cross-contamination.

We used MALDI mass spectrometry (PerSeptive Voyager STR) to determine the number of PEG molecules attached to lysozyme in each collected fraction. The sample crystals were prepared in a 3,5-dimethoxy-4-hydroxycinnamic acid matrix. The MALDI-time of flight (TOF) operation conditions were set as follows: the mode of operation was linear, the polarity was positive, the acceleration voltage was 25 000 V, and the delayed extraction time was 750 ns. Analysis of the mass spectra gave mass/charge ratios of 35 500 and 56 200 for peaks 1 and 2, respectively, and 21 300 for the mPEG-propionaldehyde alone. The width of the MALDI peaks was ~6000 mass/charge (*m/e*) units. Given the 21 300 molecular weight of PEG and the 14 600 molecular weight of lysozyme, the molecular weights of peaks 1 and 2 correspond to mono- and di-PEG-modified lysozyme, respectively. Although we could not follow PEG elution from the size exclusion chromatography (SEC) column via absorbance changes, we did not see a peak at 21 300 in the MALDI profiles of the PEGylated protein fractions, indicating no homopolymer PEG contamination. After size exclusion chromatography, the mono-PEG and di-PEG fractions were concentrated with 3 kDa molecular weight cutoff (MWCO) Centricon Plus filters (Millipore). The concentrated fractions were then dialyzed overnight against the experimental buffer.

It is difficult to determine the location of the PEG modification on the protein, and others have pursued several different experimental strategies toward this aim.[37−39] We attempted to confirm that the location of the PEG modification for mono-PEGylated lysozyme was the N-terminus by subjecting whole proteins and tryptic digests of unmodified and mono-PEGylated proteins to reverse-phase high performance liquid chromatography (HPLC) and MALDI mass spectrometry analysis. We used a reverse-phase C18 column (Waters) to separate lysozyme and mono-PEG−lysozyme. The column was cleaned with acetonitrile for 30 min and then water for 30 min before each experiment. The column was then equilibrated against a 35% acetonitrile solution. We monitored the elution of unmodified and PEGylated protein on a 35−55% acetonitrile gradient over 50 min. We used a 100 μL sample loop and a flow rate of 1 mL/min. Mono-PEG−lysozyme eluted from the column as a single peak, consistent with one primary location for PEG modification.

For tryptic digests, we followed a standard protocol.[4] We reduced lysozyme, mono-PEG−lysozyme, and di-PEG−lysozyme solutions with DTT in 10 mM phosphate buffer, pH 7.2, with 150 mM KCl. We added 42 μL of a 5 mg/mL DTT solution to 100 μL of protein solution (0.06−5 mg/mL). We then added 38 μL of iodoacetate solution (10 mg/mL) to the reduced protein solution. The solutions were incubated at room temperature for 6 h and then dialyzed twice in 8 kDa MWCO tubing (Fisher Scientific) against the phosphate buffer. We then added trypsin to obtain a trypsin/sample mass ratio of 1:30 and incubated at 37 °C overnight.

The HPLC tryptic digest maps for unmodified and PEG-modified protein were similar. We further analyzed the digests with MALDI using an α-cyano-4-hydroxycinnamic acid matrix. A comparison of the ratio of MALDI peaks for digests of unmodified, mono-PEGylated, and di-PEGylated lysozyme suggested that mono-PEGylated lysozyme was primarily N-terminally modified, with slight modification at lysine residues 33 and 97. Di-PEGylated lysozyme was likely modified at the N-terminus and at residue 33, with slight modification at residues 97 and 116.

Size of the PEG−Lysozyme Conjugates. We measured the hydrodynamic diameters of unmodified lysozyme, nominal 20 kDa PEG homopolymer, and PEGylated lysozyme using a Malvern Zetasizer dynamic light scattering apparatus.[40] Malvern's version of the autoCONTIN algorithm was used to fit measured autocorrelation functions to a distribution of decay times, from which a distribution of diffusion coefficients was calculated. Apparent hydrodynamic diameters were calculated from the diffusion coefficients using the Stokes−Einstein equation for spherical particles. Samples were dissolved in pH 7.2, 10 mM phosphate buffer containing 150 mM KCl and filtered with 0.2 μm filters prior to measurement, with results listed in Table 1.

Optical Reflectometry. Details of the optical reflectometry technique, the standard methods of data analysis, and the particular instrument used here have been described elsewhere.[14,21−22,41] Conversion from raw reflectivity data to surface excess concentrations was based on the two-homogeneous-layer striated interface optical model, consisting of the oxide layer and the adsorbed layer separating the semi-infinite solution and the semi-infinite silicon slab. We used values of 0.18 and 0.134 cm^3/g for the refractive index increment of lysozyme[18] and PEG,[42] respectively. The amount of material required to measure the refractive index increment of the PEGylated conjugates exceeded that which we could produce economically. Hence, the refractive index increments of the PEGylated conjugates were estimated as 0.154 and 0.146 cm^3/g for mono-PEG−lysozyme and di-PEG−lysozyme, respectively, using a mass average of the increments for PEG homopolymer and lysozyme for each conjugate molecule.

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Table 1. Molecular Weights as Measured by MALDI Mass Spectroscopy and Hydrodynamic Sizing as Determined by Dynamic Light Scattering[a]

	unmodified lysozyme	PEG−lysozyme	di-PEG−lysozyme	PEG
molecular weight (g/mol)	14 600	35 500	56 200	21 300
diffusion coefficient (cm^2/s)	$(1.32 \pm 0.2) \times 10^{-6}$	$(4.4 \pm 0.4) \times 10^{-7}$	$(3.03 \pm 0.3) \times 10^{-7}$	$(6.2 \pm 0.5) \times 10^{-7}$
diameter of equivalent sphere (nm)	3.3 ± 0.4	9.9 ± 0.9	14.4 ± 1.2	6.9 ± 0.5
area/molecule (nm^2)	9.1	77.0	162.9	37.4
dimensions of cylinder (nm)		6.9×10.5		

[a] The error values correspond to the width of the light scattering peak.

