

Preparation of Mixed Self-Assembled Monolayers (SAMs) That Resist Adsorption of Proteins Using the Reaction of Amines with a SAM That Presents Interchain Carboxylic Anhydride Groups

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This paper describes a procedure for preparing mixed self-assembled monolayers (mixed SAMs) on gold that resist the nonspecific adsorption of proteins from solution. This method was tested using α -amino derivatives of ω -hydroxy- and ω -methoxy-oligo(ethylene glycols): $\text{H}_2\text{N}(\text{CH}_2\text{CH}_2\text{O})_n\text{CH}_3$ and $\text{H}_2\text{N}(\text{CH}_2\text{CH}_2\text{O})_n\text{H}$ ($n = 3, 6$). Mixed SAMs were prepared by allowing these amines to react with a SAM presenting interchain carboxylic anhydride groups. The resistance of the resulting surfaces to adsorption of several proteins—carbonic anhydrase (EC 4.2.1.1), ribonuclease A (EC 3.1.27.5), lysozyme (EC 3.2.1.17), and fibrinogen—was examined using surface plasmon resonance (SPR) spectroscopy. These mixed SAMs resist the nonspecific adsorption of proteins approximately as effectively as single-component SAMs prepared using the conventional method involving chemisorption of oligo(ethylene glycol)-terminated alkanethiols on gold. Characterization of the mixed SAM that presents a 1:1 mixture of $-\text{OCNH}(\text{CH}_2\text{CH}_2\text{O})_6\text{CH}_3$ and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups by polarized infrared external reflectance spectroscopy indicates that the ethylene glycol units are in an amorphous conformation. A model surface for use in studies of biospecific adsorption was synthesized by reacting the anhydride groups with a mixture of $\text{H}_2\text{N}(\text{CH}_2\text{CH}_2\text{O})_6\text{H}$ and $\text{H}_2\text{N}(\text{CH}_2\text{CH}_2\text{O})_6\text{CH}_2\text{CONH}(\text{CH}_2)_6\text{NHCOC}_6\text{H}_4\text{SO}_2\text{NH}_2$; the resulting system was examined for its ability to bind bovine carbonic anhydrase by SPR. The values of the relevant constants were $k_{\text{off}} = 0.0054 \text{ s}^{-1}$, $k_{\text{on}} = 13 000 \text{ M}^{-1} \text{ s}^{-1}$, and $K_d = 0.42 \mu\text{M}$. These values agree with values obtained by other means. The reaction of amines with SAMs that present interchain carboxylic anhydrides provides an experimentally simple route to the formation of mixed SAMs that resist the nonspecific adsorption of proteins or that adsorb a protein of interest biospecifically.

Introduction

This paper describes an experimentally convenient method to synthesize mixed self-assembled monolayers (mixed SAMs) of alkanethiolates on gold that present functional groups that either (i) resist nonspecific adsorption of proteins to their surfaces or (ii) promote biospecific adsorption of specific proteins. SAMs have been widely used in studying the biocompatibility of materials,¹ in the development of biosensors, and in cell biology.^{2–9} We chose oligo(ethylene glycols) (EG_n) as the test functional groups to incorporate into SAMs to prevent nonspecific adsorption of proteins, because these groups have been used successfully in other studies of SAMs that resist protein adsorption^{10–12} and because poly(ethylene glycol) (PEG)

has been recognized for its ability to render surfaces biocompatible, and it is often used for that purpose.^{13–19}

In principle, there are two strategies to prepare SAMs that present EG_n groups on their surfaces: (i) to prepare EG_n -terminated alkanethiols and adsorb them on gold;¹⁰ (ii) to introduce EG_n groups into the SAMs after their assembly. The first method has been extensively exploited.^{2,10–12,20} We have described the second procedure as a route to mixed SAMs but have not applied it to the preparation of surfaces that are inert or resistant to the adsorption of proteins (surfaces that, for brevity, we call “inert”).²¹ These two methods provide complementary routes to inert surfaces.

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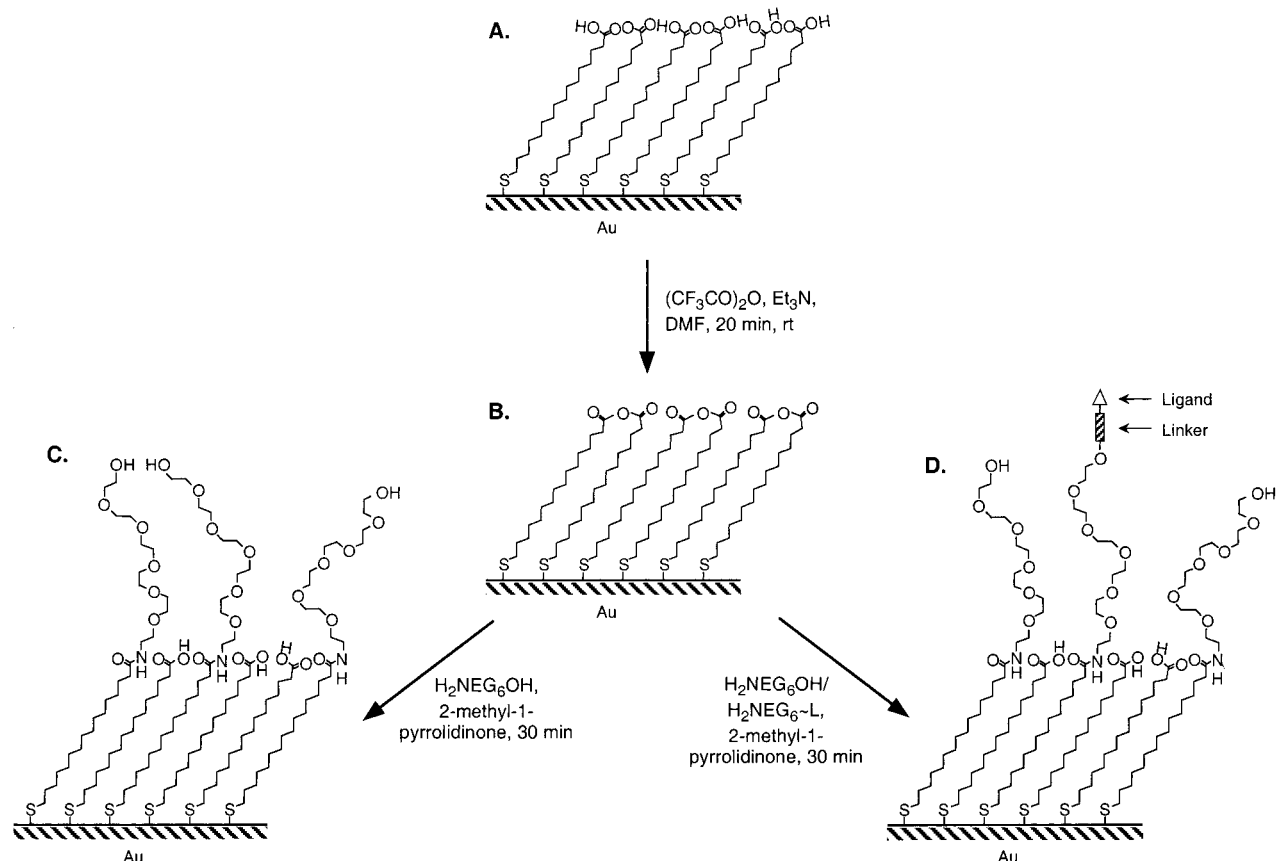


Figure 1. Schematic representation of the preparation of SAMs presenting EG_n and $\text{EG}_n\sim\text{L}$ (where \sim represents a linker and L is a ligand) by the anhydride method. In this method, a reactive SAM that is terminated in interchain carboxylic anhydride groups is reacted with amines (see text for details). The scheme suggests the composition of the SAM but not the conformation of the groups in it.

