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Self-assembled monolayers (SAMs) of ω-functionalized long-chain alkanethiolates on gold films are excellent model systems with which to study the interactions of proteins with organic surfaces. Monolayers containing mixtures of hydrophobic (methyl-terminated) and hydrophilic [hydroxyl-, maltose-, and hexa(ethylene glycol)–terminated] alkanethiols can be tailored to select specific degrees of adsorption: the amount of protein adsorbed varies monotonically with the composition of the monolayer. The hexa(ethylene glycol)–terminated SAMs are the most effective in resisting protein adsorption. The ability to create interfaces with similar structures and well-defined compositions should make it possible to test hypotheses concerning protein adsorption.

UNDERSTANDING THE MECHANISM of protein adsorption at surfaces (*1, 2*) is an important element of research in protein chromatography (*3*), clinical diagnostics (*4*), biomedical materials (*5*), and cellular adhesion (*6*). No system is available that permits the structure and properties of the interface to be controlled in detail sufficient for the investigation of hypotheses concerning protein adsorption at the molecular level. We report a study of protein adsorption at interfaces between SAMs and aqueous buffer solutions. The results indicate that the organic interfaces prepared by the self-assembly of long-chain

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alkanethiols onto gold are suitable model systems for the study of protein adsorption at interfaces.

We prepared the SAMs by the chemisorption of alkanethiols from 0.25 mM solutions in ethanol or methanol onto thin (200 ± 20 nm) gold films supported on silicon wafers (*7*). In SAMs derived from ω-substituted alkane-1-thiols [$R(CH_2)_nSH$, $n \geq 10$, where R is a small functional group], the molecules pack densely on the gold surface in a predominantly *trans*-extended conformation, with the axes of the polymethylene chains at an average cant of ≈30° from the surface normal (*8*). The internal domains of these monolayers are pseudo-crystalline; the chain termini are less ordered (*9*). One can control the interfacial properties of these monolayers by changing the tail group, R. SAMs comprising mixtures of two or more components can be prepared by adsorption from solutions containing mixtures of these components: the components of such "mixed SAMs" are not segregated into macroscopic islands (*10*). This combination of a uniform substrate and the ability to control the composition—and to some degree the structure—of the interface at the molecular scale have made SAMs excellent systems with which to study the physical-organic chemistry of organic interfaces.

We used five alkanethiols, $R(CH_2)_{10}SH$: R = $HOCH_2$–, **1** (*10*); R = Glc–α(1,4)–Glc–β(1)–O–, Glc = glucose, **2** (*11*); R = $HO(CH_2CH_2O)_6CH_2$–, **3** (*12*); R = H–, **4** (*13*); and R = CH_3–, **5** (*10*). The SAMs derived from **1**, **2**, and **3** model three materials that resist the adsorption of proteins: hydroxylated polymers such as poly(hydroxyethyl methacrylate) (*14*), agarose (*15*), and polymers containing poly(ethylene oxide) (*16*), respectively. For each model system, we prepared a series of mixed SAMs (*10*) from a hydrophilic alkanethiol (*17*) (**1**, **2**, or **3**) and a hydrophobic alkanethiol (**5** with **1** and **3**; **4** with **2**). The structures of these mixed SAMs are shown schematically in Fig. 1. We calculated the mole fraction of hydrophilic alkanethiolate in each mixed SAM, χ, by normalizing the intensity of the O(1*s*) x-ray photoelectron peak obtained from the mixed SAM to that of a SAM containing only the hydrophilic component and by assuming that this normalized intensity is directly proportional to the number of oxygen atoms in the SAM. In the case of SAMs formed from mixtures of **3** and **5**, the intensity of the O(1*s*) peak is linearly proportional to the ellipsometric thickness of the SAM (*12*); this observation is strong evidence that our assumption is valid for the other two cases.