For both reflectometry and TIRF experiments, the temperature was held constant at 25 ± 1 °C. Buffer was pumped through the flow cell for at least 30 min prior to baseline collection for all experiments. Adsorption took place in rectangular slit flow cells, with solutions undergoing fully developed laminar flow at a 32 s^{-1} wall shear rate.

TIRF. The basic principles of TIRF[30,43,44] have been described elsewhere. For this study, we used a modular Spex Fluorolog-3 (Jobin Yvon Horiba) fluorescence spectrometer. A TIRF flow cell manufactured by Bioelectrospec was mounted into the spectrometer as described previously.[16] Excitation and emission slit widths were both fixed at 7 nm. We monitored intrinsic tryptophan fluorescence during adsorption at excitation and emission wavelengths of $\lambda_{ex} = 295$ nm and $\lambda_{em} = 320$ nm, respectively. The tryptophan intensity is proportional to the surface concentration of lysozyme only, since PEG does not fluoresce. Hence, we used TIRF to follow the adsorption of lysozyme.

Exchangeability refers to the replacement on the surface of one type of preadsorbed molecule by a different type of molecule that is in solution. We monitor the exchangeability of PEG−lysozyme and unmodified lysozyme by fluorescently labeling unmodified lysozyme with fluorescein isothiocyanate (FITC) and challenging an adsorbed layer of one species with a solution containing the other species. Optical reflectometry measurements show that FITC−lysozyme and unmodified lysozyme are identical with respect to adsorption and reversibility on silica. There is no evidence for labeling-induced artifacts. The FITC labeling procedure for unmodified lysozyme was described previously.[16,18] The fluorescein excitation and emission wavelengths were set to $\lambda_{ex} = 488$ nm and $\lambda_{em} = 520$ nm, respectively.

In the exchange experiments, PEGylated lysozyme was not labeled with FITC but un-PEGylated lysozyme had an FITC label. Thus, the tryptophan emission traces the total protein surface excess concentration, while the FITC emission is a reporter for the un-PEGylated protein.

Because fluorescein fluorescence exhibits pH sensitivity, and therefore electrostatic potential sensitivity, monitoring fluorescein emission during exchange experiments on negatively charged silica surfaces also provides information about the average proximity of the FITC moiety to the surface. Details of the TIRF technique that exploits this phenomenon are provided elsewhere.[16,18] Here, we combine the conventional TIRF exchange experiments with the pH-sensitive fluorophore TIRF approach[18] to monitor reorientation dynamics within the PEG-modified lysozyme layer.

Atomic Force Microscopy. In situ force measurements between adsorbed layers on opposing silica surfaces were performed with a Nanoscope III atomic force microscope (Digital Instruments, now Veeco Metrology). The fluid cell volume was ~0.1 mL, and solutions were manually introduced to the fluid cell by syringe. Force measurements were conducted and analyzed according to well-established procedures developed elsewhere.[26,27] Forces were calculated from the measured cantilever deflection by applying Hooke's law with a predetermined cantilever spring constant. The force curves were normalized by the tip radius of curvature. We used the nominal tip radius and cantilever spring constant as reported by the manufacturer. We considered these approximations to be acceptable because the shapes of the force curves, not the force magnitudes, were used to estimate the adsorbed layer thickness, and results are only compared for measurements made with the exact same cantilever.

Changes in the separation distance were determined directly from the calibrated piezoelectric drive displacement and the measured cantilever deflection. As is customary, we took the onset of the constant compliance regime of the force curve to represent the zero apparent separation distance between the opposing surfaces.[26] This would not necessarily correspond to direct contact between the tip and the substrate, because constant compliance occurs whenever the force gradient exceeds the cantilever spring constant. Hence, some of the protein−polymer conjugate could be trapped between the tip and the substrate at the onset of constant compliance, resulting in uncertainty in the absolute separation distance.

To measure the force between opposing layers of adsorbed mono-PEGylated lysozyme, we injected approximately 20 flow cell volumes of a 0.1 μM solution of mono-PEG−lysozyme into the fluid cell and allowed 1 h for adsorption to both the silica slide and the cantilever tip. We then collected force curves at several different locations on the slide over several hours. After rinsing with buffer, we then replaced the dilute solution with 20 flow cell volumes of a 10 μM solution of mono-PEG−lysozyme and allowed it to equilibrate for 30 min prior to collecting new force curves. We continued to collect force curves at different locations on the slide for the next several hours.

Results and Discussion

Size of the Conjugates. Awareness of the dimensions and structure of the adsorbing protein facilitates the interpretation of adsorption isotherms. The diffusion coefficients and equivalent sphere hydrodynamic diameters are summarized in Table 1. The hydrodynamic diameters of mono-PEGylated and di-PEGylated lysozyme are approximately the sum of the diameters of one lysozyme molecule and one or two PEG chains. The protective effect of PEGylation has been described as originating from a shell of PEG chains surrounding the protein,[11,18] but in the case of mono-PEGylation, it is unlikely that a single 20 kDa chain would be able to surround the protein. The dimensions of the conjugates suggest that the 20 kDa PEG chains exist as relatively unperturbed random coil domains adjacent to the lysozyme molecule, rather than as a protein shroud. Table 1 also lists the cross-sectional area of the equivalent spherical molecule calculated from the hydrodynamic diameter. We used these area-per-molecule values in order to estimate area fractional surface coverages corresponding to different points on the adsorption isotherms below. We also modeled mono-PEG−lysozyme as a cylinder, using the friction coefficient, f,

$$f = 6\pi\eta_0 \frac{L/2}{\ln q + 0.312 + 0.565q^{-1} - 0.100q^{-2}} \quad (1)$$

for a short cylinder, where L is the length of the cylinder and q is the cylinder's aspect ratio (L/D), where D is the diameter of the cylinder, valid for $2 < q < 30$.[46,47] We fixed D as 6.9 nm, or the diameter of an unperturbed PEG coil in solution, and calculated $L = 10.5$ nm from the diffusion coefficient reported in Table 1. The footprint of this cylinder lying with the long axis parallel to the adsorbing surface

