A Strategy for the Generation of Surfaces Presenting Ligands for Studies of Binding Based on an Active Ester as a Common Reactive Intermediate: A Surface Plasmon Resonance Study

Joydeep Lahiri, Lyle Isaacs, J oe Tien, and George M. Whitesides*
Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138

This paper describes the immobilization of ten proteins and two low-molecular-weight ligands on mixed self-assembled monolayers (SAMs) of alkanethiolates on gold generated from the tri(ethylene glycol)-terminated thiol 1 (HS(CH2)11(OCH2CH2)3OH) (χ(1) = 1.0–0.0) and the longer, carboxylic acid-terminated thiol 2 (HS(CH2)11(OCH2CH2)6OCH2CO2H) (χ(2) = 0.0–1.0). The immobilization was achieved by a two-step procedure: generation of reactive N-hydroxysuccinimidyl esters from the carboxylic acid groups of 2 in the SAM and coupling of these groups with amines on the protein or ligand. Because this method involves a common reactive intermediate that is easily prepared, it provides a convenient method for attaching ligands to SAMs for studies using surface plasmon resonance spectroscopy (and, in principle, other bioanalytical methods that use derivatized SAMs on gold, silver, and other surfaces). These SAMs were resistant to non-specific adsorption of proteins having a wide range of molecular weights and isoelectric points. The pH of the coupling buffer, the concentration of protein, the ionic strength of the solution of protein, and the molecular weight of the protein all influenced the amount of the protein that was immobilized. For the proteins investigated in detail—carbonic anhydrase and lysozyme—the highest quantities of immobilized proteins were obtained when using a low ionic strength solution at a value of pH approximately one unit below the isoelectric point (pI) of the protein, at a concentration of ~0.5 mg mL⁻¹. Comparisons of the kinetic and thermodynamic constants describing binding of carbonic anhydrase and vancomycin to immobilized benzene sulfonamide and N-o-Ac-Lys-o-Ala-o-Ala groups, respectively, on mixed SAMs (by methods described in this paper) and in the carboxymethyl dextran matrix of commercially available substrates yielded (for these systems) essentially indistinguishable values of $K_d$, $k_{off}$, and $k_{on}$.

This paper describes coupling of ligands and proteins to mixed self-assembled monolayers (SAMs) of alkanethiolates on gold derived from the tri(ethylene glycol) ((EG)6OH)-terminated thiol 1 (Chart 1) and the hexa(ethylene glycol)—carboxylic acid ((EG)6CO2H)-terminated thiol 2 (Figure 1), and studies of biospecific binding to these SAMs, after derivatization with appropriate ligands. The method involves the generation of activated N-hydroxysuccinimidyl (NHS) esters of 2 and the reaction of these active esters with molecules (proteins or small molecules) having amino groups.¹–⁴ We have developed this method specifically for use with surface plasmon resonance (SPR) spectroscopy, but it should be useful for other forms of surface spectroscopy as well.⁵–⁸ SPR is a useful analytical tool for the study of biomolecular recognition at surfaces⁹–¹⁰ because it can monitor interactions between proteins and immobilized ligands in real time. SPR is an optical technique that senses the refractive index of a medium near a thin film of metal deposited on a glass substrate (Figure 2). The angle of minimum intensity of reflected light, the resonance angle ($\Theta_{res}$), is affected by changes in the refractive index of the medium near the surface of the metal film. Such changes in refractive index occur, for example, when a protein adsors to a surface. The BIAcore 1000—the instrument used in these studies—can detect the binding of protein to immobilized ligand at the level of less than 1% of a complete monolayer.
Currently, there are two commonly used methods for the generation of surfaces or surface films presenting ligands for use in SPR studies: (1) derivatization of the commercially available carboxymethyl dextran-coated substrates (CM5 chips) with protein or low-molecular-weight ligands and (2) organic synthesis of small molecule ligands covalently linked to thiols, followed by incorporation of these thiols into mixed SAMs. The dextran substrates are convenient to use because a variety of chemical methods for immobilizing proteins or low-molecular-weight ligands to dextran have been developed and do not have ambiguities associated with nonspecific binding, exclusion of large proteins from the interior of the gel, ambiguities in the influence of mass transport on the values of $k_{on}$ and $k_{off}$, and high cost. Mixed SAMs made from thiols presenting ligands are useful for SPR studies because they are structurally well-characterized on a molecular scale, can be tailored to be highly resistant to nonspecific adsorption (by incorporating (EG) groups in the SAM) and do not have ambiguities associated with interfacial partitioning.


Figure 1. Schematic drawing of the common reactive intermediate method used to immobilize ligands. The top panel illustrates the immobilization of ligands by (1) activation of the surface carboxylic acid groups with NHS and EDC to form the NHS ester and (2) displacement of the NHS ester with an amino group on the ligand (ε-amino groups of lysine residues of proteins or primary amino groups of low-molecular-weight ligands) to form an amide bond. The lower panel illustrates the differences between the common intermediate method and the method involving the synthesis of alkanethiol-based ligands. In the common intermediate method, a SAM bearing carboxylic acid groups is formed by the cochemisorption of alkanethiols and NHS, followed by activation with NHS and EDC, and then used to form mixed SAMs. The methylene chains of the alkanethiols in the SAM are drawn in the all-trans conformation; this conformation has been observed in SAMs of long-chain alkanethiols on gold. The oligo(ethylene glycol) chains are depicted with little or no ordering; the detailed conformation in these SAMs is not firmly established (see ref 27).

Figure 2. Schematic representation of the experiment. Light (p-polarized, 760 nm) is incident on the backside of a glass slide on which Ti (15 Å) and Au (380 Å) have been evaporated. The instrument (BIAcore) measures the angle at which a minimum in intensity is observed (resonance angle), $\theta_m$. Changes in $\theta_m$ are caused by changes in the refractive index of the medium adjacent to the thin metal film that occur, for example, when a protein binds to a ligand immobilized at the interface.

of large proteins because binding occurs at the surface of the SAM. The disadvantage of using such SAMs is that synthesis of ligand-presenting thiols is laborious and impractical (or even impossible) in the case of peptides, proteins, or other sensitive biomolecules. By providing a structurally well-defined surface that
is highly resistant to nonspecific adsorption, but one that is appropriate for immobilization of proteins and ligands with sensitive functional groups, the common intermediate method described here combines the advantages (and circumvents some of the disadvantages) of these two established methods.