We examined the adsorption of five well-characterized proteins, ribonuclease A (RNase A), pyruvate kinase, fibrinogen, lysozyme, and chymotrypsinogen (*18*), on

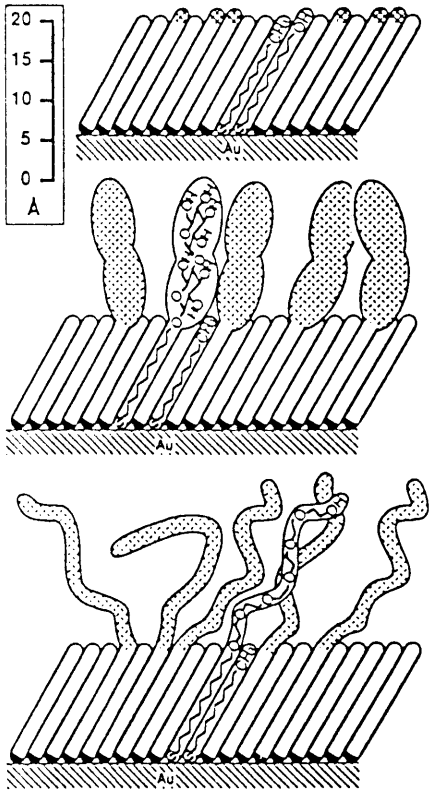

Fig. 1. Schematic representation of the structures of mixed monolayers of $HO(CH_2)_{11}SH$ and $CH_3(CH_2)_{10}SH$ (top), of Glc–α(1,4)–Glc–β(1)–$O(CH_2)_{10}SH$ and $CH_3(CH_2)_9SH$ (middle), and of $HO(CH_2CH_2O)_6(CH_2)_{11}SH$ and $CH_3(CH_2)_{10}SH$ (bottom). The ethylene glycol chains in the lower structure are flexible but probably prefer a helical conformation when in contact with water (*32*). The areas of the hatched regions are roughly proportional to the cross-sectional areas of the polar tail groups. The scale bar is approximate and applies to all three illustrations.