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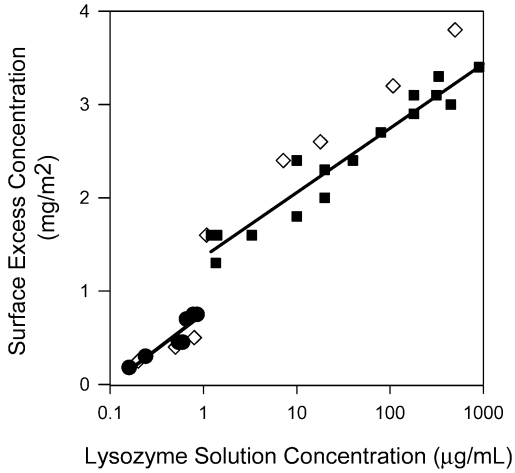

Figure 2. Isotherm for lysozyme shown in filled squares and the isotherm for a mixture of lysozyme and 20 kDa homopolymer PEG (1:1 mole ratio) shown in open diamonds. The lines are linear regressions of the corresponding data in two regimes of the lysozyme isotherm.

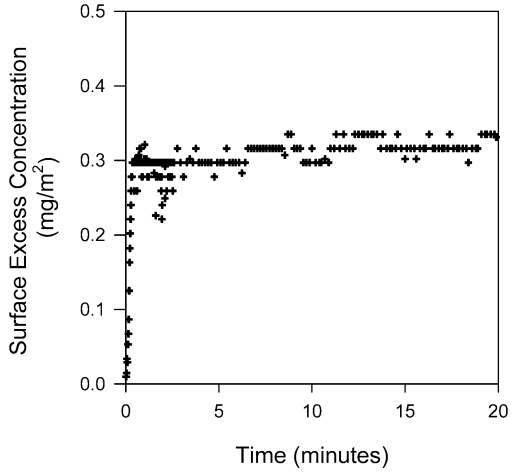

Figure 3. Adsorption kinetic profile of 20 μg/mL poly(ethylene glycol) in pH 7.4, 5 mM TEA buffer.

would be 72.5 nm^2, and that lying with the long axis perpendicular to the adsorbing surface would be 37.4 nm^2.

The remainder of this section is organized as follows. First, we examine the extent of adsorption of the individual components of the lysozyme and PEG homopolymer system to provide a baseline for comparison. Then, we examine the extent of adsorption of conjugates, including the effects of ionic strength variation. The isotherms are interpreted in terms of conjugate orientation, based entirely on geometrical arguments. To independently test those interpretations, we then examine force curves to probe conjugate orientations. Finally, we examine the effects of PEGylation on the tenacity of adsorption, expressed in terms of adsorption irreversibility and exchangeability.

Lysozyme Adsorption Isotherms. The adsorption isotherm for lysozyme on silica in 5 mM, pH 7.4 TEA buffer at 25 °C is shown in Figure 2. This isotherm displays two approximately linear regimes separated by a sharp transition at a solution concentration of ~1 μg/mL. Such transitions are commonly attributed to lateral interactions[48] that induce either conformational changes for proteins with a flexible structure[48] or changes in orientation for proteins with a rigid structure.[16,18]

PEG Homopolymer Adsorption. Figure 3 indicates the extent of PEG adsorption to silica at a bulk concentration of 20 μg/mL with pH and ionic strength conditions identical to those used for the lysozyme adsorption. Although we did not measure the complete adsorption isotherm for homopolymer PEG, it is well-known that the adsorption isotherm for PEG on silica is of the high affinity type, with surface excess concentration nearly independent of solution concentration.[42] PEG adsorbs rapidly to reach a stable adsorption plateau. The initial rate of adsorption ($d\Gamma/dt = 2 \times 10^{-6}$ mg/(cm^2·s)) at 20 μg/mL is consistent with the Levêque solution to the convective−diffusive transport equation,[29,30] using the diffusion coefficient for PEG that we measured with dynamic light scattering, a shear rate of 32 s^{-1}, and a flow cell entrance to observation point distance of 1.25 cm. Others have also observed that PEG adsorption onto silica is transport-limited up to at least 90% of the final surface excess concentration for laminar slit flow.[24] The plateau surface excess concentration, 0.35 mg/m^2, is consistent with the literature.[11] At the same solution concentrations, PEG adsorption reaches a plateau more quickly than does lysozyme adsorption. Since both PEG and lysozyme adsorb to silica in pH 7.4, 5 mM TEA buffer, one cannot easily predict which species would dominate the adsorption behavior of a PEG−lysozyme conjugate. Pursuing the block copolymer analogy, PEG−lysozyme conjugates consist of highly soluble blocks, each of which is surface active on silica. The question is which block(s) anchor(s) the conjugate to the silica surface.

Competitive Coadsorption Isotherm for Lysozyme and PEG Homopolymer. The coadsorption isotherm of a solution containing free PEG (20 kDa) and unmodified lysozyme is overlaid on the single-component lysozyme isotherm in Figure 2. These solutions were prepared in pH 7.4, 5 mM TEA buffer with a 1:1 mole ratio of PEG and lysozyme. Since we do not know the composition of the adsorbed layer, we analyzed the reflectometry signal using the refractive index increment of lysozyme to express the sum of the adsorbed amounts of PEG and lysozyme as an optically equivalent surface excess concentration of lysozyme. This allowed us to at least compare the plots qualitatively. The PEG + lysozyme competitive coadsorption isotherm is very similar to the lysozyme adsorption isotherm. Most importantly, the onset of the sharp transition in the isotherm is not affected by the presence of PEG homopolymer.