We prepared mixed SAMs comprising 1 and 2 by chemisorption of these alkanethiols onto the gold surface. By changing the ratio of 1 to 2, we were able to control the surface density of ligands immobilized at the surface. Our investigations were designed to demonstrate the following: (1) the ability of mixed SAMs comprising 1 and 2 to resist nonspecific adsorption of proteins, (2) the effectiveness of reactions that activate the carboxylic acid group of 2 for nucleophilic coupling (leading to the formation of amide bonds) to proteins and low-molecular-weight ligands, and (3) the biospecific recognition of molecules in solution by ligands immobilized by this process. The studies of biospecific recognition involved the interaction of immobilized benzenesulfonamide with bovine carboxylic anhydride II (CA) and that of immobilized N-α-Ac-Lys-D-Ala-D-Ala (6) (N-α-Ac-KdAdA) with vancomycin.

**EXPERIMENTAL SECTION**

**Materials.** All materials and reagents were used as received. Protein A (P-6650), concanavalin A (C-2010), fibrinogen (F-3879), cytochrome c (C-7752), superoxide dismutase (S-2515), ribonuclease A (R-4875), aldolase (A-7145), vancomycin (V-2002), and N-α-Ac-KdAdA (A-6950) were obtained from Sigma. Bovine carboxylic anhydride II, trypsin, ovalbumin, and lysozyme were obtained from Worthington. Anti-DNP IgG (04-8500) was obtained from Zymed Laboratories. Sodium dodecyl sulfate (SDS) was obtained from Bio-Rad. NHS, ethylene dichloride (EDC), and p-carboxybenzenesulfonamide were purchased from Aldrich. Dextran-coated CM5 chips were purchased from BIAcore. Silicon wafers were purchased from Silicon Sense (Nashua, NH). Compounds 1, 3, and 5 were prepared as described previously.

**Preparation of Substrates and Mixed SAMs.** Substrates for SPR measurements were prepared by electron beam evaporation of Ti (1.5 nm) and then Au (38 nm) onto glass cover slides (0.2 mm thick, No. 2, Corning). For ellipsometric and IR measurements, we evaporated Ti (2 nm) and Au (200 nm) onto Si wafers. The metallized substrates were cut into rectangles about 1 cm by 1.5 cm. These chips were immersed in solutions of mixtures of the appropriate thiols (2 mM total concentration) for 12 h, then rinsed, rinsed with ethanol, and dried under a stream of nitrogen.

**Buffers and Solutions.** Phosphate-buffered saline (PBS: 10 mM phosphate, 138 mM NaCl, and 2.7 mM KCl) was prepared in distilled, deionized water. Solutions of NHS (0.10 M) and EDC (0.4 M) were prepared in distilled, deionized water immediately before use. Proteins to be immobilized were dissolved in sodium phosphate buffer (25 mM) and adjusted to the appropriate pH. Proteins used in the binding and protein resistance studies were dissolved in PBS. Solutions of proteins containing soluble inhibitors were prepared in PBS and kept at room temperature before use. Low-molecular-weight ligands 3 and 6 (2 mg mL⁻¹ in pH 8.0) were dissolved in pH 8.0 25 mM phosphate buffer. Ethanolamine was immobilized using a 1 M solution of the compound at pH 8.5. SDS was prepared as a solution (10 mg mL⁻¹ in PBS).

**Surface Plasmon Resonance Spectroscopy.** The BIAcore 1000 instrument was used for all SPR studies described in this paper. Our substrates were incorporated into the BIAcore cassette by removing the manufacturer’s substrate and gluing our chip into the cassette using a two-part epoxy (Devcon). The cassettes containing our substrates were then docked into the BIAcore 1000.

**Typical Immobilization Procedure.** For immobilization of protein, we used a flow rate of 5 μL min⁻¹. We prepared solutions of NHS (0.1 M) and EDC (0.4 M) in distilled deionized water. Activation of the surface was achieved by equilibration with PBS, followed by transformation of the surface carboxylic acid groups into NHS esters by passing a mixture of 0.05 M NHS and 0.20 M EDC in H₂O over the surface for 7 min (the mixture is prepared by the automated robotics of the BIAcore 1000 using the DILUTE command). The system was then returned to PBS (2 min), and the solution of the protein or ligand to be immobilized was injected over the surface (7 min) resulting in amide bond formation by displacement of the NHS esters. The system was returned to PBS, and excess NHS esters were deactivated by washing (5–10 min) with pH 8.6 sodium phosphate buffer (25 mM).

**Removal of Irreversibly Bound Material.** Surfaces bearing low-molecular-weight ligands 3 and 6 containing irreversibly adsorbed protein was cleaned by washing with SDS (10 mg mL⁻¹ in PBS) for 3 min. After this cleaning procedure, we passed buffer at a high rate of flow over the surface of the SAM using the UNCLOG command.

**Kinetics.** For kinetic measurements we used the KINJECT command. The data were collected at 10 Hz and a flow rate of 20 μL min⁻¹ for CA and 10–50 μL min⁻¹ for vancomycin. The BIAcore 1000 output is given in resonance units (RU, 10 000 RU °). We convert the output to changes in the resonance angle (Δθ). The data were analyzed by standard procedures using KaleidaGraph and BIAevaluation software packages.