Inert surfaces have a range of applications in biosurface chemistry: in sensors,^{22,23} to pattern cells,^{2,3,24} and as coatings for biomaterials.^{25,26} SAMs that present $-\text{EG}_n\text{OH}$ and $-\text{EG}_n\text{OCH}_3$ groups ($n = 6$) provide the most inert interfaces that are now available. The mechanism by which SAMs terminating in EG_n groups resist the nonspecific adsorption of protein has not been entirely established. Single-component SAMs that present EG_n groups are not unique in their ability to resist adsorption of proteins: mixed SAMs containing as little as 50% of $\text{HS}(\text{CH}_2)_{11}\text{-EG}_6\text{OH}$ (mixed with $\text{HS}(\text{CH}_2)_{11}\text{CH}_3$) show good resistance to the adsorption of proteins, and SAMs presenting $-\text{CH}_2\text{-CH}_2\text{CH}_2\text{S}(=\text{O})-$ groups are also effective in resisting protein adsorption.²⁷ Theoretical calculations performed by Andrade and DeGennes suggested that the protein resistance of grafted poly(ethylene glycol) (PEG, $n = 80-120$) increases with increasing chain length and surface density of the polymer.²⁸ The high resistance to protein

adsorption of SAMs that present short oligo(ethylene glycol) groups ($n \leq 6$) suggests that the mechanism of protein resistance may differ between SAMs and grafted PEG polymers ($n = 80-120$).²⁸ Grunze et al. have correlated resistance to the adsorption of proteins for the EG_n -terminated SAMs in contact with water with IR signatures of the EG_n groups of these SAMs in air and concluded that densely packed SAMs that present EG_n groups in the fully extended, all trans conformation adsorb proteins more strongly than do less densely packed SAMs with the same terminal groups in amorphous or helical conformations. They suggested that resistance to protein adsorption is not due to the molecular compositions or structures per se, but rather the ability of these molecules to organize (or, to accommodate organized) water on their surface.²⁹

These studies have all used SAMs prepared by adsorbing fully functionalized alkanethiols onto a surface. These preparations are experimentally inconvenient: each thiol requires independent synthesis and characterization, and these syntheses are usually technically complicated. Here, we demonstrate a straightforward method to prepare mixed SAMs presenting EG_n groups and carboxylic acid moieties that are resistant to the nonspecific adsorption of proteins. This method comprises two steps:²¹ (i) formation of a reactive SAM that presents interchain carboxylic anhydride groups (Figure 1A,B);²¹ and (ii) reaction of the anhydride groups with appropriate reactive groups ($\text{H}_2\text{N}(\text{CH}_2\text{CH}_2\text{O})_n\text{R}$; $\text{R} = \text{H}$ or CH_3 and $\text{H}_2\text{N}(\text{CH}_2\text{CH}_2\text{O})_n\text{-L}$; $\text{L} = \text{ligand}$) (Figure 1B–D).²¹ In this paper, unless we specify otherwise, we use the term “mixed SAM” to refer

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to SAMs formed by the reaction of an amine with a SAM that presents interchain anhydrides.

We wished to examine these mixed SAMs for four reasons: (i) The ease with which they can be prepared makes them good alternates to SAMs prepared from functionalized thiols. (ii) Since it is easy to introduce a large number of functional groups into these mixed SAMs, they are attractive as systems with which to screen for functional properties. (iii) The convenience with which these SAMs can be prepared makes them attractive as substrates for experiments involving *biospecific* adsorption, and we wished to illustrate the procedures involved in this type of study (Figure 1D). (iv) Since alkanethiolate groups in the SAMs presenting both EG_n and EG_n-L groups are necessarily mixed 1:1 with alkanethiolates terminating in CO_2H/CO_2^- groups, they offered the chance to determine the ability of a new type of mixed SAM (albeit one terminating in EG_n groups) to resist protein adsorption and, thus, to clarify the molecular mechanisms underlying resistance to protein adsorption.

Here, we have compared the resistance to protein adsorption of mixed SAMs that present EG_n moieties prepared by the anhydride procedure to those of single-component SAMs that present EG_n moieties, as a preliminary step in a more extensive study of the relationship between the structure of SAMs and resistance to protein adsorption.³⁰ In using the anhydride method to make mixed SAMs, one gains enormously in convenience: these mixed SAMs can be prepared from commercially available materials (i.e., $H_2N(CH_2CH_2O)_3H$ and $HS(CH_2)_{15}CO_2H$) using straightforward procedures. This paper compares the ability of these mixed SAMs and single-component SAMs to resist the adsorption of proteins.

We studied the wettability and the adsorption of proteins to mixed SAMs that present different lengths of EG_n ($n = 1-6$) to measure the effect of the CO_2H/CO_2^- groups on the properties of the surfaces. We expected mixed SAMs presenting longer oligomers of EG_n groups ($n = 3, 6$) to screen the effects of the underlying CO_2H/CO_2^- groups and to lead to less adsorptive surfaces than mixed SAMs that present short oligomers of EG_n groups ($n = 1$). We used X-ray photoelectron spectroscopy (XPS) and polarized infrared external reflectance spectroscopy (PIERS) to characterize these mixed SAMs and surface plasmon resonance spectroscopy (SPR) to examine the nonspecific adsorption of proteins to their surfaces.^{20,31}

The most important conclusions from this work are that the inertness of these mixed SAMs is comparable to that of single-component SAMs and that these mixed SAMs can be used as the basis for procedures for screening organic functional groups for their ability to resist the adsorption of proteins from solution.

Results and Discussion

Preparation of the Reactive SAMs. SAMs of alkanethiolates were prepared by immersing the gold substrates overnight in a 2 mM ethanolic solution of a thiol. The anhydride-terminated SAMs were prepared according to the procedure reported previously:²¹ SAMs of 16-mercaptohexadecanoic acid³² were formed overnight in a 2 mM solution of ethanol/water/acetic acid (85/10/5, v/v/v), rinsed with ethanol, dried under a stream of nitrogen, and immersed in a freshly prepared solution of

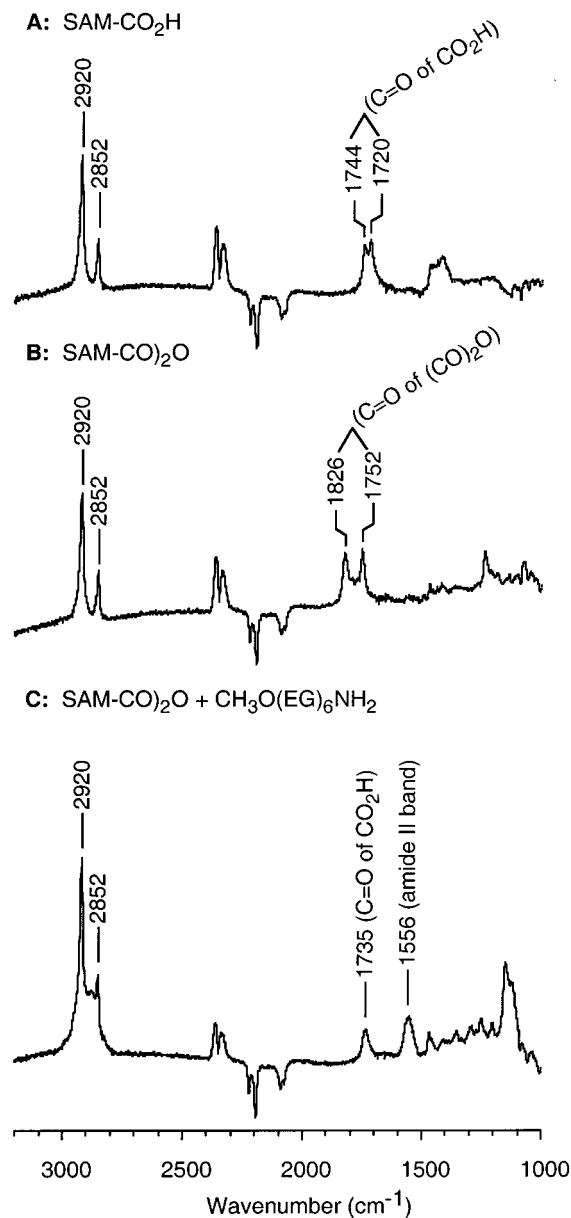


Figure 2. PIERS spectra of (A) the SAM of 16-mercaptohexadecanoic acid ($HS(CH_2)_{15}CO_2H$) on gold, (B) the SAM presenting the corresponding interchain carboxylic anhydride groups, and (C) the SAM presenting a $\sim 1:1$ mixture of CH_3OEG_6NHCO- and carboxylic acid groups. Two $C=O$ stretching bands are observed for the carboxylic acid in (A); the band at 1744 cm^{-1} arises from the free carboxylic acid while the band at 1720 cm^{-1} arises from the hydrogen bonded carboxylic acid.³⁰

0.1 M trifluoroacetic anhydride and 0.2 M triethylamine in anhydrous *N,N*-dimethylformamide for 20 min at room temperature. The substrates were then removed from the anhydride solution, rinsed with methylene chloride, and dried in a stream of nitrogen.

