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Control of Protein Adsorption: Molecular Level Structural and Spatial Variables

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Protein rejection by oligo(ethylene oxide) (OEO)-modified surfaces is described here in terms of changes in free energy (system = protein + surface) due to oligomer-oligomer interactions that extend over a lateral area greater than the contact area. From the experimental protein adsorption obtained over a wide range of coverages by self-assembly of $HS(CH_2)_3O(CH_2CH_2O)_5CH_3$,[1] abbreviated ***A*** hereafter, on gold, we show that the protein adsorption reaches a minimum at coverages where the opposing conditions of effective screening of the underlying substrate and significant conformational mobility of the bound oligomers are both present.[2]

Biofouling processes begin with deposition of proteinaceous or glycoproteinaceous films[3] on metal or oxide surfaces immersed in physiological fluids or other aqueous (e.g., marine) environments. The control of surface fouling and biofouling presents a challenge of enormous economic importance for a wide variety of medical, biotechnology, maritime, and energy industries. Substrates modified with poly(ethylene oxide) (PEO, $HO(CH_2CH_2O)_xH$, $x \geq 12$) or OEO ($x = 3-11$) chains are well-known to exhibit high resistance to protein adsorption[4] and are currently the most promising candidates for fouling-resistant materials. However, the molecular basis of the protein resistance of PEO- and OEO-modified surfaces is not well-understood.[5] Systematic characterization of the precise chemical structure of the protein-OEO interface is impeded because the OEO interfacial structure is known to vary with changes in the length,[4f,6] packing density,[4a,7] and order and conformation[4b,h] of the chains.

The protein adsorption of fibrinogen (Fb) and bovine serum albumin (BSA) onto polycrystalline gold coated with ***A***, from bare Au (0% coverage) to a complete, near-single-phase self-assembled monolayer (SAM) (~100% coverage, where the OEO segments adopt the highly ordered, 7/2 helical conformation) is shown in Figure 1.[8] Both test proteins exhibit similar adsorption curves. Minimal protein adsorption levels occur at ~60% coverage and remain constant or increase slightly from ~60 to ~80% coverage. In this range of coverage, BSA adsorption is below the detection limit, within the experimental error of the surface plasmon resonance (SPR) device, whereas Fb adsorption is finite throughout. From ~80 to 100% coverage, protein adsorption increases from the minimum for both BSA and Fb. The following paragraphs describe the regions of the data in Figure 1 from low to high oligomer coverage.

At low coverage of ***A***, Fb and BSA adsorption can occur directly onto the Au, accessible as bare Au patches or as Au ineffectively screened by striped-phase OEO or collapsed sites. Both proteins exhibit decreasing adsorption with increasing oligomer coverage from 0% to ~55% as the increasing oligomer concentration more

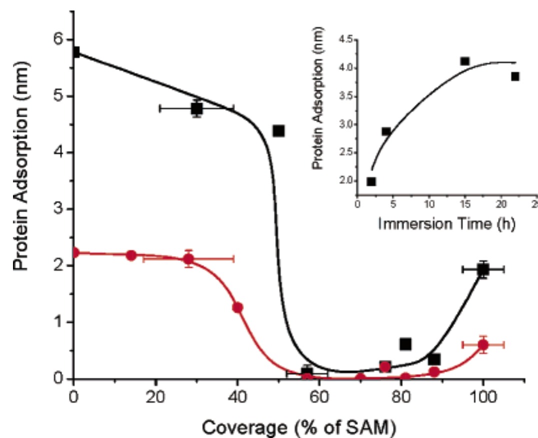

Figure 1. Surface plasmon resonance adsorption data of fibrinogen (black ■) and bovine serum albumin (red ●) as a function of coverage of ***A*** from 0% coverage to complete SAM (~3 h in 0.5 mM ***A*** in water). Inset: Fb adsorption at longer immersion times.