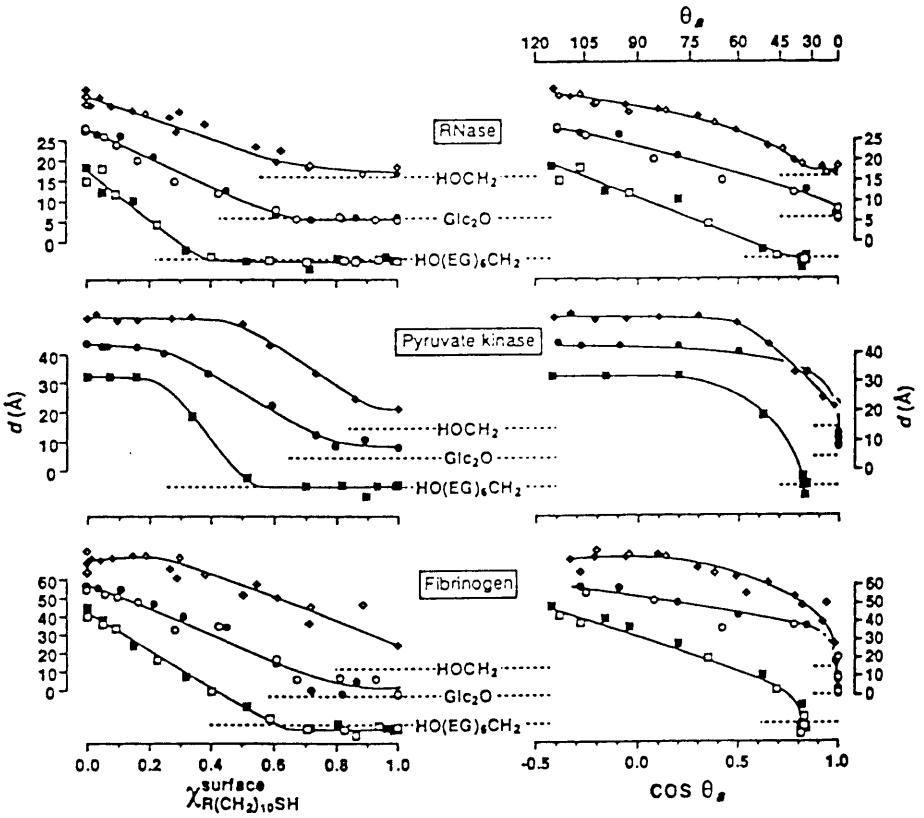

Fig. 2. Adsorption of proteins to mixed SAMs varies monotonically with the composition of the SAM. The thickness, *d*, of the adsorbed film of RNase A (top), pyruvate kinase (middle), and fibrinogen (bottom) on mixed SAMs containing $HO(CH_2)_{11}SH$ and $CH_3(CH_2)_{10}SH$ (diamonds, R = $HOCH_2$), Glc–α(1,4)–Glc–β(1)–$O(CH_2)_{10}SH$ and $CH_3(CH_2)_9SH$ (circles, R = Glc_2O, Glc = glucose), or $HO(CH_2CH_2O)_6(CH_2)_{11}SH$ and $CH_3(CH_2)_{10}SH$ (squares, R = $HO(EG)_6CH_2$, EG = ethylene glycol, $-OCH_2CH_2-$) is plotted as a function of the composition (left) and wettability (right) of the SAM. The filled and hollow symbols represent data derived from two independent experiments. The values of *d* were determined by ellipsometry and represent the average of three measurements made at different positions on a single sample. The standard deviations of the observed values of *d* are no larger than the symbols representing the data. The values of χ, the mole fraction of $R(CH_2)_{10}S$ on the surface, were measured before protein adsorption. Each value is the intensity of the O(1*s*) x-ray photoelectron peak of the SAM, normalized to $\chi^{surface}_{R(CH_2)_{10}SH} = 1$ for a SAM containing only $R(CH_2)_{10}S$. The values of θ_a are the maximum advancing contact angles of water (*10*, *30*) on the SAM before protein adsorption. The data are offset vertically for clarity; the dashed lines show the location of $d = 0$ Å (no adsorbed protein) for each series of mixed SAMs. The solid curves organize the data visually but do not represent an attempt to model the data.

these mixed SAMs (*19*). The results for RNase, fibrinogen, and pyruvate kinase are summarized in Fig. 2 (*20*). We measured the thickness, *d*, of the adsorbed protein film on each SAM by ellipsometry, treating the film as a homogeneous layer of uniform thickness with a refractive index of 1.45 (*21*). Any difference between the real refractive index of the adsorbed protein and 1.45 results in a systematic error in the calculated thickness but does not change the relative values or the conclusions. The calculated values of thickness are accurate to within ≈25% (*22*).

The data in Fig. 2 point to several conclusions. (i) The system comprising proteins adsorbed on SAMs of alkanethiolates on gold generates reproducible data concerning the extent of protein adsorption. The standard deviations of measurements of *d* taken on several independently prepared samples are within the range of 1 to 4 Å, near the 1 to 2 Å limit of ellipsometry. The N(1*s*) photoelectron signals from adsorbed films of chymotrypsinogen correlate well with the values of *d* determined by ellipsometry (*23*). This observation suggests that variability in the refractive indices of the adsorbed proteins, which would cause nonuniform errors in the calculation of *d*, are not important in this system. (ii) SAMs containing high concentrations of 3 prevent adsorption of the five proteins examined, including fibrinogen. SAMs containing high concentrations of 2 nearly eliminate the adsorption of fibrinogen and pyruvate kinase and prevent adsorption of the other proteins examined. (iii) The observed value of the thickness of the adsorbed protein layer on the hydrophobic, methyl-terminated surface (4 or 5; $\chi = 0$ in Fig. 2) corresponds approximately to that expected for a monolayer of native protein (*24–27*). Consistent with others' observations (*28*), multilayers of protein appear not to form. (iv) There is only a general correlation between the interfacial free energy of the SAM [as measured by cos θ_a, the cosine of the maximum advancing contact angle of water on the SAM (*29*)] and *d*. Although within a set of SAMs derived from the same components more hydrophobic surfaces adsorb greater quantities of protein, the thickness of the adsorbed protein film at any given interfacial free energy differs for each hydrophilic component. For example, when $\theta_a = 34°$, proteins do not adsorb to SAMs containing $HO(CH_2CH_2O)_6$– groups but do adsorb to SAMs containing Glc–α(1,4)–Glc–β(1)–O– or $HOCH_2$– groups. The same effect is observed when the values of *d* for different proteins on SAMs of equal receding contact angle, θ_r, are compared.