Attaching EG_n Groups to the Reactive SAMs. We incorporated H_2NEG_nOR ($n = 3, 6$; $R = H$ or CH_3)^{33,34} into SAMs by immersing the anhydride-terminated SAM in a 10 mM solution of amines in anhydrous 2-methyl-1-pyrrolidinone (NMP). PIERS was the most useful spectroscopic tool for studying the chemical transformations on the surface. Figure 2A shows the PIERS spectrum of the

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SAM of 16-mercaptohexadecanoic acid on gold. After treatment of the SAM of carboxylic acid with trifluoroacetic anhydride, the C=O stretches for the SAM of carboxylic acids at 1744 and 1720 cm^{-1} were converted into two bands at 1826 and 1752 cm^{-1} that are characteristic of a carboxylic anhydride (Figure 2B).^{35,36} After reaction of these anhydride groups with $\text{CH}_3(\text{OCH}_2\text{CH}_2)_6\text{NH}_2$, two absorption bands appeared at 1735 cm^{-1} (which we assigned to a C=O stretch of a carboxylic acid) and at 1556 cm^{-1} (which we assign to the amide II band) (Figure 2C).³⁷ The observation that each of these PIERS spectra is relatively simple suggests that conversions of the terminal functional groups proceed in good yields.²¹

We have also used XPS and contact angles of water to characterize these mixed SAMs. XPS spectra of the mixed SAMs showed a N(1s) photoelectron peak at 399.9 eV; this observation indicates the presence of amide bonds in the SAM. The advancing contact angle of distilled, deionized water (pH = 7) on the mixed SAM was $\theta_{\text{aH}_2\text{O}} = 46^\circ \pm 3^\circ$ and the receding contact angle was $\theta_{\text{rH}_2\text{O}} = 37^\circ \pm 3^\circ$; single-component SAMs made from $\text{HS}(\text{CH}_2)_{11}\text{EG}_6\text{OCH}_3$ had $\theta_{\text{aH}_2\text{O}} = 45^\circ \pm 3^\circ$ and $\theta_{\text{rH}_2\text{O}} = 37^\circ \pm 3^\circ$. The fact that these contact angles are experimentally indistinguishable suggests that the EG_6OCH_3 groups effectively screen the $\text{CO}_2\text{H}/\text{CO}_2^-$ groups from contact with water. The presence of the carbonyl stretch of the amide II band at 1556 cm^{-1} in PIERS and the N(1s) photoelectron peak at 399.9 eV in XPS is compatible with the hypothesis that $\text{H}_2\text{N}(\text{CH}_2\text{CH}_2\text{O})_6\text{CH}_3$ groups were covalently attached to the SAM by amide bonds.

The Ionization State of the Carboxylic Acid Groups. A question in mixed SAMs containing carboxylic acid groups that are in contact with water is the state of ionization of these groups. We wished to understand the influence of the ionization on the properties of the SAM. Mixed SAMs that present a 1:1 mixture of $-\text{CON}(\text{CH}_2\text{CH}_2\text{O})_n\text{R}$ (R = H or CH_3 ; $n = 1, 3, \text{ or } 6$) and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups have enough free volume to allow the incorporation of water. The dielectric constant of a mixture of oligo-(ethylene glycol) and water should have a value somewhere between that of neat tri(ethylene glycol) ($\epsilon = 23.7$)³⁸ and that of a 1:1 v/v mixture of ethylene glycol and water ($\epsilon = 65$).³⁹ The length of the EG_n groups presented on the mixed SAMs and the packing of the EG_n groups, largely determines the value of the dielectric constant in the vicinity of the $\text{CO}_2\text{H}/\text{CO}_2^-$ groups. The extent of ionization of the CO_2H to CO_2^- in the mixed SAM in turn depends on the value of the dielectric constant at this interface (it is more difficult, energetically, to ionize the carboxyl acid groups as oligo(ethylene glycol) displaces water because the dielectric constant of the interface decreases).

We have not made spectroscopic measurements on the state of ionization of the carboxylic acid groups of mixed SAMs in contact with water and do not know how to do so. Measurements of the wettability as a function of pH on systems of mixed SAMs presenting EG_nOH groups of different oligomer lengths provide limited but useful

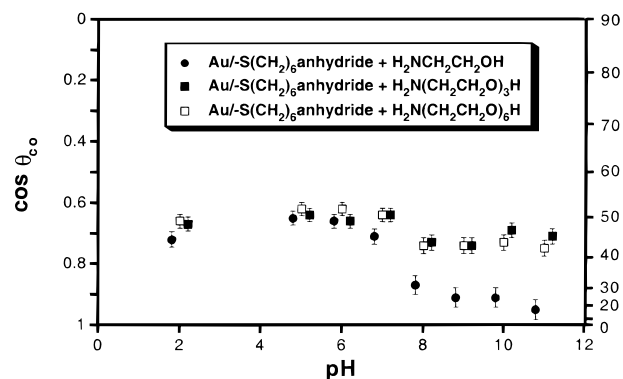


Figure 3. Graph of the cosine of the sessile contact angle of the mixed SAMs presenting a 1:1 mixture of $-\text{CON}(\text{CH}_2\text{CH}_2\text{O})_n\text{H}$ ($n = 1, 3, \text{ or } 6$) and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups as a function of different values of pH of aqueous buffer solutions measured under cyclooctane ($\cos \theta_{\text{co}}$). Integral values of pH were used (pH = 2, 5–11) for the contact angle measurements; the points are offset in the graph for clarity.

information about the ionization state of the $\text{CO}_2\text{H}/\text{CO}_2^-$ groups (Figure 3). Figure 3 shows a graph of the cosine of the sessile contact angle of the mixed SAMs presenting EG_nOH groups as a function of different values of pH of aqueous buffer solutions measured under cyclooctane³¹ ($\cos \theta_{\text{co}}$). Mixed SAMs that present EG_nOH groups with $n = 3, 6$ have similar wettabilities over the range of pH values investigated (pH = 2–11, $\cos(\theta_{\text{co}}) = 0.62\text{--}0.75$ for EG_3OH and $0.64\text{--}0.74$ for EG_6OH). The wettability of mixed SAMs that present EG_1OH increases at high values of pH (pH = 6, $\cos \theta_{\text{co}} = 0.71$; pH = 8, $\cos(\theta_{\text{co}}) = 0.87$) suggesting that the underlying $\text{CO}_2\text{H}/\text{CO}_2^-$ groups are exposed and are becoming ionized; the inflection point of the pH titration curve occurs at approximately pH 7; this value is similar to that determined for exposed carboxylic acid groups on other surfaces.^{40,41} It is apparent that mixed SAMs that present EG_nOH groups ($n = 3, 6$) are more effective than EG_1OH groups in preventing the underlying $\text{CO}_2\text{H}/\text{CO}_2^-$ groups from contributing to the surface properties of the mixed SAM.⁴²

Adsorption of Proteins. We used SPR to compare the ability to resist the nonspecific adsorption of proteins of single-component SAMs and mixed SAMs that present oligo(ethylene glycol) groups. In our experiment, single-component SAMs were prepared by the conventional method of dipping the gold substrate in an ethanolic solution of thiols; mixed SAMs were prepared by allowing the anhydride groups to react with amines (Figure 1C). We used SPR to measure the amount of adsorbed protein.⁴³ Our protocol for measuring the adsorption of proteins to these SAMs comprised sequential flow of three solutions over the surface of the SAM: a solution of 10 mM phosphate-buffered saline (PBS) solution (138 mM NaCl, 2.7 mM KCl, and pH = 7.4 at 25 °C), a solution of protein (~1 mg/mL) in the same buffer, and again the PBS buffer.

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(42) The sessile contact angles of buffer solutions under cyclooctane were measured immediately after the addition of the solution to the surface because the wettability of the mixed SAMs increased as a function of time. For example, the contact angle of a pH 11 buffer solution on a mixed SAM that presents $-\text{COHN}(\text{CH}_2\text{CH}_2\text{O})_6\text{H}$ groups changes from $\cos(\theta_{\text{co}}) = 0.71$ at time zero to $\cos(\theta_{\text{co}}) = 0.95$ after 10 min.

(43) SPR is an optical technique that detects refractive index changes at the surface of a thin film of metal. The adsorption of protein to a SAM on gold is an example of the interactions that can be measured in situ by SPR.

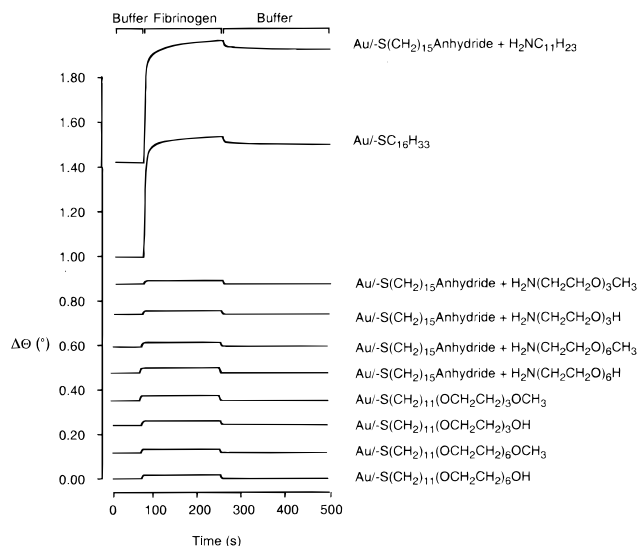


Figure 4. SPR sensorgrams showing the adsorption and desorption of fibrinogen on single-component SAMs prepared by the adsorption of oligo(ethylene glycol)-based alkanethiolates onto a gold surface and mixed SAMs prepared by the reaction of an oligo(ethylene glycol)-based amine with a surface that presents interchain carboxylic anhydride groups. The sensorgrams are offset vertically for clarity.