**Ellipsometry.** A commercially available ellipsometer (AutoEL II, Rudolph, Inc.) was used for all the experiments. The silicon substrates were cut into squares 1.5 cm by 1.5 cm, washed with distilled H₂O and EtOH, and dried under a stream of nitrogen. We determined the optical constants for each substrate. The substrates were immersed in a mixture of thiols 1 and 2 ([1] + [2] = 2 mM in EtOH) for 12 h to form the SAM. Ellipsometric thicknesses were determined using the previously obtained optical constants. Immobilization of 3 to the mixed SAM comprising 1 and 2 was performed in glass scintillation vials according to the following procedure: (1) the substrate was equilibrated with PBS, (2) the surface carboxylic acid groups were transformed into activated NHS esters by immersing the substrate in a solution of 0.05 M NHS and 0.20 M EDC in water for 7 min, (3) the substrate was washed with PBS for 2 min, and (4) the substrate was immersed in a solution of 3 (2 mg mL⁻¹ in 25 mM phosphate buffer (pH 7.0) for 20 min). We determined the optical constants for each substrate. The substrates were immersed in a mixture of thiols 1 and 2 ([1] + [2] = 2 mM in EtOH) for 12 h to form the SAM. Ellipsometric thicknesses were determined using the previously obtained optical constants. Immobilization of 3 to the mixed SAM comprising 1 and 2 was performed in glass scintillation vials according to the following procedure: (1) the substrate was equilibrated with PBS, (2) the surface carboxylic acid groups were transformed into activated NHS esters by immersing the substrate in a solution of 0.05 M NHS and 0.20 M EDC in water for 7 min, (3) the substrate was washed with PBS for 2 min, and (4) the substrate was immersed in a solution of 3 (2 mg mL⁻¹ in 25 mM phosphate buffer (pH 7.0) for 20 min).
Figure 3. Plot of $\chi(2)$ in solution versus the thickness of the SAM measured by ellipsometry and of $\chi(2)$ in solution versus $\chi(2)$ in the SAM. We calculate the mole fraction of 2 in the mixed SAMs by comparing the ellipsometric thicknesses measured for the mixed SAMs with the ellipsometric thicknesses of the homogenous SAMs formed from 1 ($\chi(1) = 1.0$) and 2 ($\chi(2) = 1.0$). There is some uncertainty in our measurements of thickness due to the unknown influence of adsorbed water vapor. Three independent measurements of ellipsometric thickness are shown at each value of $\chi(2)$ in solution.

Figure 4. (A) Comparison of the amount of protein adsorbed on a SAM consisting of 1, a mixed SAM with $\chi(2) = 0.02$ (dashed line), and the CM dextran layer of the commercial BIAcore CM5 chip (dashed line); and to ethanolamine-derivatized SAM (SAM-EA) and dextran (dextran-EA) (solid lines). The coupling of ethanolamine was accomplished by injecting a 1 M solution of ethanolamine (pH 8.5) over the SAM (or gel) that was first activated by NHS/EDC (see Experimental Section and Figure 6 for immobilization procedure). The mixture of proteins used in this experiment contained superoxide dismutase, lysozyme, ovalbumin, trypsin, ribonuclease A, CA, aldo-, and fibrinogen, each at a concentration of ~0.5 mg mL$^{-1}$. The terms $\Delta\theta_{\text{loose, dex-EA}}$, $\Delta\theta_{\text{tight, dex-EA}}$, and $\Delta\theta_{\text{total, dex-EA}}$ refer to loosely bound, tightly bound, and total amounts of nonspecifically adsorbed protein to ethanolamine-derivatized dextran, respectively. (B) Resistance of a mixed SAM ($\chi(2) = 0.02$) to nonspecific adsorption of proteins of different molecular weight and pI. Lysozyme, ovalbumin, carbonic anhydrase, and fibrinogen are each at a concentration of 1.0 mg mL$^{-1}$ in PBS.

RESULTS AND DISCUSSION

Preparation and Characterization of Mixed SAMs. Mixed SAMs comprising 1 and 2 were prepared by immersing the gold-coated substrates in solutions of mixtures of 1 and 2 of the stated mole fraction overnight; the substrates were then removed, rinsed with ethanol, and dried under a stream of nitrogen. The mole fractions of all the SAMs reported in this paper refer to the mole fractions of the solutions used to prepare the SAM. That is, we did not determine the composition of most of the SAMs directly, but refer to them by the concentrations of the solutions from which they were made. On the basis of earlier studies, we believed that the compositions of the SAMs would be similar but not identical.

The incorporation of different thiols into the SAM from a mixture of thiols in solution depends on the chemical structure of the thiols.\textsuperscript{16,29,38} We wished to determine, therefore, the mole fraction of 1 and 2 in SAMs formed from a solution containing 1 and 2 at a given mole fraction. For this purpose, we measured the thickness of a series of SAMs formed from solutions of 1 and 2 at different ratios. Figure 3 shows a plot of $\chi(2)$ in solution versus the thickness of the SAM; we convert this plot into $\chi(2)$ in solution versus $\chi(2)$ in the SAM by assuming that the measured


ellipsometric thickness depends linearly on mole fraction. At all mole fractions, 2 was incorporated into the SAM in greater than statistical proportions; this difference was most pronounced at $\chi(2) \sim 0.4$.