From this limited set of data, it is premature to infer mechanisms of adsorption of proteins at interfaces. The observation that adsorption increases as hydrophobicity increases (for a given set of components) is expected and consistent with the idea that hydrophobic interactions are important in protein adsorption. The observation that $HO(CH_2CH_2O)_6$– groups are especially effective in preventing protein adsorption suggests that steric stabilization—a phenomenon commonly used to explain the stability of colloidal suspensions in the presence of polymers (*30*)—is important in preventing protein adsorption (*31*). The extent to which entropic repulsion (*30*) contributes to the steric stabilization is not clear and may vary with χ: the steric requirements of packing in the SAM should reduce the conformational entropy of the $HO(CH_2CH_2O)_6$– groups as their concentration in the SAM increases. We believe that SAMs are the best defined systems now available for examining the interactions of proteins and surfaces and that they will provide the means to test many of the current hypotheses regarding the mechanisms of these interactions.

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10. We use "macroscopic" to mean sufficiently large, perhaps more than 10 nm in diameter, that the properties of the monolayer are determined by molecules of each component in environments indistinguishable from the environment in a pure monolayer of that component. A number of experimental results suggest that macroscopic islands do not form [C. D. Bain, J. Evall, G. M. Whitesides, *J. Am. Chem. Soc.* 111, 7155 (1989); C. D. Bain and G. M. Whitesides, *ibid.*, p. 7164]. The advancing contact angles of water on SAMs containing mixtures of long and short hydroxyl-terminated chains [$HO(CH_2)_{10}SH$ and $HO(CH_2)_{11}SH$, respectively] reach a pronounced maximum ($\theta_{adv} = 40°$) near $\chi^{surface}_{HO(CH_2)_{11}SH} = 0.5$ [C. D. Bain and G. M. Whitesides, *Science* 240, 62 (1988)]. Because pure SAMs of either hydroxyl-terminated chain are wet by water ($\theta_{adv} < 15°$), this increase in the contact angle is inconsistent with the presence of macroscopic islands, which would also wet, and supports the hypothesis that the chains in two-component SAMs are well mixed at length scales near molecular dimensions.
11. β-(9-Decenyl)maltoside heptaacetate was prepared from commercial maltose octaacetate and 9-decen-1-ol [P. Rosevar, T. VanAken, J. Baxter, S. Ferguson-Miller, *Biochemistry* 19, 4108 (1980)]. β-(10-Mercaptodecyl)maltoside octaacetate was prepared by the photochemical addition of thiolacetic acid to the olefin (*12*). We achieved deacetylation by allowing the acetate to stand in a 2:1:1 mixture of methanol, water, and triethylamine. The products were purified by flash chromatography (silica gel, methanol-chloroform) after each step. The overall yield was ≈60% from maltose octaacetate.
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17. We use the term "hydrophilic/hydrophobic alkanethiol" as a convenient shorthand for "alkanethiol that forms a hydrophilic/hydrophobic interface when adsorbed on gold."
18. α-Chymotrypsinogen A (type II from bovine pancreas, Sigma), lysozyme (E.C. 3.2.1.17, grade III from chicken egg white, Sigma), RNase A (E.C. 3.1.27.5, type III-A from bovine pancreas, Sigma), pyruvate kinase (E.C. 2.7.1.40, type PK-3 from rabbit muscle, Biozyme), and fibrinogen (fraction I from human plasma, Sigma) were used as received.
19. The following protocol was used in these experiments: the SAM (7) was rinsed with ethanol and blown dry with nitrogen. It was immersed in an unstirred solution of protein (1 mg/ml in 10 mM aqueous phosphate buffer, pH 7.5, 23°C, 1 hour) under air. The SAM was then removed from solution, rinsed six times with 2-ml aliquots of distilled, deionized water applied as a stream from a Pasteur pipette, and blown dry with a stream of nitrogen passed through another Pasteur pipette at a pressure of 10 psi (70 kPa) above atmospheric pressure. Using this protocol, we found that our results were insensitive to small variations in the rinsing procedure and in the time of immersion.
20. Our choice of proteins for Fig. 2 was made to illustrate representative adsorption isotherms. A suitably scaled plot of the data obtained from lysozyme would superimpose upon the data from fibrinogen. Likewise, the data for chymotrypsinogen on the series prepared from 2 and from 3 are nearly indistinguishable from the data for RNase; chymotrypsinogen and pyruvate kinase show behavior nearly indistinguishable from each other on mixed SAMs prepared from 1.
21. The refractive index of an adsorbed protein film is effectively constant once the adsorption plateau is reached [P. A. Cuypers *et al.*, *J. Biol. Chem.* 258, 2426 (1983)]. We use $n = 1.45$ because it is near the average of the reported values of *n* for a number of proteins [range, 1.34 to 1.71 (*33*)] and it corresponds to the value of *n* used in earlier studies of SAMs (*7*).
22. Choosing $n = 1.33$ or $n = 1.71$ causes a 25% increase or decrease, respectively, in the calculated values of *d*, relative to the values obtained for $n = 1.45$.
23. The photoelectron signal observed from an atom in a thin film is proportional to $1 - e^{(-d/\lambda \sin\theta)}$, where *d* is the thickness of the film, λ is the escape depth of the photoelectron through the film, and sin θ is the angle between the surface normal and the analyzer [C. D. Bain and G. M. Whitesides, *J. Phys. Chem.* 93, 1670 (1989)]. The value of λ for protein films is