Figure 4 shows SPR sensorgrams for the adsorption of fibrinogen to SAMs. This protein has a high molecular weight (340 kDa) and it readily adsorbs to most surfaces. Both mixed SAMs and the single-component SAMs that presented EG_n (*n* = 3 or 6) groups adsorbed negligible amounts of fibrinogen. As expected, both mixed SAMs and the single-component SAMs that present long alkyl chains irreversibly adsorbed significant quantities of fibrinogen; approximately 100% of a monolayer of protein adsorbed on a single-component SAM of *n*-hexadecanethiol (HDT).^{11,44} More than 90% of the irreversibly adsorbed protein could be removed from these surfaces by rinsing them with a buffered solution of sodium dodecyl sulfate (50 mM). Thus, the amounts of protein adsorbed on single-component SAMs presenting $-\text{S}(\text{CH}_2)_{11}\text{EG}_n\text{OH}$ groups, and on mixed SAMs presenting a 1:1 mixture of $-\text{COHN}(\text{CH}_2\text{CH}_2\text{O})_6\text{CH}_3$ and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups were comparable, although the density of the oligo(ethylene glycol) groups in the mixed SAMs was only half that of the single-component SAMs. Mixed SAMs presenting a 1:1 mixture of $-\text{S}(\text{CH}_2)_{11}\text{EG}_6\text{OH}$ and $-\text{S}(\text{CH}_2)_{10}\text{CH}_3$ groups were also as resistant to the adsorption of proteins as a single-component SAM presenting $-\text{S}(\text{CH}_2)_{11}\text{EG}_3\text{OH}$ groups.¹² The amount of protein adsorbed on single-component SAMs presenting *n*-alkyl chains was also comparable to that of mixed SAMs presenting a 1:1 mixture of *n*-alkyl chains and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups. We conclude that the long alkyl chains of the mixed SAMs largely screen the buried $\text{CO}_2\text{H}/\text{CO}_2^-$ groups.

We compared semiquantitatively the amount of protein adsorbed to single-component SAMs that present EG_nOR groups (*R* = H, *n* = 1, 3, or 6; *R* = CH₃, *n* = 3 or 6) and mixed SAMs that present a 1:1 mixture of $-\text{COHN}(\text{CH}_2\text{CH}_2\text{O})_n\text{R}$ (*R* = H or CH₃, *n* = 3 or 6) and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups relative to the amount of protein adsorbed to a single-component SAM of HDT (Figure 5). Single-component SAMs of HDT irreversibly adsorb approximately a monolayer of protein under our experimental conditions.^{11,44} Figure 5 reports the amount of protein adsorbed to the

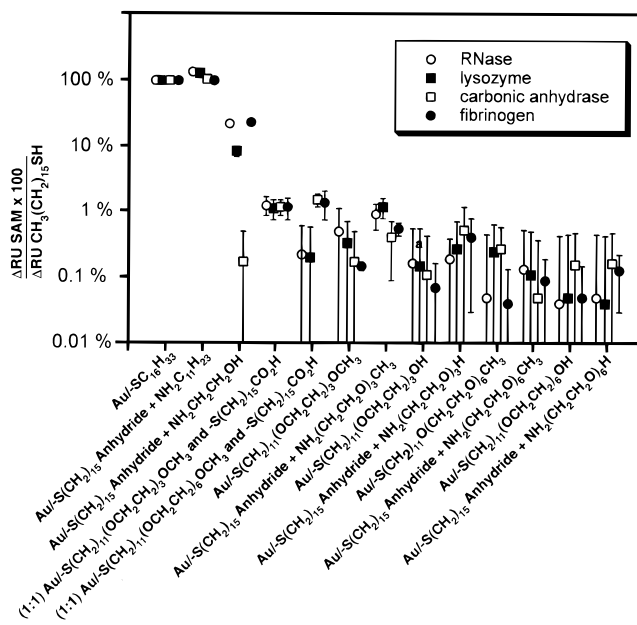


Figure 5. Comparison of the amount of four model proteins (fibrinogen, RNase, lysozyme, and carbonic anhydase) that adsorbed to single-component SAMs that present $-\text{S}(\text{CH}_2)_{11}\text{EG}_n\text{OR}$ (*R* = H or CH₃, *n* = 3 or 6) groups and mixed SAMs presenting a 1:1 mixture of $-\text{COHN}(\text{CH}_2\text{CH}_2\text{O})_n\text{R}$ (*R* = H or CH₃, *n* = 3 or 6) and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups. We report the values relative to the amount of protein adsorbed to a single-component SAM of *n*-hexadecanethiolates (HDT). The uncertainty in our measurements increased as the amount of adsorbed protein decreased; the uncertainty reported is the larger value of either the standard deviation of the average of two or more runs or the uncertainty of our measurement ($\pm 5 \Delta\text{RU} \times 100 / \Delta\text{RU}_{\text{HDT}}$). The points are horizontally offset in the graph for clarity.

SAM of interest ($\Delta\text{RU}_{\text{SAM}}$, RU = reflectance units measured by SPR) relative to the amount that adsorbed on a single-component SAM of HDT ($\Delta\text{RU}_{\text{HDT}}$). We express this value as a percentage determined by eq 1 (Figure 5). The values of the percentages of adsorbed protein that we report in this paper are all determined with eq 1.

$$\left(\frac{\Delta\text{RU}_{\text{SAM}} \times 100}{\Delta\text{RU}_{\text{HDT}}}\right) = \text{\% of a monolayer of protein adsorbed (resonance unit, RU = } 10^{4\circ}\text{)} \quad (1)$$

We compared four proteins: ribonuclease A (RNase A, 13.7 kDa), lysozyme (Lys, 14.3 kDa), carbonic anhydrase (CA, 30 kDa), and fibrinogen (Fib, 340 kDa). Both single-component SAMs and mixed SAMs that presented hexa-(ethylene glycol) units terminated in either H or CH₃ completely resisted the nonspecific adsorption of these four model proteins. These SAMs adsorbed only 0.05–0.2% of a monolayer of the test proteins; these values correspond approximately to the detection limit of the analysis. Similarly, single-component SAMs that presented tri(ethylene glycol) units terminated in either H or CH₃ adsorbed between 0.05 and 0.3% of a monolayer of the test proteins. Mixed SAMs that presented $-\text{COHN}(\text{CH}_2\text{CH}_2\text{O})_3\text{R}$ groups (*R* = H or CH₃) and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups adsorbed between 0.2 and 1.2% of a monolayer of the test proteins. Although tri(ethylene glycol)-based single-component SAMs (0.3%) adsorbed approximately four times less of our test proteins than tri(ethylene glycol)-based mixed SAMs (1.2%), these differences are negligible. Similar quantities of lysozyme (pI = 10.9) and CA (pI = 5.9) adsorbed to the mixed SAMs that presented $-\text{COHN}(\text{CH}_2\text{CH}_2\text{O})_n\text{R}/\text{CO}_2\text{H}$ groups (*n* = 3 or 6; *R* = H or CH₃)

(44) Lahiri, J.; Isaacs, L.; Tien, J.; Whitesides, G. M. *Anal. Chem.* **1999**, *71*, 777–790.

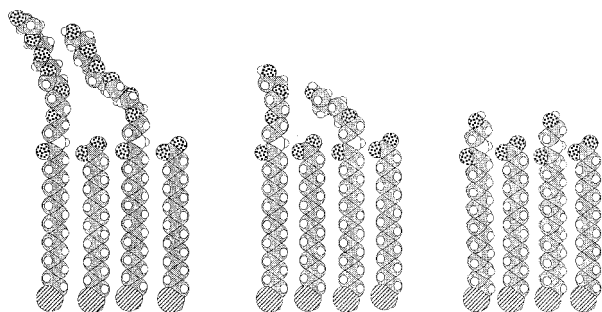


Figure 6. Space filling molecular model illustration of mixed SAMs presenting a 1:1 mixture of $-\text{CON}(\text{CH}_2\text{CH}_2\text{O})_n\text{H}$ ($n = 1, 3, \text{ or } 6$) and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups. The models depict the ability of longer oligomers of EG_n to sterically screen the underlying $\text{CO}_2\text{H}/\text{CO}_2^-$ groups.

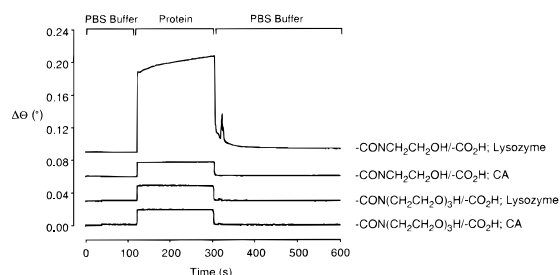


Figure 7. SPR sensorgrams showing the adsorption and desorption of lysozyme and CA on mixed SAMs presenting a 1:1 mixture of $-\text{CON}(\text{CH}_2\text{CH}_2\text{O})_n\text{H}$ ($n = 1 \text{ or } 3$) and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups.

suggesting that lysozyme (positively charged at pH 7.4) does not engage in Coulombic/ion exchange interactions with the $\text{CO}_2\text{H}/\text{CO}_2^-$ groups. Overall, mixed SAMs that present a 1:1 mixture of $-\text{COHN}(\text{CH}_2\text{CH}_2\text{O})_3\text{R}$ ($\text{R} = \text{H}$ or CH_3 , $n = 3$ or 6) and $\text{CO}_2\text{H}/\text{CO}_2^-$ demonstrate protein resistance comparable to that of single-component SAMs presenting the same groups. Mixed SAMs that present hexa(ethylene glycol) groups are slightly better than mixed SAMs that present tri(ethylene glycol) groups at preventing protein adsorption, probably because their longer length more effectively screens the effects of the $\text{CO}_2\text{H}/\text{CO}_2^-$ groups. Figure 6 schematically illustrates that long oligomers of EG_n ($n = 3$ or 6) can sterically block the underlying $\text{CO}_2\text{H}/\text{CO}_2^-$ groups.