At high solution concentrations in the presence of PEG, the surface concentrations are higher than those for single-component lysozyme adsorption by an amount that approximates the amount of PEG adsorption from a protein-free solution, suggesting the formation of a mixed layer. While PEGylated surfaces are known to repel proteins, lysozyme is able to adsorb to silica simultaneously with PEG adsorption from solution.

Adsorption Isotherms for PEGylated Lysozyme. In contrast to PEG homopolymer coadsorption with lysozyme, PEG modification of lysozyme significantly changes the shape of the adsorption isotherm. Figure 4 compares the isotherms of unmodified, mono-PEGylated, and di-PEGylated lysozyme in pH 7.4, 5 mM TEA buffer at 25 °C. The mono-PEG−lysozyme isotherm retains the general shape of the lysozyme isotherm, but the transition occurs at a solution concentration that is approximately

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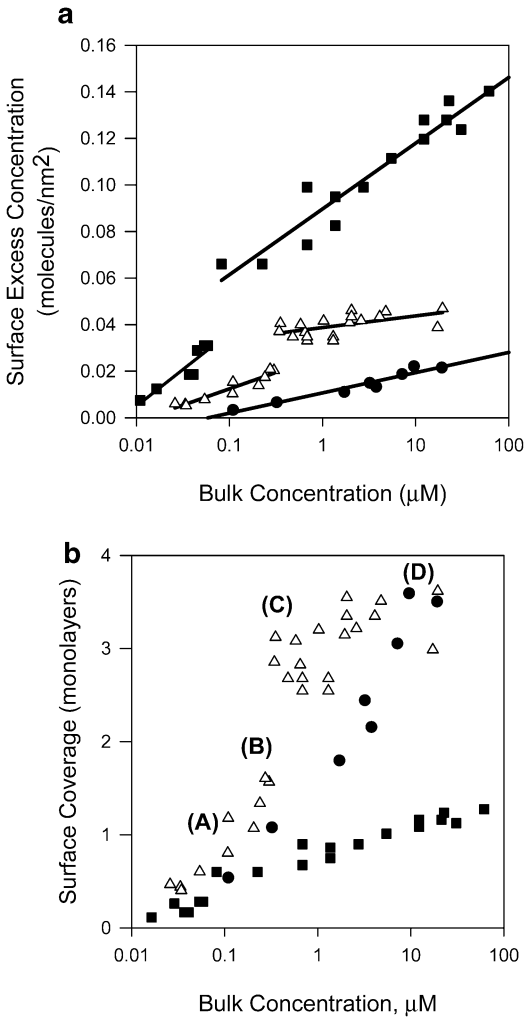

Figure 4. (a) Adsorption isotherms in 5 mM, pH 7.4 TEA buffer: unmodified lysozyme (filled squares); mono-PEG−lysozyme (open triangles); di-PEG−lysozyme (filled circles). The lines are linear regressions of the corresponding data. (b) The surface coverage in monolayers is calculated from the surface excess concentration assuming that each of the molecules is a sphere with the hydrodynamic diameter reported in Table 1. The letters A, B, C, and D denote the position in the mono-PEGylated lysozyme isotherm where mean molecular center-to-center distances are calculated for the schematic illustrations shown in Figure 5.

6-fold greater. The di-PEGylated species does not exhibit a transition over the bulk concentration range accessible to us.

When comparing adsorption isotherms for the unmodified and PEGylated lysozyme, we prefer to express the bulk concentrations on a molar basis and the surface excess concentration in terms of the number of molecules per unit area, where we consider one conjugate as one molecule. In this way, we can directly indicate the effect of PEGylation on the amount of protein that resides on the surface for solutions containing equal protein concentrations or chemical potentials. Figure 4a shows that the number of molecules per unit area is significantly lower for PEG−lysozyme conjugates than for unmodified lysozyme.

We compute the apparent area fraction surface coverage from the total number of molecules per unit area, using our measured hydrodynamic diameters to compute the cross-sectional area of the molecules (Table 1). Figure 4b shows that the surface coverage at which the mono-PEG−lysozyme isotherm begins the transition is close to one monolayer. After the transition, the isotherm appears to plateau at three equivalent monolayers of coverage. This implies either multilayer adsorption or a decrease in the projected cross-sectional area, the "footprint", of the conjugate in a monolayer.

If we now consider the conjugate molecules as short cylinders with the dimensions specified in Table 1, the footprint of the conjugate will depend on its orientation. In the side-on configuration, the footprint of the conjugate is almost identical to the footprint of an equivalent sphere. However, in the end-on configuration, the footprint is significantly smaller than the area of the equivalent sphere, suggesting the possibility that the large apparent areal coverages correspond to an end-on orientation, rather than a multilayer.

We note that the average center-to-center distance between adsorbed mono-PEG−lysozyme conjugates at one apparent monolayer of coverage (Figure 4b, point A) is 9.9 nm. Just before the sharp transition (Figure 4b, point B), the average center-to-center distance between molecules is 8 nm. This is slightly smaller than twice the radius of mono-PEG−lysozyme molecules. Such a situation is easily accommodated by a mono-PEG−lysozyme conjugate lying parallel to the surface, with both lysozyme and PEG occupying surface area. Just above the transition (Figure 4b, point C), the average center-to-center distance between molecules is 5.6 nm. This is slightly smaller than the hydrodynamic diameter of the PEG random coil. This distance decreases to 5.2 nm at the highest solution concentrations (Figure 4b, point D). Referring to the schematic illustration in Figure 5, we suggest that as the center-to-center distance decreases below the size of the conjugate molecule, the conjugates orient their long axes perpendicular to the surface. Above the transition, steric interactions among the PEG chains become the limiting factor that determines the amount of conjugate adsorbed to silica. In other words, the isotherm plateau would correspond to a single monolayer of molecules that exclude the same area as a relatively unperturbed PEG random coil. Packing to an extent where the PEG random coils are separated by approximately one diameter would correspond to a mushroom layer in the block copolymer analogy. At the highest solution concentrations, the center-to-center distance is smaller than the size of the PEG chain, suggesting that some chain stretching does occur at the highest surface concentration. An alternate possibility is multilayer adsorption, although this is unlikely because the N-terminal face of lysozyme is covered by a large PEG chain. This face is involved in the lysozyme dimerization process[49,50] and would presumably be involved in the multilayer buildup. Additionally, PEG chains extending from the surface would sterically repel other conjugate molecules, rather than encourage multilayer adsorption.