not known, determination of an accurate value of λ requires continuous films of different thicknesses. This criterion is not met by the probably discontinuous films formed in this study. Thus, only a qualitative comparison of results from ellipsometry and x-ray photoelectron spectroscopy (XPS) is possible at this time. Using the values of d obtained by ellipsometry and the intensities of the N(1s) peaks, we found that least-squares fitting to the above equation yielded a fit with $r^2 = 0.98$, $n = 11$.

24. The averages and standard deviations of the observed thicknesses of adsorbed protein films on methyl-terminated SAMs were 21 ± 1 Å (RNase), 53 ± 3 Å (fibrinogen), and 38 ± 1 Å (pyruvate kinase). Each value represents an average of 18 measurements. Three measurements were made from different positions on each of six independently prepared samples to derive these values.
25. RNase A (molecular weight ≈13,700) forms monoclinic crystals with one molecule per unit cell. The parameters of the unit cell are $a = 30$ Å, $b = 38$ Å, $c = 53$ Å, $\beta = 106°$ [A. Wlodawer, L. A. Svensson, L. Sjoelin, G. L. Gilliland, *Biochemistry* **27**, 2705 (1988)].
26. Pyruvate kinase is a tetrameric enzyme of identical subunits; the dimensions of the tetrameric molecule are 75, 95, and 125 Å. The molecular weight of each subunit is 54,600 in yeast and 57,900 in cat muscle. (In this study we used enzyme isolated from rabbit muscle.) The shape of each subunit is approximated by an ellipsoid 75 Å long with a maximum transverse diameter of 45 Å [H. Muirhead, *Biol. Macromol. Assem.* **3**, 143 (1987)]. We do not know whether the molecule retains its tetrameric structure upon adsorption.
27. Human fibrinogen (molecular weight ≈340,000) is a structurally complex protein containing several domains connected by more flexible segments [J. A. Shafer and D. L. Higgins, *Crit. Rev. Clin. Lab. Sci.* 26, 1 (1988)]. It is, therefore, difficult to provide specific molecular parameters for comparison with our experimental results.
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30. Steric stabilization of hydrophobic colloids in aqueous solution occurs when a hydrophilic polymer is adsorbed at the colloid-water interface. This polymer prevents flocculation of two colloid particles in two ways. First, approach of the particles to a distance such that the strength of the attractive hydrophobic interaction rises above kT (where k is the Boltzmann constant and T is temperature) is inhibited enthalpically by changes in the configuration of the polymer and perhaps by its desolvation as it is compressed. Second, for coiled polymers, loss of conformational entropy due to the approach of the two particles to one another creates a repulsive force that helps to oppose the attractive hydrophobic interaction. We refer to the second effect as "entropic repulsion," consistent with D. H. Everett [*Basic Principles of Colloid Science* (Royal Society of Chemistry, London, 1988), pp. 45–50].
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