**Resistance of Mixed SAMs Prepared from 1 and 2 to Nonspecific Adsorption of Proteins.** As a first step in establishing the feasibility of the common intermediate strategy, we needed to demonstrate the ability of mixed SAMs comprising 1 and 2 to resist nonspecific adsorption of proteins. A mixed SAM containing a relatively low mole fraction of 2 ($\chi(2) = 0.02$) was selected for this demonstration since the immobilization and binding studies described below were performed using similar SAMs. Figure 4a shows the sensorgrams obtained when a solution containing a mixture of eight proteins was injected over a SAM containing 1 only, a mixed SAM ($\chi(2) = 0.02$), a mixed SAM ($\chi(2) = 0.02$) with covalently immobilized ethanolamine (that is, one in which the molecules of 2 terminated in CONHCH$_2$CH$_2$OH groups), a carboxymethyl dextran gel (the BIAcore CM 5 chip), and a dextran gel with covalently immobilized ethanolamine (with CO$_2$H groups also converted to CONHCH$_2$CH$_2$OH groups). Procedures for reaction of the active ester with ethanolamine (as described below and in the Experimental Section) were similar to those used for proteins and other ligands. For the homogeneous SAM consisting of 1, we observed a fast increase in $\Delta \Theta$ (within ~ 5 s) and no further increase in the value of $\Delta \Theta$ thereafter; the fast increase reflects differences in bulk refractive index ($\Delta n$) between the solution of protein and the buffer. When the solution of protein was replaced by buffer, there was a fast drop in the value of $\Delta \Theta$ (within 5 s) back to its original value, i.e., the value preceding injection of the protein. An almost indistinguishable sensorgram was also obtained for the mixed SAM derivatized with ethanolamine. The lack of any further increase in $\Delta \Theta$ after the initial fast increase, and the fast drop in the value of $\Delta \Theta$ back to its original value, suggest that there was no observable nonspecific binding to these neutral SAMs. For the underviratized mixed SAM, we observed an increase in the value of $\Delta \Theta$ (after injection of protein) that was slightly greater than that observed for the ethanolamine-derivatized SAM; we infer that there was a small amount of nonspecific adsorption (0.1%) due to the carboxylate groups in these SAMs. We hypothesize that this nonspecific binding was because of electrostatic interactions between the positively charged proteins in the mixture (e.g., trypsin and lysozyme) and the carboxylate groups.18 The amounts of nonspecific adsorption to carboxymethyl dextran and to dextran derivatized with ethanolamine were higher; the amount of nonspecific binding to dextran with derivatized ethanolamine (~0.6%) was, however, half that with carboxymethyl dextran (~1.2%). While most of the nonspecific adsorption to these dextran gels was reversible (labeled $\Delta \Theta_{\text{dose, dextran}}$ for protein loosely bound to the ethanolamine-derivatized dextran), some of the adsorption was irreversible (labeled $\Delta \Theta_{\text{light, dextran}}$ for the ethanolamine-derivatized dextran). The large amounts of nonspecific adsorption to these dextran-based chips, though mostly reversible, can be problematic. In fact, nonspecific adsorption consisting of loosely bound protein can be as problematic as (if not more problematic than) irreversibly bound protein, especially in kinetic studies with fast rates of association and dissociation, such as those between antibodies and antigens ($k_{\text{on}} \sim 10^6$–$10^7$ M$^{-1}$ s$^{-1}$).20,37

Figure 4b shows the sensorgrams obtained when solutions of lysozyme, ovalbumin, carbonic anhydrase, or fibrinogen were passed over a mixed SAM ($\chi(2) = 0.02$). None of these proteins, with their widely different molecular weights (MW) and isoelectric points (pI), showed significant amounts of nonspecific adsorption. These mixed SAMs do show significant levels of nonspecific adsorption with cytochrome c. The interaction of cytochrome c with surfaces presenting carboxylic acid groups is well documented; this interaction is mediated by a patch of five surface-exposed lysine residues in the vicinity of its heme group.38,39

Figure 5 shows sensorgrams obtained when solutions of cytochrome c and lysozyme (each at 0.5 mg/mL, in PBS) to mixed SAMs with increasing mole fractions of 2 ($\chi(2) = 0.02$; 0.10; 0.20; 0.40). The inset shows a plot of the difference in $\Delta \Theta$ ($\Delta\Delta \Theta$) between the mixed SAM and the homogeneous SAM consisting of 1 (measured at $t = 600$ s) versus $\chi(2)$. The nonspecifically bound proteins could be dissociated from the surface by washing it with 0.1 N HCl.

![Figure 5](image)

**Figure 5.** Binding of cytochrome c and lysozyme (each at 0.5 mg/mL, in PBS) to mixed SAMs with increasing mole fractions of 2 ($\chi(2) = 0.02$; 0.10; 0.20; 0.40). The inset shows a plot of the difference in $\Delta \Theta$ ($\Delta\Delta \Theta$) between the mixed SAM and the homogeneous SAM consisting of 1 (measured at $t = 600$ s) versus $\chi(2)$. The nonspecifically bound proteins could be dissociated from the surface by washing it with 0.1 N HCl.

pl than cytochrome c. We infer that nonspecific adsorption of proteins onto mixed SAMs comprising 1 and 2 and containing low mole fractions of 2 is not generally observed but can become problematic for proteins that contain localized patches of positively charged residues.

**Immobilization of Proteins to Mixed SAMs by NHS/EDC-Mediated Peptide Coupling.** This section describes the general procedure for immobilizing proteins onto mixed SAMs by peptide bond-forming reactions mediated by NHS/EDC. It also describes the effects of $\chi(2)$, the concentration of protein, the molecular weight of the protein, the pH of the coupling buffer, and the presence of added salt (NaCl) on the amount of protein immobilized on the mixed SAMs.

Coupling Procedure. The coupling was carried out by first injecting an aqueous solution containing NHS and EDC over the SAM to transform the carboxylic acid groups of 2 into the activated NHS esters and then injecting a solution of the protein over the reactive surface. The surface was washed with PBS after each injection, and unreacted NHS esters were hydrolyzed by washing the surface with pH 8.6 phosphate buffer. We chose pH 8.6 buffer for NHS ester deactivation based on the short half-life (0.75 min) of NHS esters of α-alkoxy acids at pH 8. Figure 6 is a sensorgram showing immobilization of carbonic anhydrase to a mixed SAM using this procedure.

**Effect of $\chi(2)$ on the immobilization of CA.** Figure 7a shows the immobilization of CA to mixed SAMs with increasing $\chi(2)$. The amount of protein immobilized does not increase linearly with the increase in $\chi(2)$; $\Delta \theta$ for the SAM with $\chi(2) = 0.02$ is ~60% of $\Delta \theta$ for the SAM with $\chi(2) = 1.0$. Figure 7b shows a schematic depiction of a mixed SAM comprising 1 and 2 ($\chi(2) = 0.02$) before and after coupling with carbonic anhydrase. This figure illustrates our inference that a mole fraction of $\chi(2) = 0.02$ is sufficient for immobilization of almost a complete monolayer of CA, since the cross-sectional area of the CA (~1300 Å²) covers a large number (>60) of thiolates in the SAM. Identical experiments using lysozyme (Table 1, entries 10−11) show a similar trend; the amount of lysozyme immobilized with $\chi(2) = 0.02$ is nearly 80% of the value observed for $\chi(2) = 1.0$.