The adsorption isotherm of di-PEGylated lysozyme is linear on the semilog plot and shows no transition in the concentration range examined. Figure 4a shows that the total number of di-PEG−lysozyme molecules per unit area is very small, yet Figure 4b shows that the apparent surface coverage exceeds one apparent monolayer coverage, based on the equivalent sphere cross-sectional area. Since there is likely some asymmetry in the shape of the di-PEG−lysozyme conjugates, they can assume different configurations on the surface that project different molecular footprints. Di-PEG−lysozyme is not necessarily

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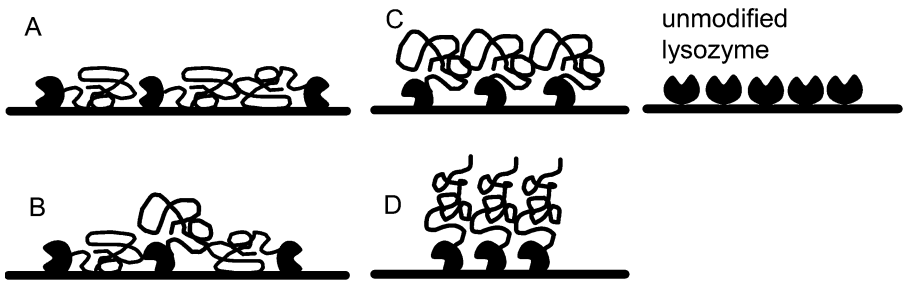

Figure 5. Schematic illustration of layer structure before and after the isotherm transition. The illustrations are based on the center-to-center distance between adsorbed molecules assuming monolayer coverage: (A) center-to-center distance of 9.9 nm; (B) center-to-center distance of 8 nm; (C) center-to-center distance of 5.6 nm; (D) center-to-center distance of 5.2 nm. The orientation preferred by unmodified lysozyme is shown for comparison.

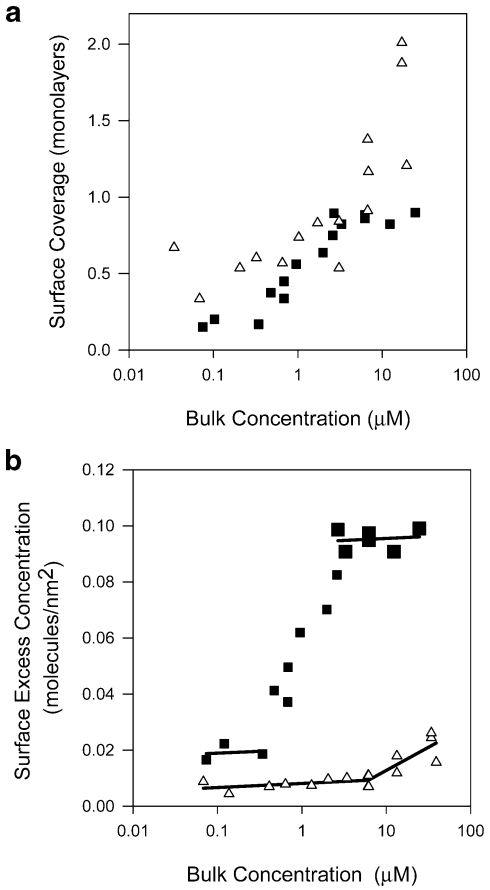

Figure 6. (a) Adsorption isotherms in 50 mM, pH 7.4 TEA buffer: unmodified lysozyme (filled squares); mono-PEG−lysozyme (open triangles). (b) The surface coverage in monolayers is calculated from the surface excess concentration assuming that each of the molecules is a sphere with the hydrodynamic diameter reported in Table 1.

forming multilayers. More work beyond the current dynamic light scattering measurements is needed to more precisely elucidate the structure of di-PEG−lysozyme. The net effect of di-PEGylation is to significantly decrease the number of lysozyme molecules adsorbed per unit area, due to the large area excluded by the PEG chains.

Effect of Electrostatic Interactions on Conjugate Adsorption Isotherms. We investigate the role of electrostatics in the adsorption of PEGylated lysozyme by increasing the ionic strength of the solution. The adsorption isotherms of unmodified and mono-PEG−lysozyme in pH 7.4, 50 mM TEA are compared in Figure 6. The unmodified lysozyme isotherm again shows a transition, now centered around a bulk concentration of 1 μM. The transition is not as steep and is shifted to a higher lysozyme solution concentration in the 50 mM buffer compared to the 5 mM buffer (compare to Figure 4). This is attributed to screening of the electrostatic attraction of lysozyme to the silica surface. The onset of the transition appears to occur at approximately the same surface coverage as that in the 5 mM buffer, but a higher solution concentration (∼chemical potential) is required to populate the surface to the same extent. Interestingly, the layer is more tightly packed at the end of the transition regime than in the low ionic strength buffer. This may be attributed to the weakened lateral repulsions within the layer.