We measured the adsorption of our four test proteins to mixed SAMs that presented $-\text{COHNCH}_2\text{CH}_2\text{OH}/\text{CO}_2\text{H}$ groups (Figure 5). The short length of the mono(ethylene glycol) group in these mixed SAMs exposes the $\text{CO}_2\text{H}/\text{CO}_2^-$ groups at the SAM-water interface (Figure 6). As expected, these mixed SAMs adsorbed more of the four test proteins (22% RNase, 23% fibrinogen, 8% lysozyme, and 1% CA) than mixed SAMs that presented longer oligomers of EG_n ($n = 3$ or 6).

Significantly more lysozyme than CA adsorbed to mixed SAMs that presented $-\text{COHNCH}_2\text{CH}_2\text{OH}/\text{CO}_2\text{H}$ groups. Figure 7 presents SPR sensorgrams illustrating the differences in weak adsorption of lysozyme and CA on mixed SAMs that present either $-\text{COHN}(\text{CH}_2\text{CH}_2\text{O})_3\text{H}$ or $-\text{COHNCH}_2\text{CH}_2\text{OH}$ groups. Here, we define the weak adsorption of proteins to a surface as a process that occurs only during the flow of protein over the surface; the weakly adsorbed molecules of protein rapidly dissociate from the surface when the solution of protein is replaced with PBS buffer. In our experiment, we observe a large change in SPR response when the positively charged lysozyme is allowed to flow over the mixed SAM that presents $-\text{COHNCH}_2\text{CH}_2\text{OH}$ and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups (Figure 7);

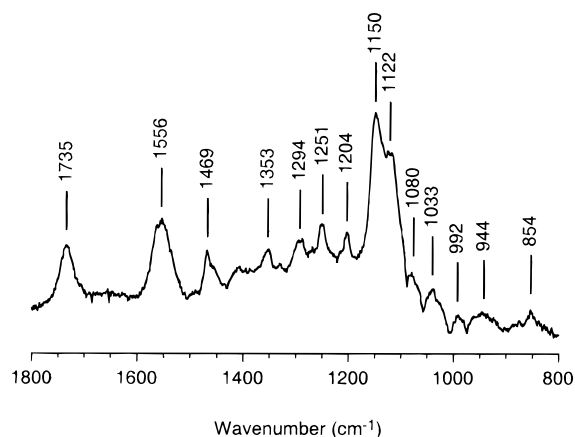


Figure 8. PIERS spectrum of a mixed SAM presenting a ~1:1 mixture of carboxylic acid and $-\text{COHN}(\text{CH}_2\text{CH}_2\text{O})_5\text{CH}_3$ groups.

this change in signal is five times larger than the change observed for the adsorption of lysozyme on a mixed SAM that presents $-\text{COHN}(\text{CH}_2\text{CH}_2\text{O})_3\text{H}$ and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups. CA (negatively charged at pH 7.4) has a small change in signal upon injection on the surface; this change is caused by the difference in refractive index between the solution of CA and that of PBS buffer. These results suggest that attractive Coulombic/ion exchange interactions are occurring between the exposed $\text{CO}_2\text{H}/\text{CO}_2^-$ groups and the positively charged lysozyme, while repulsive interactions may arise between the exposed $\text{CO}_2\text{H}/\text{CO}_2^-$ groups and the negatively charged CA.⁴⁵

We measured the amount of adsorption of our four test proteins to a 50% mixed SAM formed by the coadsorption of $\text{HS}(\text{CH}_2)_{15}\text{CO}_2\text{H}$ (1 mM in ethanol) with either $\text{HS}(\text{CH}_2)_{11}\text{EG}_3\text{OCH}_3$ or $\text{HS}(\text{CH}_2)_{11}\text{EG}_6\text{OCH}_3$ (1 mM in ethanol) thiols; these two surfaces adsorbed similar quantities of the four test proteins (0.2–1.5%). This amount of adsorption is slightly greater than the amount of the test proteins that adsorbed to mixed SAM formed by the reaction of $\text{H}_2\text{N}(\text{CH}_2\text{CH}_2\text{O})_6\text{CH}_3$ with a surface that presents carboxylic anhydride groups (0.05–0.12%) and approximately the same as the amount that adsorbed to mixed SAMs formed by the reaction of $\text{H}_2\text{N}(\text{CH}_2\text{CH}_2\text{O})_3\text{CH}_3$ with the anhydride-presenting SAM (0.4–1.2%) (Figure 5). This result suggests that the 50% mixed SAM formed by the coadsorption of $\text{HS}(\text{CH}_2)_{11}\text{EG}_6\text{OCH}_3$ and $\text{HS}(\text{CH}_2)_{15}\text{CO}_2\text{H}$ thiols may be phase-separated or that the mole fraction of thiols in solution used to form the SAM is not the same as the mole fraction of thiols on the surfaces.

Conformation of the $-\text{S}(\text{CH}_2)_{15}\text{CONH}(\text{CH}_2\text{CH}_2\text{O})_6\text{CH}_3/$ $-\text{S}(\text{CH}_2)_{15}\text{CO}_2\text{H}$ Groups in Mixed SAMs. We used PIERS to study the conformation of the $-\text{S}(\text{CH}_2)_{15}\text{CONH}(\text{CH}_2\text{CH}_2\text{O})_6\text{CH}_3/$ $-\text{S}(\text{CH}_2)_{15}\text{CO}_2\text{H}$ groups in the mixed SAMs. Figure 8 shows a PIERS spectrum of this mixed SAM in the range of 1800–800 cm^{-1} ; Table 1 compares its absorption bands to that of PEG in crystalline and molten states. In the crystalline or helical conformation, the ethylene glycol unit adopts a trans conformation

(45) The adsorption of lysozyme and fibrinogen to the mixed SAMs presenting a 1:1 mixture of $-\text{COHN}(\text{CH}_2\text{CH}_2\text{O})_n\text{H}$ ($\text{R} = \text{H}$, $n = 1, 3, \text{ or } 6$) groups at pH 10 in a 50 mM Cs_2CO_3 buffer resulted in similar amounts of adsorbed protein as measured in PBS buffer at pH 7.4. Mixed SAMs that present $-\text{COHN}(\text{CH}_2\text{CH}_2\text{O})_n\text{H}$ ($\text{R} = \text{H}$, $n = 3$ or 6) groups adsorb less than 1% of a monolayer of fibrinogen while mixed SAMs that present $-\text{COHNCH}_2\text{CH}_2\text{OH}$ groups adsorb 30% of a monolayer of fibrinogen. The values of protein adsorption for lysozyme ($\text{pI} = 10.9$) at both high and low pH to mixed SAMs that present $-\text{COHNCH}_2\text{CH}_2\text{OH}$ are similar; we could not measure the adsorption of lysozyme at $\text{pH} \geq 11$ (such that it would be negatively charged) because the manufacturer of our SPR instrument (Biacore) does not recommend exposing the instrument to $\text{pH} > 10$ for extended periods of time.

Table 1. Infrared Absorption Frequency and Assignment of Poly(ethylene glycol) in the Crystalline and Molten States, and $-\text{CONH}(\text{CH}_2\text{CH}_2\text{O})_6\text{CH}_3$ in the Mixed SAMs on Gold

wavenumber, cm^{-1}				
crystal-	molten	$-\text{CONH}(\text{CH}_2\text{CH}_2\text{O})_6\text{CH}_3$		assignment
1364				CH_2 wag
	1352	1353		CH_2 wag
	1326			CH_2 wag
	1296	1294		CH_2 twist
1280				CH_2 twist
	1249	1251		CH_2 twist
1236				CH_2 twist
		1204		OCH_3 rock
1149		1150		C–C stretch, CH_2 rock
	1140			C–O, C–C stretch, CH_2 rock
1119		1122		C–O, C–C stretch
	1107			C–O, C–C stretch, CH_2 rock
1102				C–O stretch
1062		1079		C–O, C–C stretch, CH_2 rock
	1038	1033		C–O, C–C stretch, CH_2 rock
	992	992		C–O, C–C stretch, CH_2 rock
963				CH_2 rock, CH_2 twist
947				C–C stretch, CH_2 rock
	945	944		C–C stretch, CH_2 rock
	855	854		C–O stretch, CH_2 rock
844				C–O, C–C stretch, CH_2 rock
	810			C–O stretch, CH_2 rock

around the C–O bonds and a unidirectional gauche conformation around the C–C bonds, i.e., TGT;⁴⁶ in the amorphous or molten state, it adopts a predominately gauche conformation around the C–C bonds while the conformation about the C–O bonds can be in either the trans or gauche conformation, i.e., TGT, GGT, or TGG.²⁹ The ethylene glycol units of single-component SAMs presenting $-\text{S}(\text{CH}_2)_{11}\text{OCNH}(\text{CH}_2\text{CH}_2\text{O})_6\text{H}$ had both helical and amorphous conformations; the conformation of the ethylene glycol units of mixed SAMs presenting $-\text{S}(\text{CH}_2)_{11}\text{OCNH}(\text{CH}_2\text{CH}_2\text{O})_6\text{CH}_3/-\text{S}(\text{CH}_2)_{15}\text{CO}_2\text{H}$ determined by PIERS is consistent with an amorphous state of PEG.