**Generality of the Immobilization Method.** We wanted to establish the generality of this method for the immobilization of proteins onto mixed SAMs. Table 1 summarizes the results for the immobilization of a panel of proteins onto mixed SAMs ($\chi(2) = 0.02$). We were able to immobilize proteins of widely different molecular weights (12 400−340 000) and pl (4.5−11). The following sections describe the influence of several variables that were important in determining the amount of protein that was immobilized.

**Influence of Molecular Weight on the Amount of Protein That Is Immobilized.** Figure 8 shows a plot of the molecular weight of the protein versus the maximum observed amount of immobilization ($\Delta \theta_{\text{immob}}$) on a SAM with $\chi(2) = 0.02$. Higher amounts of immobilization are obtained with proteins of higher molecular weight. This observation is intuitively reasonable, since

(43) Shearwater Polymers, Inc. Catalog, Polyethylene Glycol Derivatives; Shearwater Polymers: Huntsville, AL, 1996.
larger proteins can form thicker layers on the SAM. We can obtain a theoretical estimate of the maximal amount of immobilization for a protein by calculating the value of $\Delta \Theta$ ($\Delta \Theta_{\text{immob}}$) corresponding to the maximum number of molecules of protein (~0.9–(10^2 M/Å^2) per millimeter square that can be close-packed in a hexagonal arrangement (eqs 1 and 2). We assume that 0.1% ~ 1

$$\Delta \Theta_{\text{immob}} = \frac{0.9(10^2 M/Å^2)}{10^{-9} N_A/M W}$$

$$r = 10^6(3M W/4\pi N_A)^{1/3}$$

ng mm^-2,^12 which corresponds to 10^-9 N_A/M W molecules. In these equations, M W and r are the molecular weight and radius (in Å), respectively, of the protein, and we assume that the proteins are hard spheres of uniform density (r ~ 1.30 g cm^-3).^45 The ratio of to $\Delta \Theta_{\text{immob}}$ to $\Delta \Theta_{\text{frac cover}}$ provides an estimate of the fractional coverage of the monolayer by the immobilized protein; for all the proteins immobilized under the given experimental conditions, the value of $\Delta \Theta_{\text{frac cover}}$ is ~ 0.4. The two proteins with the highest values of $\Delta \Theta_{\text{frac cover}}$ (0.88 and 0.94) are carbonic anhydrase and lysozyme, proteins for which the conditions for immobilization were optimized. We have not optimized conditions for the other proteins listed in Table 1.


### Table 1. Tabulation of the Amount of Protein Immobilized to Mixed SAMs Comprising 1 and 2 Using NHS/EDC-Mediated Coupling Reactions

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<th>entry</th>
<th>protein</th>
<th>$\chi(2)$a</th>
<th>MWb</th>
<th>plb</th>
<th>conc (mg mL^-1)</th>
<th>pH of coupling</th>
<th>NaCl additivesc</th>
<th>hard-sphere radius (Å)d</th>
<th>$\Delta \Theta_{\text{immob}}$ (10^4 deg)</th>
<th>$\Delta \Theta_{\text{frac cover}}$ (10^4 deg)</th>
<th>$\Delta \Theta_{\text{frac cover}}$ (10^4 deg)</th>
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<td>1466 ± 150</td>
<td>3800</td>
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<td>4.5–5.5</td>
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<td>4.0</td>
<td>25.3</td>
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<td>0.5</td>
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<td>47.0</td>
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<td>4.0</td>
<td>23.2</td>
<td>1936 ± 24</td>
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<td>20.9</td>
<td>3150 ± 50</td>
<td>3300</td>
<td>95</td>
<td></td>
</tr>
</tbody>
</table>

a A mole fraction of $\chi(2) = 0.02$ is implied unless otherwise noted.

b References 45 and 46. c Unless stated otherwise, no additional components were added to the solution of protein in 25 mM sodium phosphate at the indicated pH. d Calculated according to eq 2. e Calculated using eq 1.

f Calculated by dividing $\Delta \Theta_{\text{immob}}$ by $\Delta \Theta_{\text{frac cover}}$. g Concanavalin exist as either a dimer (52 000) or a tetramer (104 000) in solution. At pH 4.0 it exists as the tetramer. h Aldolase exists in solution as a tetramer (161 000). i In this experiment, the additive containing solution was passed over the surface for 2 min followed by a solution of protein containing no additive for 7 min. j The amount of protein immobilized in these experiments slightly exceeds $\Delta \Theta_{\text{frac cover}}$. We assign a value of $\Delta \Theta_{\text{frac cover}} = 100$ in these situations. k Not reported.
The observation that we can obtain values of $\Delta \Theta_{\text{frac.cover}}^{\text{immb}}$ that are close to unity is not compatible with a mechanism for immobilization based on irreversible random sequential adsorption of protein to the SAM. Simulations of processes based on irreversible random sequential adsorption suggest that the maximal coverage obtainable during adsorption is $\sim 55\%$ higher coverages are not obtained because of steric overlap with preadsorbed molecules. In principle, it is possible to obtain higher fractional coverage during random sequential adsorption if equilibrium is achieved prior to adsorption; this scenario is probably unlikely (though it cannot be ruled out; see Results and Discussion on pH dependence of immobilization) for the irreversible coupling of proteins to SAMs presenting reactive NHS esters. We hypothesize that this discrepancy is due to the assumption that $0.1 \sim 1$ ng mm$^{-2}$; this assumption is based on the change in the resonance angle that represents the average for binding of protein in a dextran gel $\sim 100$ nm thick. In reality, molecules of protein nearer the gold film make a greater contribution to the SPR signal than those further away, because the evanescent electromagnetic field exponentially decays with a decay length estimated to be $\sim 25$–$50\%$ of the wavelength of the incident light. In a mixed SAM, binding or immobilization of protein occurs at a fixed distance from the gold film, within $\sim 25$ nm of the surface of the gold (even for large proteins such as antibodies); therefore, a SPR signal of $0.1^\circ$ would correspond to less than $1$ ng mm$^{-2}$ of adsorbed protein.