Due to limitations on the amount of material we can produce, the PEGylated lysozyme isotherm is truncated at a solution concentration of 25 μM. Nonetheless, we still observe a transition in the isotherm, at higher mono-PEG−lysozyme solution concentrations than those in the 5 mM buffer, and we again observe coverages in excess of one apparent monolayer based on the projected area of the equivalent spherical conjugate. The same arguments applied to the data acquired with the 5 mM ionic strength buffer would indicate that the conjugates orient their long axis away from the surface at high surface concentrations, with the area per molecule limited by the PEG dimensions. Thus, the main effect of increasing ionic strength is merely to shift the isotherm, and the underlying transitions in conjugate orientations, to higher bulk concentrations.

Force Curves. Interpretation of the adsorption isotherm based on geometric considerations suggests that the preferred orientation of mono-PEG−lysozyme conjugates changes between low and high surface concentrations. To independently test the proposed difference in orientation, we use AFM force measurements between two opposing silica surfaces covered by adsorbed mono-PEG−lysozyme. If the measured forces are dominated by steric interactions, then information about the layer thickness may be inferred from the data. The layer thickness would be expected to approximate the 6.9 nm PEG dimensions at low concentrations (pretransition) where we propose the conjugate lies with its long axis more or less parallel to the surface. At high solution concentrations (post-transition), one would expect a thicker layer, reflecting the full 10.5 nm long axis of the cylindrical mono-PEG−lysozyme conjugate (see Table 1) oriented perpendicular to the surface.

Figure 7 shows representative advancing and retracting force curves between the conjugate-covered tip and slide after at least 30 min of incubation in a 0.1 μM mono-PEG−lysozyme solution. Upon retraction, a weak local minimum at very small separation (as seen in Figure 7) is observed in ∼50% of the force curves collected. This may indicate weak bridging of conjugates between the surfaces. This is not observed in any of the approaching force curves.

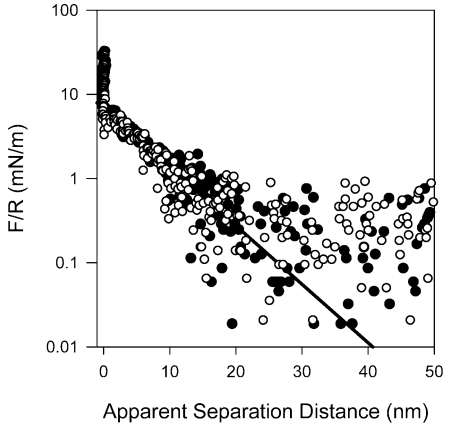

Figure 7. Semilog plot of the force normalized by the nominal tip radius of curvature as a function of apparent separation distance after adsorption from a 0.1 μM mono-PEG−lysozyme solution: forces measured upon approach (solid circles) and retraction (open circles). The linear regime is fitted by linear regression.

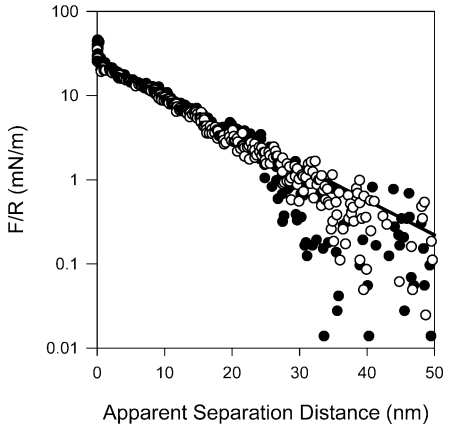

Figure 8. Semilog plot of the force normalized by the nominal tip radius of curvature as a function of apparent separation distance after adsorption from a 10 μM mono-PEG−lysozyme solution: forces measured upon approach (solid circles) and retraction (open circles). The linear regime is fitted by linear regression.

The long range force on approach can be fit to a single-exponential decay, with an average decay length of 5.4 ± 1.4 nm, (averaged over 20 force curves). The Debye length expected for the 5 mM TEA solution is 4.2 nm. Thus, it is difficult to decouple a possible long range steric force from the double-layer repulsion. The normalized force begins to exceed 1 mN/m at an apparent separation distance of ~10 nm, roughly twice the hydrodynamic diameter of the PEG chain. Fully recognizing the uncertainty in apparent separation distances, the relatively short ranged force measured at low coverage is consistent with a monolayer of mono-PEG−lysozyme conjugate lying "flat" with its long axis parallel to the adsorbing surface.

Figure 8 shows representative advancing and retracting force curves when a 10 μM solution of mono-PEG−lysozyme is injected into the flow cell and allowed to equilibrate for 30 min. This is deemed a sufficient equilibration time for this bulk concentration, based on separate optical reflectometry measurements showing that 85% of the adsorption plateau is reached within 1 min. The force is of considerably longer range than that measured in the less concentrated conjugate solution. The normalized force reaches 1 mN/m at a separation distance of ~30 nm. No local minima are observed in any of the retracting force curves. Thus, the layers are sufficiently dense to prevent conjugate bridging. The occurrence of bridging at low coverages, but not at high coverages, is consistent with published force measurements for PEG homopolymers adsorbed on glass.[26,27]

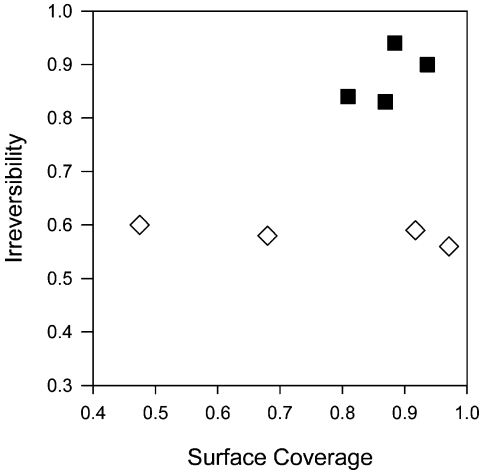

Figure 9. Fraction of protein remaining on the surface after 17 min of desorption into pure buffer reported as a function of areal surface coverage: unmodified lysozyme (solid squares); PEG−lysozyme (open diamonds).