Attachment of Benzenesulfonamide to the Surface. Binding of CA. We prepared a SAM that can recognize and adsorb CA biospecifically by reacting the anhydride groups with a mixture of $\text{H}_2\text{N}(\text{CH}_2\text{CH}_2\text{O})_6\text{H}$ and the benzene sulfonamide derived from $\text{H}_2\text{N}(\text{CH}_2\text{CH}_2\text{O})_6\text{CH}_2\text{CONH}(\text{CH}_2)_6\text{NHCOC}_6\text{H}_4\text{SO}_2\text{NH}_2$ (compound **13**) (Figure 9). Figure 9C shows the SPR sensorgram obtained for the adsorption and desorption of CA ($5 \mu\text{M}$ in PBS buffer) to a mixed SAM that presents benzene sulfonamide **13** covalently immobilized on its surface. The binding of CA was largely reversible ($>90\%$). Analysis of the rate of dissociation and association of CA from the mixed SAM presenting benzenesulfonamide groups resulted in a value of $k_{\text{off}} = 0.0054 \text{ s}^{-1}$ and a value of $k_{\text{on}} = 13\,000 \text{ M}^{-1} \text{ s}^{-1}$ (Table 2). The equilibrium dissociation constant ($K_{\text{d}} = k_{\text{off}}/k_{\text{on}}$) was $0.42 \mu\text{M}$. The kinetic and thermodynamic parameters for the binding of CA to our mixed SAMs that present benzene sulfonamide are comparable to those found for other, structurally similar mixed SAMs (Table 2).^{44,47}

We demonstrate that the mixed SAMs presenting benzene sulfonamide groups bound CA biospecifically with a competitive inhibition experiment. The binding of CA to this mixed SAM was completely inhibited by the

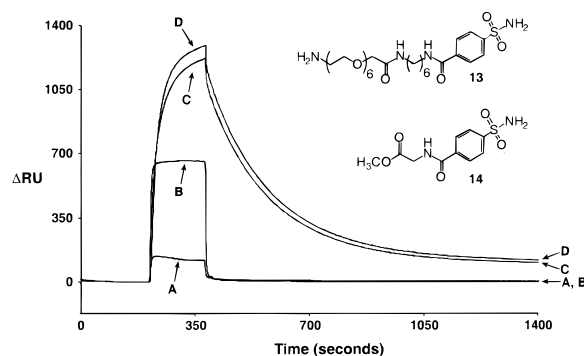


Figure 9. SPR sensorgrams for the adsorption of proteins to a mixed SAM prepared by the reaction of $\text{H}_2\text{N}(\text{CH}_2\text{CH}_2\text{O})_6\text{H}$ and the benzene sulfonamide ligand **13** (95:5 mol:mol, 10 mM final concentration) with a surface that presents carboxylic anhydride groups: A, CA ($10 \mu\text{M}$) with 1 mM soluble benzene sulfonamide ligand ($\text{H}_2\text{NSO}_2-1,4-\text{C}_6\text{H}_4\text{CONHCH}_2\text{CO}_2\text{CH}_3$, compound **14**); B, a mixture of proteins including fibrinogen, RNase, lysozyme, and myoglobin (0.2 mg/mL each); C, CA ($5 \mu\text{M}$); D, same mixture of proteins as in B with added CA ($5 \mu\text{M}$).

addition of 1 mM benzene sulfonamide **14** ($\text{H}_2\text{NSO}_2\text{C}_6\text{H}_4-\text{CONHCH}_2\text{CO}_2\text{CH}_3$, $K_{\text{d}} = 63 \mu\text{M}$)⁴⁸ to the CA-containing solution (Figure 9A). The mixed SAM presenting **13** and $-\text{CONH}(\text{CH}_2\text{CH}_2\text{O})_6\text{H}/-\text{CO}_2\text{H}$ groups also did not adsorb a detectable amount of protein when exposed to a mixture of fibrinogen, myoglobin, lysozyme, and RNase (Figure 9B); the addition of $5 \mu\text{M}$ CA to the mixture of proteins resulted in a biospecific response (Figure 9D). We prepared mixed SAMs that presented **13** and $-\text{CONH}(\text{CH}_2\text{CH}_2\text{O})_6\text{H}/-\text{CO}_2\text{H}$ groups because the longer hexa(ethylene glycol) groups reduced the non-specific adsorption of our mixture of proteins relative to the structurally similar mixed SAM that presented **13** and $-\text{CONH}(\text{CH}_2\text{CH}_2\text{O})_3\text{H}/-\text{CO}_2\text{H}$ groups (data not shown). The longer hexa(ethylene glycol) groups were more effective than the shorter tri(ethylene glycol) groups in reducing the nonspecific adsorption of proteins, probably because they are better at screening the effects of the underlying $\text{CO}_2\text{H}/\text{CO}_2^-$ groups.

Conclusions

The reaction of an amine with a surface that presents interchain anhydrides (Figure 1) is a versatile and synthetically convenient method to generate mixed SAMs that can be used to study the adsorption of proteins. The inertness of mixed SAMs that present $-\text{CON}(\text{CH}_2\text{CH}_2\text{O})_6\text{R}$ ($\text{R} = \text{H}$ or CH_3) and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups is comparable to that of single-component SAMs presenting EG_6OR ($\text{R} = \text{H}$ or CH_3) groups; that is, essentially no protein adsorbs to either SAM ($<0.2\%$ of a monolayer of the four test proteins adsorbed). Mixed SAMs based on $-\text{CON}(\text{CH}_2\text{CH}_2\text{O})_3\text{R}$ ($\text{R} = \text{H}$ or CH_3) and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups adsorb slightly more protein ($<1.2\%$) than single-component SAMs presenting EG_3OR ($\text{R} = \text{H}$ or CH_3) groups ($<0.3\%$).

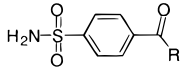
The ability of mixed SAMs that present a 1:1 mixture of $-\text{CON}(\text{CH}_2\text{CH}_2\text{O})_6\text{R}$ ($\text{R} = \text{H}$ or CH_3) and $\text{CO}_2\text{H}/\text{CO}_2^-$ groups to resist the nonspecific adsorption of proteins completely (within the limits of detection of the analysis) is compatible with the work of Prime et al., who showed that mixed SAMs formed by coadsorption of two thiols that present a 1:1 mixture of $-\text{S}(\text{CH}_2)_{11}\text{EG}_6\text{OR}$ ($\text{R} = \text{H}$ or CH_3) and $-\text{S}(\text{CH}_2)_{11}\text{CH}_3$ groups also completely resist the adsorption of proteins.¹² Using shorter oligo(ethylene

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Table 2. Thermodynamic and Kinetic Constants for Binding of CA to Benzene Sulfonamide Ligands^a

		k_{on} ($\text{M}^{-1} \text{s}^{-1}$)	k_{off} (s^{-1})	K_{d} (μM)
1	–OH, measured by capillary electrophoresis ⁴⁴	<i>b</i>	<i>b</i>	1.25
2	–NH(CH ₂) ₆ NHCOCH ₂ OEG ₆ (CH ₂) ₁₁ S–/Au by coadsorption of two thiols ⁴⁷	19000	0.0054	0.26
3	–NH(CH ₂) ₆ NHCOCH ₂ OEG ₆ (CH ₂) ₁₁ S–/Au by coupling to an <i>N</i> -hydroxysuccinimidyl ester ⁴⁴	9400	0.0054	0.56
4	–NH(CH ₂) ₆ NHCOCH ₂ (OCH ₂ CH ₂) ₆ NHCO(CH ₂) ₁₅ S–/Au by reaction with carboxylic anhydride groups	13000	0.0054	0.42

^a Entry 4 corresponds to the values determined by SPR in this paper ^b Not measured.

glycol) groups to form mixed SAMs by either our method or coadsorption of alkanethiols does not generate surfaces that are protein resistant. If the oligomer length of these mixed SAMs is reduced to one ethylene glycol unit, both types of mixed SAMs become protein adsorptive; in both cases, increasing the number of oligomer units to six forms mixed SAMs that completely resist the adsorption of proteins. These results suggest that long oligomers of EG_{*n*} groups (*n* ≥ 3) can screen the contribution of the underlying CO₂H/CO₂[–] groups to give a mixed SAM that has the same ability to resist the adsorption of proteins as a single-component SAM presenting only EG_{*n*} groups (Figure 6). As we have observed previously, while mixed SAMs that present EG₃ groups provide a high degree of resistance to the adsorption of proteins, mixed SAMs that present EG₆ groups are better (EG₆ typically adsorbs 1–10 times less protein than EG₃).