**Effect of the Concentration of Protein.** For CA and lysozyme, increasing the concentration of protein increases the amount of protein immobilized (Table 1), over the concentration range studied (up to $0.5$ mg mL$^{-1}$); this observation suggests that, at these concentrations, the coupling reaction does not go to completion in the $7$ min allotted for the reaction. If desired, it may be possible to drive the reaction to completion by the use of a higher concentration of protein or by allowing the coupling reaction to proceed for a longer period of time.

**Effect of pH of the Coupling Buffer.** The levels of immobilization for a panel of ten proteins were investigated at pH $8.0$ and at a pH one unit below the pI of the protein (Table 1). Moreover, we chose to immobilize three proteins—CA, lysozyme, and concanavalin A—at several different values of pH (Table 1). We found that the pH of the coupling buffer greatly influenced the amount of protein that was immobilized. We were particularly surprised that lysozyme (pI $11$) showed highest levels of immobilization at pH $10.0$ whereas both concanavalin A (pI $4.5$–$5.5$) and CA (pI $5.9$) showed highest levels of immobilization at pH $4.0$. On the basis of these observations, we conclude that the highest levels of immobilization for proteins are obtained when the buffer used during coupling has a pH approximately one unit below the pI of the protein (pI$-\sim 1$), that is, in a regime in which the protein has a slight net positive charge. This result is nonintuitive, since a higher proportion of lysine $+$-amino groups are protonated at lower values of pH than at higher values of pH; this protonation makes them less available as nucleophiles.

We do not have a certain explanation for this influence of pH on the amount of protein that is immobilized. The hypothesis that the NHS ester undergoes buffer-catalyzed hydrolysis can be ruled out because highest levels of immobilization for lysozyme are obtained at pH $10.0$. Moreover, if the buffer were catalyzing the decomposition of NHS esters, the level of immobilization would be independent of the protein used.

A similar pH dependence on the amount of immobilization is observed in the carboxymethyl dextran matrix of the CM5 chips. It has been proposed that favorable electrostatic interactions between the positively charged protein (at values of pH below the pI of the protein) and the negatively charged matrix are responsible for this effect. Two observations suggest that a similar hypothesis—that is, favorable electrostatic interactions between the protein (below its pI) and the SAM prior to coupling—might explain the pH dependence observed on SAM s. (i) We have investigated the influence of added salt in the buffer during coupling; entries 5e, 5f, and 10e–i (Table 1) present the results of the immobilization of CA and lysozyme in the presence of added NaCl. Addition of NaCl dramatically reduced the amount of protein that was immobilized but did not appear to deactivate the NHS ester. (ii) We also studied the adsorption of CA, dissolved in phosphate buffer (25 mM) at several different values of pH (from 4 to 8.7), to mixed SAM s (underivatized) presenting high densities of carboxylic groups ($\chi(2) \sim 0.60$). The highest amount of adsorption was observed at pH $4$; there was a $\sim 15$-fold decrease

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(49) Lukosz, W. Biosens. Bioelectronics 1997, 12, 175.
(53) When solutions containing NaCl but no protein (entries 10g and 10i) were passed over the SAM before allowing that SAM to react with the solution containing both NaCl and protein, we observed similar levels of immobilization to experiments where the solution containing NaCl and protein were reacted with the SAM directly (entry 10d).
in the amount of adsorption as the pH was increased from 4 to 8.7. Moreover, the amount of adsorption decreased upon adding NaCl. While these observations support the hypothesis of favorable electrostatic interactions between the protein and the SAM, there remains an ambiguity before coupling, the carboxylic acid groups are quantitatively converted to NHS esters (see below), and we expect the resulting surfaces to be largely neutral (at least originally). Moreover, the coupling of the positively charged protein might make the surface (especially at low $\chi(2)$ overall positively charged. Obviously, if hydrolysis (or the possible formation of acyl phosphates) of the NHS esters is fast relative to coupling of the protein, this hypothesis would still be valid.\(^{54}\)

**Immobilization of Benzenesulfonamide Ligands to Mixed SAMs by NHS/EDC-Mediated Coupling and Estimation of Coupling Yields.** We coupled the benzenesulfonamide ligand \(3\) to the mixed SAMs made from \(1\) and \(2\) for studies of binding to carbonic anhydrase (Figure 9). By comparing results obtained by coupling (of \(3\) to mixed SAMs of \(1\) and activated \(2\)) to those obtained using \(1\) and \(5\), we could determine the efficiency of the coupling reaction.

Ellipsometry. From a plot of the thickness determined by ellipsometry for mixed SAMs prepared from \(1\) and \(2\) as well as for those same mixed SAMs after NHS/EDC-mediated coupling with \(3\), we can estimate the coupling yields (yield\(_{\text{coupling}}\)) by dividing the observed increase in ellipsometric thickness (\(\Delta T_{\text{obs}}\)) after coupling by the predicted increase in ellipsometric thickness (corresponding to quantitative coupling) (\(\Delta T_{\text{predict}}\)) i.e., yield\(_{\text{coupling}} = (\Delta T_{\text{obs}}/\Delta T_{\text{predict}}) \times 100\) (Figure 10). The value of \(\Delta T_{\text{predict}}\) can be estimated using eqs 3 and 4, where \(\chi(2)_{\text{SAM}}\) is the mole fraction of \(2\) in a mixed SAM with thickness \(T_{\text{mixedSAM}}\), \(\Delta T_{5-2}\) is the difference in ellipsometric thickness (~21 Å) between a SAM consisting only of \(2\) (~41 Å), and \(\Delta T_{2-1}\) is the difference in ellipsometric thickness (~20 Å) between a SAM consisting only of \(5\) (~61 Å) and a SAM consisting only of \(2\). At values of \(\chi(2) < 0.4\), the values of yield\(_{\text{coupling}}\) are >80% there is, however, a rapid decrease in the coupling yields at \(\chi(2) >0.4\) (the coupling yield is only ~50% at \(\chi(2) \sim 0.6\)).\(^{56}\)

**Polarized Infrared External Reflectance Spectroscopy.** The molecular level details of the immobilization of the benzenesulfon-
Figure 10. Yield of coupling of 3 (yield coupling (%)) to mixed SAMs versus $\chi(2)$. These yields are based on the increase in ellipsometric thickness of the mixed SAMs after coupling with 3. The error bars indicate the error in estimation of yields based on an uncertainty of ±1 Å in measurements of ellipsometric thickness; the expected increase in thickness is smaller at lower $\chi(2)$ than at higher $\chi(2)$; hence, the uncertainty in estimations of yield are higher at lower values of $\chi(2)$ than at higher values of $\chi(2)$.