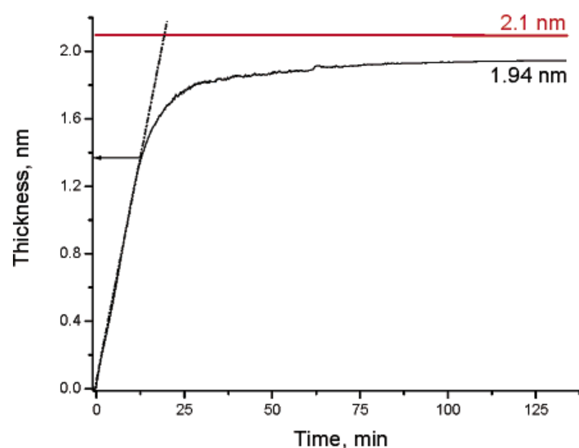

Figure 2. Kinetics of ***A*** SAM formation measured by SPR in nonflow conditions (0.005 mM ***A*** in water). The horizontal red line denotes the spectroscopic ellipsometry (SE) measured thickness of ***A*** (ref 1).

effectively screens the underlying substrate. The onset of minimal adsorption at ~60% coverage is similar to that found in an earlier system[6] and indicates an underlying substrate fully screened by loosely packed oligomers.

The kinetics of SAM formation (Figure 2) shows deviation from diffusion controlled kinetics (dashed line) at 1.4 nm, indicating that unimpeded access to the Au is no longer available to unbound oligomers. This indicates that the surface rejects small molecules in addition to the larger proteins at approximately the same range of coverage.

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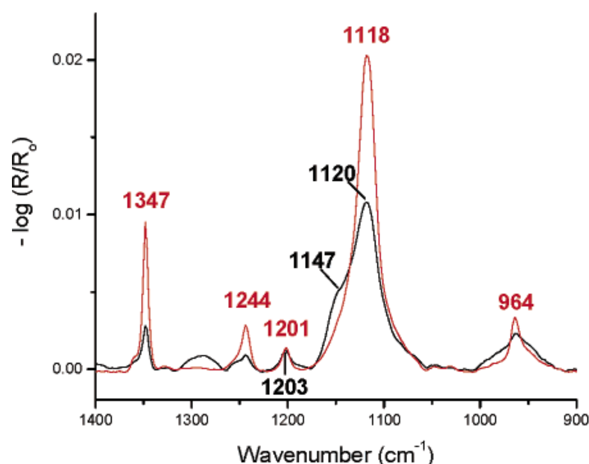

Figure 3. RAIRS spectra of ***A*** from 1400 to 900 cm^{-1} after 1 s (black spectrum: ~60% coverage) and 3 h (red spectrum: ~100% coverage) from 0.5 mM solution. SE thickness of the above films is 1.2 ± 0.1 nm and 2.1 ± 0.1 nm, respectively.

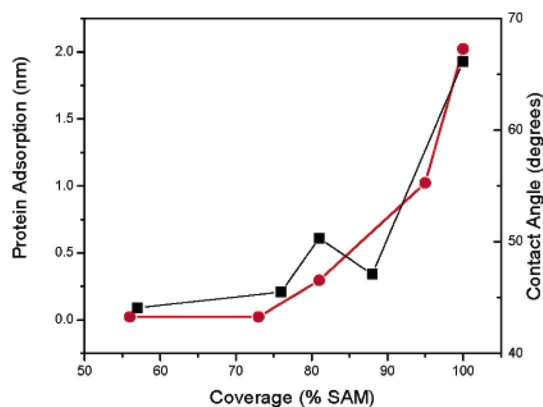

Figure 4. Plot of Fb adsorption (black ■) and CA data (red ●) from ~55 to 100% coverage of ***A***. All CA values are the average of at least four measurements taken at different locations on the film-coated substrate.

Reflection-absorption infrared spectroscopy (RAIRS) data at ~60 and 100% coverages of ***A*** are shown in Figure 3. At ~60% coverage, the bands at 1347, 1244, 1118, and 964 cm^{-1}—characteristic of conformationally restricted OEO segments in the 7/2 helical conformation oriented normal to the substrate and in contact with their neighbors[1,9]—are attenuated and similar to disordered films of analogous compounds,[4a,b,h] indicating the absence of widespread order and of conformationally restricted oligomers. These disordered oligomers are free to adopt different conformations, similar to PEOs.[10] As the coverage increases, conformational mobility decreases because of nucleation and growth of oligomer domains in the highly ordered 7/2 helical conformation.[11] The onset and range of optimal protein resistance measured here clearly define coverages that satisfy the opposing needs of screening the underlying substrate and allowing oligomer conformational mobility.