We have also demonstrated that this method could be used to prepare a mixed SAM that presents benzene-sulfonamide groups and binds CA biospecifically. This mixed SAM was prepared by allowing the anhydride groups to react with a mixture of H₂N(CH₂CH₂O)₆H and H₂N(CH₂CH₂O)₆CH₂CONH(CH₂)₆NHCOC₆H₄SO₂NH₂ (compound 13). This method is attractive for the preparation of SAMs for studies of biospecific adsorption because it uses amine-terminated ligands (here, (HS(CH₂)₁₁EG₆OCH₂CONH(CH₂)₆NHCOC₆H₄SO₂NH₂) that are either commercially available or less cumbersome to synthesize than complex thiols containing ligands.

This method will be useful in applications where SAMs can be conveniently modified *after* their assembly either to make them resist the adsorption of proteins or to generate surfaces that specifically adsorb a protein of interest. This method is particularly useful for the screening of a number of functional groups on the surface of a SAM while exploring structure–property relationships.

Experimental Section

Materials. Carbonic anhydrase II (bovine), fibrinogen, ribonuclease A (bovine pancreas), lysozyme (egg white), myoglobin (horse heart), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) were purchased from Sigma (St. Louis, MO). Anhydrous 1-methyl-2-pyrrolidinone (NMP) and 10% Pd/C were purchased from Aldrich (Milwaukee, WI). Anhydrous *N,N*-dimethylformamide (DMF) was purchased from EM Science (Gibbstown, NJ). Absolute ethanol was purchased from Pharmco-products (Brookfield, CT). 1-Undecylmercaptan was purchased from Pfaltz and Bauer (Waterbury, CT). H₂N(CH₂CH₂O)₃H was a gift of Huntsman Corp. (now a unit of Texaco). The buffers used in the wettability measurements were prepared from pHydron capsules to give 50 mM solutions of: pH 2, potassium acid phthalate and sulfamic acid; pH 5, potassium acid phthalate and sulfamic acid; pH 6, potassium acid phthalate; pH 7, sodium phosphate; pH 8, sodium phosphate; pH 9, sodium carbonate; pH 10, sodium carbonate; pH 11, sodium carbonate and sodium phosphate (Micro Essentials Laboratory, Brooklin, NY). The ¹H NMR spectra were recorded at 400 MHz on a Bruker spectrom-

eter. Chemical shifts are reported in parts per million referenced with respect to residual solvent (CHCl₃ = 7.26 ppm).

General Method. The following compounds were synthesized as previously described: 16-mercaptohexadecanoic acid,³² HS-(CH₂)₁₁OEG₃OH,¹⁰ HS(CH₂)₁₁OEG₃OCH₃,²⁹ HS(CH₂)₁₁OEG₆OH,^{11,12} and HS(CH₂)₁₁OEG₆OCH₃.^{11,12} 16-Mercaptohexadecanoic acid has just become commercially available from Aldrich and Analytical micro-Systems GbR; Wieshuberstr. 3, 93059 Regensburg, Germany (info@micro-systems.de).

Preparation of the Reactive SAM Presenting Interchain Carboxylic Anhydride. Gold substrates for PIERS spectroscopy were prepared by e-beam evaporation of 5 nm of Ti, followed by 200 nm of Au, onto test grade, (100) single-crystal silicon wafers. Gold substrates for SPR spectroscopy were prepared by e-beam evaporation of 1.5 nm of Ti, followed by 38 nm of Au, onto 50 × 18 mm² glass coverslips. The gold substrates were incubated, overnight, in a 2 mM solution of mercaptohexadecanoic acid in ethanol/water/acetic acid (85/10/5, v/v/v), rinsed with ethanol, and dried under a stream of nitrogen. The cleaned substrate was then placed in a freshly prepared solution of 0.1 M trifluoroacetic anhydride (TFAA) and 0.2 M triethylamine in anhydrous DMF without stirring for 20 min at room temperature. The substrates were removed from the TFAA solution, rinsed thoroughly with CH₂Cl₂, and dried in a stream of nitrogen. The resulting substrates (which present interchain carboxylic anhydride groups) were used immediately by immersion in a solution containing an appropriate amine.

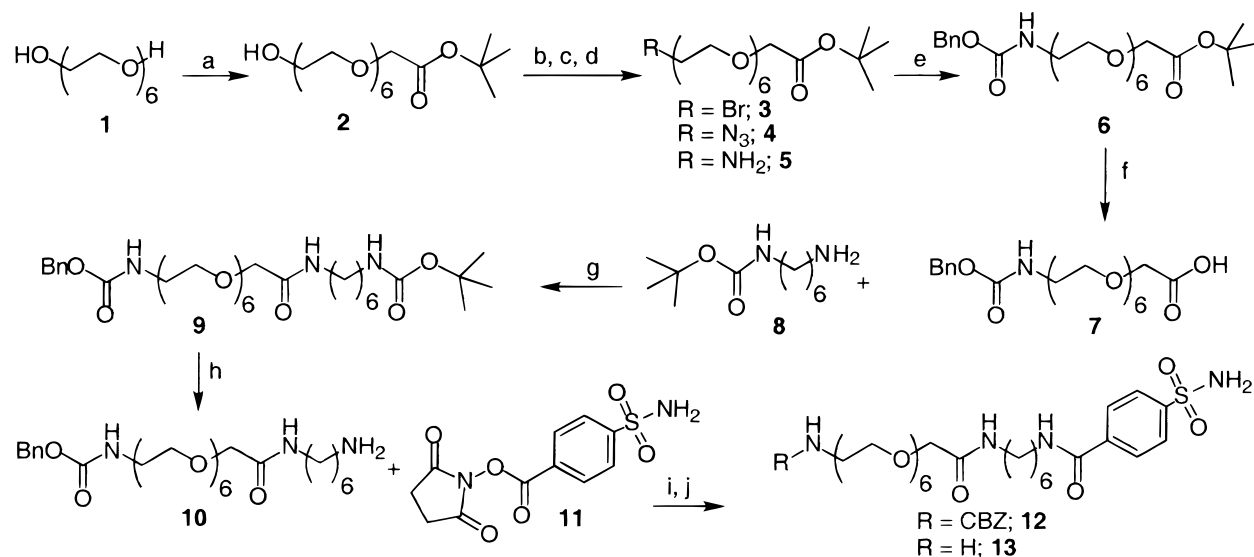
Surface Plasmon Resonance Spectroscopy. SPR was performed with a Biacore 1000 instrument. Gold-coated glass substrates presenting the SAMs to be analyzed were mounted in SPR cartridges as previously described.^{20,31} The protocol for measuring the adsorption of protein to SAMs included the following: (i) flowing a solution of 40 mM SDS (in PBS) over the SAM for 3 min followed by PBS buffer for 2 min; (ii) flowing PBS buffer for 1 min before substituting the flow with a solution of protein (1 mg/mL in PBS) for 3 min and then injecting PBS buffer for 4 min (Figure 4). We used a flow rate of 5 μL/min for all experiments.

Characterization. PIERS spectra were obtained in single reflection mode using a Digilab Fourier transform infrared spectrometer (BioRad, Cambridge, MA).⁴⁹ The p-polarized light was incident at 80° relative to the surface normal of the substrate, and a mercury–cadmium–telluride (MCT) detector was used to detect the reflected light. A spectrum of a SAM of *n*-hexadecanethiolate-*d*₃₃ on gold was taken as a reference. Typically, 1024 scans were averaged to yield spectra with excellent signal-to-noise ratios. XPS spectra were collected on an SSX-100 spectrometer (Surface Science Instruments) using monochromatic Al Kα X-rays ($\lambda = 8.3 \text{ \AA}$). The spectra were referenced to Au(4f_{7/2}) at 84.00 eV. High-resolution core-level spectra were recorded with a 50-eV pass energy, 300-μm spot size with an acquisition time of 30 min. Contact angles of water were determined on a Ramé-Hart model 100 goniometer at room temperature under ambient laboratory conditions. Data presented here were averages of the measurements of at least three spots on each of three individually prepared samples.

H₂N(CH₂CH₂O)_{*n*}H⁵⁰ (*n* = 3, 6) and H₂N(CH₂CH₂O)_{*n*}CH₃⁵¹ were synthesized by following established procedures.

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Scheme 1. Synthesis of $\text{H}_2\text{N}(\text{CH}_2\text{CH}_2\text{O})_6\text{CH}_2\text{CONH}(\text{CH}_2)_6\text{NHCOC}_6\text{H}_4\text{SO}_2\text{NH}_2$ (Compound 13)^a

^a Key: (a) KH, *tert*-butyl bromoacetate, reflux, overnight, 44%; (b) CBr_4 , PPh_3 , CH_2Cl_2 , 5 h, room temperature (rt), 91%; (c) sodium azide, DMF, 80 °C, overnight, 57%; (d) Pd/C, H_2 , ethanol, rt, 16 h, 96%; (e) benzoyl chloroformate, sodium hydroxide (3.0 M), ethyl acetate, rt, overnight, 76%; (f) TFA, CH_2Cl_2 , rt, overnight, 99%; (g) EDAC, TEA, DMF, rt, overnight, 54%; (h) TFA, CH_2Cl_2 , rt, 1 h, 98%; (i) DIPEA, DMF, rt, overnight, 54%; (j) Pd/C, H_2 , ethanol, rt, overnight, 64%.