The long range force at high surface coverage decays exponentially until an apparent separation distance of ~25 nm is reached. The average decay length from 20 force curves is 9.4 ± 0.5 nm. This is clearly larger than the Debye length, suggesting that the force is dominated by steric interactions. The significantly longer ranged steric repulsion observed above the isotherm transition is consistent with the proposed differences in conjugate orientation, wherein the conjugates lie with the long axis parallel to the adsorbing surface at low solution concentrations and perpendicular to the adsorbing surface at high solution concentrations.

Since the onset of steric repulsion begins at an apparent separation distance slightly in excess of twice the conjugate long axis length (21 nm) at high surface concentrations, it is likely that the PEG chains are stretched. This stretching is consistent with the observation that the center-to-center distance between adsorbed molecules in Figure 4b, point D, is smaller than the hydrodynamic diameter of the PEG chain.

Reversibility of Adsorption. The above results indicate how lateral interactions among adsorbed PEG−lysozyme conjugates determine the preferred molecular orientation and the form of the adsorption isotherm. Here, we consider how PEG grafting affects another important characteristic of protein adsorption—the tendency to display effectively irreversible adsorption. We measure the adsorption reversibility of unmodified and PEGylated lysozyme, as well as PEG homopolymer, by rinsing the adsorbed layer with pure buffer. Adsorption and desorption experiments are conducted in 50 mM TEA buffer because the reversibility of electrostatically driven adsorption is amplified in higher ionic strength buffers. We report the irreversibility of adsorption as the fraction of protein that remains bound to the surface after 17 min of desorption into pure buffer, since the desorption rate for unmodified lysozyme becomes immeasurably small by this time.

The adsorption irreversibility of unmodified lysozyme and of mono-PEG−lysozyme is plotted as a function of the area fraction surface coverage in Figure 9. The unmodified lysozyme reversibility experiments are conducted at areal coverages between 0.8 and 1 monolayer.

Results indicate that unmodified lysozyme is 85−95% irreversibly adsorbed to silica.

PEG adsorption is completely irreversible over the time scale of desorption used in our experiments. Other groups have also observed extremely slow desorption of PEG from silica.[51] Hence, one might expect that the PEG−silica affinity might make PEGylated lysozyme adsorption even more irreversible than unmodified lysozyme, but this turns out not to be the case.

The mono-PEG−lysozyme reversibility experiments are conducted at surface coverages less than or equal to one apparent monolayer, where we have argued that the long conjugate axis is oriented parallel to the adsorbing surface. It is cost prohibitive to produce enough PEGylated protein to measure adsorption or desorption above the expected isotherm transition in the 50 mM buffer. Figure 9 shows that the adsorption irreversibility of mono-PEGylated lysozyme in this orientation is ~60%, significantly less than either unmodified lysozyme or homopolymer PEG.

The most likely explanation for this counterintuitive observation is that the charged regions of lysozyme that interact preferentially with the silica surface are different upon PEGylation. Previously, we showed that the N-terminal face of unmodified lysozyme is preferentially oriented toward the silica surface.[16,18] Since PEG predominantly modifies the N-terminus of lysozyme, the amino acid patch in the unmodified protein that ordinarily interacts preferentially with silica is obstructed in the PEGylated conjugate. Thus, the conjugate is anchored by a portion of the lysozyme molecule that has a smaller affinity for the surface. A comparison to the preferred orientation for unmodified lysozyme is sketched in Figure 5.

Exchangeability of PEGylated and Unmodified Lysozyme. To explore the possibility that a monolayer of conjugate adsorbed with the PEG chains extended into solution might form a protein resistant surface, we monitor the exchangeability of unmodified and PEG-modified lysozyme using tryptophan fluorescence in TIRF, as shown in Figure 10a. In these experiments, the un-PEGylated lysozyme is labeled with FITC. The mono-PEGylated lysozyme has no FITC. The tryptophan signal is proportional to the surface concentration of lysozyme only and does not reflect the presence of PEG at the interface. We initially expose a 1.3 μM mono-PEG−lysozyme solution in 5 mM TEA buffer to the quartz slide in the TIRF apparatus and observe an increase in the tryptophan fluorescence, of course with no change in FITC fluorescence. This bulk solution concentration corresponds to a dense monolayer of mono-PEGylated protein, oriented with the long axis perpendicular to the surface and the PEG chain dangling into solution. Then, after a buffer rinse from approximately 5000 to 6500 s, we challenge the preadsorbed mono-PEG−lysozyme layer with a solution of 0.7 μM non-PEGylated (but FITC-labeled) lysozyme. This concentration produces a dense monolayer of lysozyme. Upon challenging the mono-PEG−lysozyme layer with un-PEGylated lysozyme, the tryptophan signal and the FITC signal both increase, indicating that unmodified lysozyme is adsorbing, despite the high density of the mono-PEG−lysozyme layer. Un-PEGylated lysozyme must displace mono-PEGylated lysozyme. This results in an increase of the lysozyme surface concentration (tryptophan signal) because un-PEGylated lysozyme excludes less area than PEGylated lysozyme. Hence, an adsorbed