On the basis of the above general surface structure and the data in Figures 1−3, we propose the following modification to the mechanism of protein resistance as originally described in ref 10. As a protein approaches the disordered surface, in which the underlying substrate is fully screened,[6] it will directly contact a small patch of oligomers,[12] compressing and restricting their conformational mobility. In response, these oligomers will press back on the protein and press on their neighbors, which, in turn, press on their neighbors, etc., increasing the lateral length scale of the mutual interaction. This confinement of the bound oligomers increases the free energy of the system ($\Delta G_{\text{system}} = \Delta G_{\text{surface layer}} + \Delta G_{\text{protein}}$), which is released with the rejection of the protein. In this mechanism, minimal protein adsorption corresponds to the maximum ΔG_{system} for a given compression. The magnitude of the possible free energy change depends on the proportional motional restriction over the varying lateral length scale. The possible conformational restriction during compression decreases as the film becomes more spatially confined and ordered. In the limit of a fully covered surface, protein contact produces minimal or no oligomer conformational change and, concomitantly, minimally perturbs the free energy of the surface ($\Delta G_{\text{surface layer}} \approx 0$), so there is no free energy change for rejection.

This mechanism postulates the cause of protein rejection to be the increase in free energy derived from the OEO chains' loss of conformational mobility both normal and parallel to the substrate surface. Normal and parallel forces have been included in recent theoretical models.[13a] Lacking a clear understanding of the possible changes in solvation of both the protein and the oligomers upon contact (as described mechanistically[10,14] and theoretically[13,15,16]), we choose to describe the rejection in terms of the oligomers only. Our mechanism is consistent with a recent report recognizing the importance of lateral packing density to protein adsorption.[4a] It is in conflict with the model in which the cause of protein resistance arises from highly ordered, helical OEO segments[4h,17] and their putative associated waters.[18] This model predicts an increase in rejection at higher coverage, contrary to the data in Figure 1.

At the highest coverages (80−100%), an increase in protein adsorption (Figure 1) is observed. Our scaled contact angle (CA) data (Figure 4) show that from 60 to 100% coverage, Fb adsorption (black ■) increases in parallel with the increasing hydrophobicity of the surface (red ●). These results are in general agreement with that found earlier[4a] but show that the CA at the onset of the loss of protein resistance is 50° ± 5° (80% coverage), much lower than 70°. Above 80% oligomer coverage, the loss of oligomer conformational mobility occurs concurrently with greater fractional methyl group coverage and, therefore, a higher CA. (A purely methyl surface exhibits CA > 105°.) Historically, CA data have been used to probe changes in surface structure to correlate with protein adsorption;[6] however, survey studies show little or no correlation between CA data and protein adsorption/resistance.[4l,19] As a result, we can consider that surface hydrophobicity and the loss of protein resistance have the same origin in the molecular structures of the oligomer-covered substrate. Because of the lack of correlation of CA with adsorption, we suggest that the molecular structural order of the oligomers is dominant and that hydrophobicity parallels but is not the cause of the loss of protein resistance.

The dominance of oligomer structural order on protein adsorption at the higher coverages is supported by the data shown in the Figure 1 inset which shows that Fb adsorption doubles on substrates with immersion times 4−24 h (0.5 mM ***A***), longer than that required to nominally reach 100% coverage (~3 h; 0.5 mM ***A***). During these longer immersion times the SAMS continue to become more ordered. This has been shown from previous RAIRS data[1] and by the fact that for helical ω-methyl-OEO SAMs, the maximum CA values of 70° ± 2° and, therefore, the final orientation of the ω-group are only attained with longer immersion times (≥12 h).[4a,b] BSA adsorption also doubles (ref 8). The significant protein adsorption increases undoubtedly reflect the sensitivity of protein adsorption to these subtle final stages of film ordering and are not due to changes in the surface hydrophobicity which are small (2° to 4°) over these immersion times.