Synthesis of $\text{H}_2\text{N}(\text{CH}_2\text{CH}_2\text{O})_6\text{CH}_2\text{CONH}(\text{CH}_2)_6\text{NHCOC}_6\text{H}_4\text{SO}_2\text{NH}_2$ (compound 13) (Scheme 1). {2-[2-(2-{2-[2-(2-Hydroxyethoxy)ethoxy]ethoxy}ethoxy)ethoxy]ethoxy}acetic acid *tert*-Butyl Ester (**2**). To a mixture of potassium hydride (340 mg of 35% KH in mineral oil, 3.0 mmol) in dry THF (50 mL) at 0 °C was added hexa(ethylene glycol) (1.3 mL, 5.2 mmol) over 5 min. The resulting mixture was allowed to warm to ambient temperature and was stirred for 1 h. To this mixture was added *tert*-butyl bromoacetate (0.44 mL, 3.0 mmol) in one portion and the mixture was refluxed overnight. The reaction mixture was cooled to 0 °C and filtered to remove potassium bromide. The filtrate was concentrated in vacuo and loaded directly onto a silica gel gravity column (100 g) and eluted with CH_2Cl_2 :MeOH 100:1 to 10:1 (v/v) to afford compound **2** as a clear oil (0.90 g, 44%). ¹H NMR (CDCl_3 , 400 MHz): δ 1.33 (s, 9H, C-(CH_3)₃), 2.99 (s, 1H, OH), 3.45 (m, 2H, HOCH₂), 3.51 (m, 16H, OCH₂CH₂), 3.55 (m, 4H), 3.88 (s, 2H, OCH₂CO₂). ¹³C NMR (CDCl_3 , 400 MHz): 27.7, 61.2, 68.6, 70.0, 70.2, 70.3, 72.2, 81.1, 169.4. HRMS-FAB: m/z 419.2248 ([M + Na]⁺, calcd for C₁₈H₃₆O₉Na 419.2257).

{2-[2-(2-{2-[2-(2-Bromoethoxy)ethoxy]ethoxy}ethoxy)ethoxy]ethoxy}acetic Acid *tert*-Butyl Ester (**3**). Carbon tetrabromide (9.0 g, 27 mmol) and triphenylphosphine (1.2 g, 4.5 mmol) were added to a solution of compound **2** (1.8 g, 4.5 mmol) in dry CH_2Cl_2 (100 mL). After the reaction mixture was stirred for 5 h, the solvent was concentrated in vacuo and the residue was loaded onto a silica gel gravity column (200 g) and eluted with CH_2Cl_2 :MeOH 100:0 to 20:1 (v/v) to afford compound **3** as a colorless oil (1.9 g, 91%). ¹H NMR (CDCl_3 , 400 MHz): 1.38 (s, 9H, C-(CH_3)₃), 3.38 (t, $J = 6.2$ Hz, 2H, BrCH₂), 3.57–3.63 (m, 20H, OCH₂CH₂), 3.71 (t, $J = 6.1$ Hz, 2H), 3.94 (s, 2H, OCH₂CO₂). ¹³C NMR (CDCl_3 , 400 MHz): 27.9, 30.2, 68.8, 70.2, 70.3, 70.4, 70.9, 81.2, 169.4. HRMS-FAB: m/z 481.1408 ([M + Na]⁺, calcd for C₁₈H₃₅O₈BrNa 481.1413).

{2-[2-(2-{2-[2-(2-Azidoethoxy)ethoxy]ethoxy}ethoxy)ethoxy]ethoxy}acetic Acid *tert*-Butyl Ester (**4**). Sodium azide (1.5 g, 23 mmol) was added to a solution of compound **3** (1.9 g, 4.1 mmol) in dry DMF (50 mL), and the resulting mixture was stirred at 80 °C overnight. The reaction mixture was concentrated in vacuo and then triturated with CH_2Cl_2 (20 mL) and filtered to remove sodium azide. The filtrate was concentrated in vacuo, and the residue was loaded onto a silica gel gravity column (200 g) and eluted with CH_2Cl_2 :MeOH 100:0 to 20:1 (v/v) to afford compound **4** as a colorless oil (1.0 g, 57%). ¹H NMR (CDCl_3 , 400

MHz): δ 1.41 (s, 9H, C-(CH_3)₃), 3.32 (t, $J = 5.0$ Hz, 2H, N₃CH₂), 3.59–3.66 (m, 22H, OCH₂CH₂), 3.96 (s, 2H, OCH₂CO₂). ¹³C NMR (CDCl_3 , 400 MHz): δ 28.0, 50.5, 68.9, 69.9, 70.4, 70.5, 70.6, 81.4, 169.5. HRMS-FAB: m/z 444.2323 ([M + Na]⁺, calcd for C₁₈H₃₅N₃O₈Na 444.2322).

Caution, sodium azide is both highly toxic and an explosive hazard; handle with care.

{2-[2-(2-{2-[2-(2-Aminoethoxy)ethoxy]ethoxy}ethoxy)ethoxy]ethoxy}acetic Acid *tert*-Butyl Ester (**5**). To a solution of compound **4** (1.0 g, 2.4 mmol) in ethanol (20 mL) was added 10% Pd/C (0.3 g) and acetic acid (0.3 mL). The resulting mixture was stirred under an atmosphere of hydrogen for 16 h and then filtered to remove Pd/C. The filtrate was concentrated in vacuo, and the residue was loaded onto a silica gel gravity column (100 g) and eluted with CH_2Cl_2 :MeOH 20:1 to 10:1 (v/v) to afford compound **5** as a oil (0.90 g, 96%). ¹H NMR (CDCl_3 , 400 MHz): δ 1.39 (s, 9H, C-(CH_3)₃), 3.15 (m, 2H, NH₂CH₂), 3.62–3.68 (m, 20H, OCH₂CH₂), 3.82 (m, 2H), 4.00 (s, 2H, OCH₂CO₂), 7.57 (s, 2H, NH₂). ¹³C NMR (CDCl_3 , 400 MHz): δ 27.9, 40.3, 66.7, 68.6, 69.5, 69.6, 69.7, 69.8, 69.9, 70.0, 70.1, 70.2, 70.4, 81.9, 169.8. HRMS-FAB: m/z 418.2400 ([M + Na]⁺, calcd for C₁₈H₃₇NO₈Na 418.2417).

{2-[2-(2-{2-[2-(2-Benzyloxycarbonylaminoethoxy)ethoxy]ethoxy}ethoxy)ethoxy]ethoxy}acetic Acid *tert*-Butyl Ester (**6**). Sodium hydroxide (2.0 mL of a 3M in water, 6.0 mmol) and benzoyl chloroformate (0.52 mL, 0.36 mmol) were added in portions to compound **5** (0.90 g, 2.3 mmol) in ethyl acetate (10 mL), and the resulting mixture was stirred overnight. The reaction mixture was concentrated in vacuo, water (20 mL) was added, the slurry was extracted with CH_2Cl_2 (3 × 100 mL), and the combined organic solutions were washed with saturated aqueous NaHCO₃ (20 mL) and brine (20 mL) and dried over anhydrous MgSO₄. The solution was concentrated in vacuo, and the residue was loaded onto a silica gel gravity column (100 g) and eluted with CH_2Cl_2 :MeOH 100:1 to 10:1 (v/v) to afford compound **6** as a colorless oil (0.93 g, 76%). ¹H NMR (MeOD, 400 MHz): δ 1.46 (s, 9H, C-(CH_3)₃), 3.29 (m, 2H), 3.51 (t, $J = 5.5$ Hz, 2H), 3.58–3.70 (m, 20H, OCH₂CH₂), 4.01 (s, 2H, OCH₂CO₂), 5.05 (s, 2H, PhCH₂O), 7.31 (m, 5H, Ph). ¹³C NMR (MeOD, 400 MHz): δ 28.3, 41.7, 67.4, 69.5, 71.0, 71.1, 71.2, 71.3, 71.4, 83.1, 128.8, 128.9, 129.5, 171.9, 181.5. HRMS-FAB: m/z 552.2786 ([M + Na]⁺, calcd for C₂₆H₄₃NO₁₀Na 552.2785).

{2-[2-(2-{2-[2-(2-Benzyloxycarbonylaminoethoxy)ethoxy]ethoxy}ethoxy)ethoxy]ethoxy}acetic acid (**7**). TFA (4 mL) was added to a solution of compound **6** (0.93 g, 1.7 mmol) in CH_2Cl_2 (20 mL), and the resulting solution was stirred overnight at ambient temperature. The resulting solution was concentrated