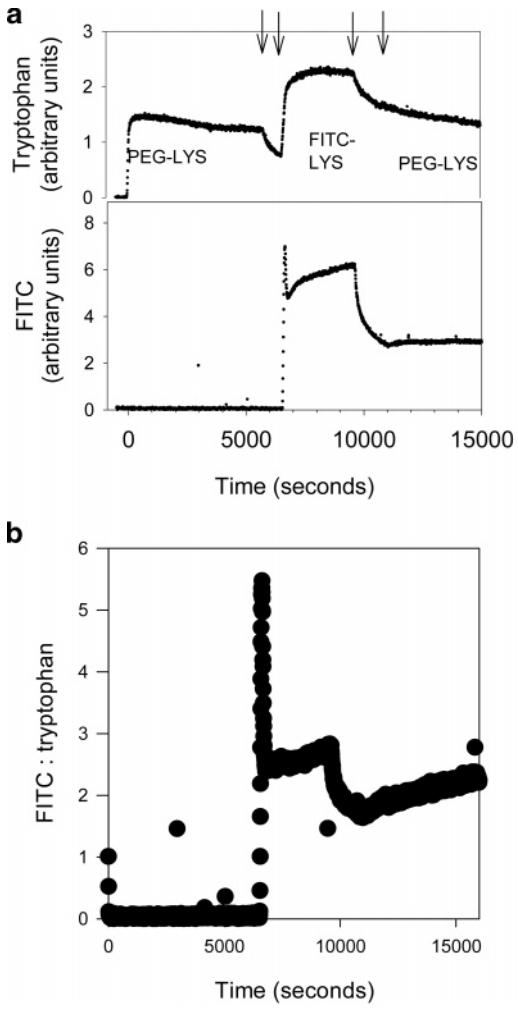

Figure 10. Exchange of PEG−lysozyme (1.3 μM) and FITC−lysozyme (0.7 μM) in pH 7.4, 5 mM TEA monitored using total internal reflection fluorescence. (a) PEG−lysozyme is adsorbed, and the layer is rinsed with buffer (first arrow) and challenged with FITC−lysozyme (second arrow). Then, the mixed layer is rinsed with buffer (third arrow) and challenged with PEG−lysozyme (fourth arrow). (b) Changes in the ratio of FITC to tryptophan fluorescence emission intensity throughout the experiment described in part a.

PEGylated lysozyme layer does not produce a protein resistant surface. The additional information contained in the FITC signal is discussed below.

Restructuring of the Adsorbed Layer upon Exchange. The FITC signal increases when FITC-labeled, un-PEGylated lysozyme displaces preadsorbed mono-PEG−lysozyme as expected. When the total amount of lysozyme on the surface crosses the threshold where we have previously observed restructuring of unmodified lysozyme layers,[16] we observe the same sort of overshoot in the FITC signal (at ~6500 s in Figure 10) that is characteristic of the layer reconfiguration described in the previous work. Hence, we believe that as un-PEGylated FITC−lysozyme displaces preadsorbed mono-PEG−lysozyme, it experiences a similar reorientation from the initial random placement on the surface to a thermodynamically preferred orientation that places the N-terminal face in contact with the silica surface, just as unmodified lysozyme would have done when adsorbing to an initially bare silica surface.

The final stage of these exchange experiments is to rinse the mixed layer with 5 mM TEA buffer from ~10 000−11 000 s, producing ~20% desorption. Then, we reintro-

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duce the original 1.3 μM mono-PEG−lysozyme solution, whereupon the tryptophan signal decreases further. One might expect the amount of adsorbed lysozyme to increase as mono-PEG−lysozyme fills voids created during the buffer rinse, yet there continues to be a net loss of protein from the surface. This can be attributed to the greater excluded area of mono-PEG−lysozyme; on average, each desorbing lysozyme molecule is replaced by less than one mono-PEG−lysozyme molecule.

The complexity of events occurring within this transient layer is highlighted by Figure 10b. We observe a significant increase in the ratio of FITC signal to tryptophan signal as the mono-PEG−lysozyme fills voids left by desorbing lysozyme. If the FITC signal were simply proportional to the FITC−lysozyme surface concentration, it would have decreased in parallel with the tryptophan signal. The most plausible explanation for this increase in FITC emission intensity that accompanies a net loss of lysozyme from the surface is a reverse reconfiguration of the lysozyme layer, whereby the N-terminus on the unmodified protein moves away from the silica surface, increasing the emission intensity from the FITC label.

What does that rollover of unmodified protein imply for the PEG−lysozyme orientation? Mono-PEG−lysozyme conjugates that may surround unmodified lysozyme molecules are not capable of sustaining the optimal orientation of the un-PEGylated lysozyme molecules. An important feature of the previous work on lysozyme orientation was that lateral electrostatic repulsions among lysozyme molecules drove the molecules to their preferred orientation. Evidently, steric repulsions from PEG chains do not have the same effect on the lysozyme orientation as do the lateral electrostatic repulsions.

Conclusions

By combining optical reflectometry with total internal reflection fluorescence spectroscopy techniques and AFM force measurements, we have observed the effects of PEGylation on the mechanism of lysozyme adsorption at the silica/water interface. The main conclusion is that the covalent attachment of PEG to lysozyme significantly alters the preferred molecular orientation. This in turn alters the adsorption isotherms and the adsorption reversibility. The net effect on the amount of lysozyme adsorption is that even at high bulk concentrations where the surface is covered by a densely packed layer of PEG−lysozyme conjugates, there are fewer lysozyme molecules per unit area compared to a dense layer of unmodified lysozyme. This is due to the large contribution of the PEG grafts to the excluded area per molecule. Changes in preferred orientation coincide with less tenacious, that is, less irreversible, adsorption of PEGylated lysozyme to silica. Finally, we note that, at the highest coverages, the PEG−lysozyme conjugates create a layer that resembles a polymer mushroom layer.

Looking to future developments in the application of protein PEGylation technology, we speculate that protein PEGylation might improve the structural stability of adsorbed proteins, hence increasing pharmaceutical bioavailability. If this postulated protective effect is true, then we may also expect PEGylation technology to increase the sensitivity and useful lifetime of biosensors or similar devices based on immobilized proteins. The modification may also allow for control of 2-D clustering upon adsorption. This study of the effects of PEGylation on adsorbed protein layer structure is a step toward the goal of better control over adsorbed protein bioactivity.

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