The ability to control substrate coverage and oligomer order, as demonstrated here, allows insights into the molecular level understanding of protein adsorption not revealed from previous PEO[4c,g]

or comparative OEO-SAM studies.[4a,h,l] First, we show that minimal protein adsorption corresponds to coverage ranges in which the onset of self-rejection is observed. Optimal coverage for protein resistance should scale with substrate occupation site densities and oligomer molecular dimensions, such as the length of the OEO segment. Indeed, previous data show this to be the case.[6] However, because a balance must be struck between adequate surface coverage and retention of OEO flexibility, we expect that as the OEO chains are lengthened, the minimum distance between the surface anchors of adjacent chains will increase. Proteins smaller than the minimum separation then can only be retarded by the OEO and eventually are able to penetrate to the binding surface, as has been discussed theoretically.[13,16] Second, our data oppose the idea that the mechanism of protein resistance of PEOs and OEO-SAMs is very different. The mechanism of protein resistance, proposed here, is similar to the excluded volume models for the longer PEOs,[10] and our protein adsorption at the high coverage ranges experimentally validates an earlier hypothesis.[15] Third, our OEO surfaces on Au are not fully resistant to Fb. (Our SPR device—sensitivity $< 5 \times 10^{-7}$ refractive index units—is more sensitive than ellipsometry and infrared methods and an order of magnitude more sensitive than commercially available SPR devices.[4k,l]) The Fb's measurable adsorption at its minimum may be due to its high surface activity or to small areas of surface coverage heterogeneity.

The relatively low surface coverage for minimal protein adsorption suggests that it should be possible to retain the general rejection properties of the surface in the presence of specific sites anchored among the rejecting oligomer matrix. In that way, nonspecific adsorption might be minimized in a straightforward and general manner for analytical techniques using, for example, microcantilevers, SPR arrays, and fluorescence detection. Although the sensitive SPR metrology indicates that complete repulsion of all proteins will be difficult, the direction of molecular design toward limiting adsorption of proteins appears clear.

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Note Added in Proof: After the acceptance of this paper, similar Fb absorption as a function of the film coverage appeared in a related paper, Zheng, J.; Li, L.; Chen, S.; Jiang, S. *Langmuir* **2004**, *20*, 8931−8938.

Supporting Information Available: Experimental details of sample preparation, SPR, RAIRS, and CA measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) Film coverage was controlled by immersion time in either 0.5 mM or 0.005 mM thiol solutions for times varying from 1 s to 1 day. Coverage of ***A*** [percent (%) of SAM] = spectroscopic ellipsometry (SE) film thickness (d)/2.1 nm (ref 1) × 100. Protein adsorption measured by surface plasmon resonance (SPR) during a 15 min adsorption time and 15 min washing time using a flow cell (volume = 2 μL) (see Supporting Information for additional SPR and experimental details). Error bars on selected data points represent the spread of values over replicate experiments and are not standard deviations. The error bar along the x-axis at 100% coverage ($d = 2.1$ nm after ~3 h in 0.5 mM solution) corresponds to an SE error of ±0.1 nm. For clarity, error bars are not drawn for all points. Larger error bars at <50% coverage are due to the rapid initial kinetics, which makes the lower coverages difficult to control. Inset shows fibrinogen adsorption versus immersion time from 4 to 24 h. BSA exhibits a similar increase in adsorption (8 nm (3 h) to 14 nm (24 h); data not shown to facilitate figure inset size). The curves in the figure and the figure inset are drawn only as an aid to the eye and are not fitted to the data.

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(12) Prior to contact with the surface, the protein is in its conformational equilibrium state. Our mechanism assumes no denaturation of the protein and that the protein-surface contact and subsequent compression result only in loss of conformational mobility of those protein moieties and atoms in direct contact with the OEO chain. This assumption is based on previous reports suggesting that reversibly adsorbed proteins on PEO/OEO chains surfaces do not denature (Yang, Z.; Galloway, J. A.; Yu, H. *Langmuir* **1999**, *15*, 8405−8411).

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(14) A successful explanation of the protein-surface interaction/adsorption has not emerged from studies with polymers because (a) it has been difficult to define the region of the surface/polymer chain that interacts with the protein[4k] and (b) the magnitude and sign of water's free energy components (enthalpy and entropy) have not been determined experimentally. The central question of PEO/protein resistance, "Is it the water or is it the PEO (PEG)?",[5] remains to be answered.

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