Table 2. Values of $K_d$, $k_{on}$, and $k_{off}$ As Measured in Solution and at Interfaces

<table>
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<th>protein</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_d$ (mM)</th>
<th>$k_{on}$</th>
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<td>6</td>
<td>Van</td>
<td>h</td>
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<sup>a</sup> Prepared using the common intermediate method to immobilize 3 or 6 on a mixed SAM prepared from a solution containing 1 and 2.
<sup>b</sup> Prepared directly from a solution containing thiols 1 and 5.
<sup>c</sup> Not reported. The values of $k_{off}$ for six different peptide-terminated 4-carbamoylbenzenesulfonamide derivatives are reported in ref 62. The values of $K_d$ ranged from 0.120 to 0.011 s$^{-1}$.<sup>d</sup> Using a mixed SAM prepared by the common intermediate method as the substrate.<sup>e</sup> Could not be determined from our experiments.<sup>f</sup> The dextran matrix of the CM5 chip was used as the substrate. The dextran rate constants could not be determined accurately because the effect of mass transport could not be eliminated.

Amide ligand were studied using PIERs. PIERs provides evidence for the presence of specific functional groups on the surface of the SAM and may provide information about the conformation of the methylene groups in the alkane chain. In PIERs, vibrational modes with transition dipole moments perpendicular to the surface show signals with maximum intensity; those vibrational modes with dipole moments parallel to the surface show weak signals. Figure 11 shows the PIERs spectra obtained for (a) a homogenous SAM of 1, (b) a homogenous SAM of 2, (c) a homogenous SAM of 3, (d) a mixed SAM comprising 1 and 2 ($\chi(2) = 0.1$), (e) a mixed SAM comprising 1 and 2 ($\chi(2) = 0.1$) after reaction with NHS and EDC, (f) a mixed SAM comprising 1 and 2 ($\chi(2) = 0.1$) after activation with NHS and EDC followed by amide bond formation with 3, and (g) a mixed SAM comprising 1 and 5 ($\chi(5) = 0.40$).

We now consider the IR spectra of the mixed SAMs before and after NHS/EDC-mediated reaction with 3. The IR spectrum (between 1500 and 1850 cm$^{-1}$) of the mixed SAM comprising 1 and 2 ($\chi(2) = 0.1$) is indistinguishable from that of a homogenous SAM comprising 2; we attribute the band at 1631 cm$^{-1}$ to the C=O stretch of the carboxylic acid groups. The frequency of this absorption is lower than that expected for an α-alcohol (-1730 cm$^{-1}$). We do not know the exact reason for this shift, although we hypothesize that intermolecular hydrogen bonding or ionization might be responsible (see below). The IR spectrum for the homogeneous SAM comprising 5 shows a strong band at 1553 cm$^{-1}$ and a weaker band at 1647 cm$^{-1}$ (Figure 11c). We assign the band at 1553 cm$^{-1}$ to the NH bending modes and the band at 1647 cm$^{-1}$ to the C=O amide stretches of 5.

Figure 11. PIERs spectra obtained for (a) a homogenous SAM of 1, (b) a homogenous SAM of 2, (c) a homogenous SAM of 3, (d) a mixed SAM comprising 1 and 2 ($\chi(2) = 0.1$), (e) a mixed SAM comprising 1 and 2 ($\chi(2) = 0.1$) after reaction with NHS and EDC, (f) a mixed SAM comprising 1 and 2 ($\chi(2) = 0.1$) after activation with NHS and EDC followed by amide bond formation with 3, and (g) a mixed SAM comprising 1 and 5 ($\chi(5) = 0.40$).

We focus on the C=O stretching region (1500–1850 cm$^{-1}$) of the spectrum, since this region is most diagnostic for the changes that occur during activation of the carboxylic acid group and formation of the amide bond.

We first examine the IR spectra of the homogeneous SAMs ($\chi(1) = 1.0$, $\chi(2) = 1.0$, and $\chi(5) = 1.0$). For these samples, the substrates were washed (after immersion in the appropriate thiols) in ethanol only and then dried under nitrogen. The spectrum for the tri(ethylene glycol)-terminated thiol 1 is, as expected, featureless in this region of the spectrum (Figure 11a). In contrast, the spectrum for the SAM comprising 2 shows a broad absorption centered at 1631 cm$^{-1}$ (Figure 11b). We assign this band to the C=O stretch of the carboxylic acid groups. The frequency of this absorption is lower than that expected for an α-alcohol (-1730 cm$^{-1}$). We do not know the exact reason for this shift, although we hypothesize that intermolecular hydrogen bonding or ionization might be responsible (see below). The IR spectrum for the homogeneous SAM comprising 5 shows a strong band at 1553 cm$^{-1}$ and a weaker band at 1647 cm$^{-1}$ (Figure 11c). We assign the band at 1553 cm$^{-1}$ to the NH bending modes and the band at 1647 cm$^{-1}$ to the C=O amide stretches of 5.

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for the product SAM (Figure 11f). The spectrum shows strong bands of approximately equal intensity at 1550 and 1663 cm⁻¹, as well as a weak band at 1733 cm⁻¹. The bands at 1550 and 1663 cm⁻¹ correspond to the NH bending modes and the C=O amide stretches of 5. The band at 1733 cm⁻¹ corresponds to the C=O stretching frequencies of carboxylic acid groups produced by hydrolisis of the NHS ester.58 Next, we measured the IR spectrum for a mixed SAM prepared from 1 and 5, χ (5) = 0.40 (Figure 11g). In this spectrum, the bands at 1550 and 1663 cm⁻¹ are of almost equal intensity and the overall spectrum is almost indistinguishable from the one measured for the SAM prepared by the common intermediate method. On the basis of the comparison between these two spectra (Figure 11f and g), we infer that the reaction of the NHS ester with the amino group on the ligand 3 proceeds in good yield (>80%). The differences in the relative intensities of the bands corresponding to the NH bend and C=O stretch between the homogeneous SAM made from 5 and those consisting of mixed SAM’s prepared by using either the common intermediate strategy or by using 1 and 5 suggest that there are differences of presently undefined nature between the benzene-sulfonamide headgroups of the SAM that is homogeneous in 5 and the mixed SAM’s.

Equations for 1:1 Binding of Ligand to Receptor. This section summarizes the basic equations we used to determine (1) equilibrium binding, (2) kinetics of dissociation, and (3) kinetics of association of a protein to a surface.10,22,32–34,59

Equilibrium Binding (Scatchard Analysis). We assume an equilibrium described in eq 5. In this section P refers to a protein, L refers to a ligand, species marked with an asterisk (*) are bound to the surface, and subscripts 0, t, and eq refer to the concentration of a given species at time = 0, time = t, and at equilibrium, respectively. At equilibrium, eq 6 is valid regardless of the influence of mass transport on the kinetics of association or dissociation. Substituting eq 7 into eq 6 and rearranging gives eq 8. According to eq 8, a plot of [P-Leq*]/[P] versus [P-Leq*] should be a straight line with slope equal to −1/Kd (a Scatchard plot).45

Kinetics of Dissociation.32 The rate of dissociation of the protein from the surface is given by eqs 9 and 10. Separating variables and integrating gives eq 11, which can also be written as eq 12. The kinetic constant, koff, can be obtained by fitting the experimental data to either eq 11 or eq 12.

Kinetics of Association.21,32–34 The rate equation for association can be written as in eq 13 (units: koff (M⁻¹ s⁻¹), [P] (M), and [L*] (mol mm⁻²)). Substituting eq 7 (mass balance) into eq 13 gives eq 14. Equation 15 defines k on; we measure k on as the slope of a plot of d[P−LΔ*]/dt versus [P−LΔ*]. We calculate the value of koff by measuring k on at several values of [P], plotting k on versus [P], and estimating the value of koff from the slope of that plot.

Studies of Binding of CA to Immobilized Benzzenesulfonamide Ligands: Comparisons between Mixed SAMs and Dextran. The CA−benzenesulfonamide interaction has been studied extensively in solution; reported values of koff in solution are ~10⁻²−10⁻¹ s⁻¹ and those for koff are 10⁻³−10⁻² M⁻¹ s⁻¹.60–65 We studied binding of CA to three different surfaces presenting benzzenesulfonamide ligands: mixed SAM’s consisting of 1 and 5;34 mixed SAMs consisting of 1 and 2 to which 3 had been covalently immobilized; and dextran-based surfaces to which 3 had been covalently immobilized. Table 2 summarizes the results of these studies.

Biospecific Binding of CA to Mixed SAMs Presenting Benzzenesulfonamide Ligands Prepared by the Common Intermediate Method. Figure 12a shows the sensorgrams obtained when solutions of CA were passed over a mixed SAM (χ (2) = 0.01) to which benzenesulfonamide 3 had been covalently immobilized. The binding observed in all cases was largely reversible (>90%). The value of koff (calculated using eqs 11 and 12) was 0.0054 s⁻¹. We determined the on-rate constant, kon, by calculating initial values of k on (eqs 14 and 15), plotting those values of k on against [CA] and using the value of the slope of the k on versus [CA] plot as a measure of kon. We estimate that kon = 9.4 × 10³ M⁻¹ s⁻¹ and Kd = 0.56 μM. Figure 12b shows the sensorgrams obtained when solutions of CA containing soluble 4-carboxybenzenesulfonamide (4) were passed over a mixed SAM to which 3 had been covalently immobilized. The presence of 4 in the solution of CA reduced the concentration of free CA; 4 binds to CA with Kd = 1.25 μM.66 Correspondingly, the initial rate of binding of CA to the SAM decreased with increasing concentrations of 4; when the concentration of 4 was 50 μM, binding to the surface was completely

\[ P + L^* \overset{k_{on}}{\rightarrow} P \cdot L^* \]  
\[ -d[P-Lt*]/dt = k_{off}[P-Lt*] \]  
\[ \ln([P-Lt*]/[P-L0*]) = -k_{off}t \]  
\[ [P-Lt*] = [P-L0*]e^{-k_{off}t} \]

inhibited. We infer, therefore, that the binding of CA to immobilized 3 is biospecific. We can estimate the value of $K_d$ for the interaction between CA and 4 based on the decrease in the initial rates of binding\(^2\) (d[CA-3])**\(t\)\)/dt) of CA to immobilized benzenesulfonamide in the presence of 4 as a competitive inhibitor \(eqs 16\); in these equations, [CA] \(_o\) is the total concentration of CA and [CA] is the concentration of free CA available for binding to immobilized benzenesulfonamide. We calculated the concentration of the complex between CA and 4 ([CA-4]) from these calculations of [CA-4] at several different concentrations of 4, we constructed a Scatchard plot to determine the $K_d$ for the interaction between CA and 4 (Figure 12b); we estimate $K_d = 0.64 \, \mu M$.

**Biospecific Binding of CA in the Dextran Matrix of the CM5 Chip.** Benzenesulfonamide (3) was covalently immobilized to the carboxymethyl dextran matrix of a CM5 chip by (1) activating the carboxylic acid groups with NHS and EDC, (2) coupling the NHS esters with a mixture of 3 and ethanolamine, and (3) reaction of any remaining NHS esters with ethanolamine (1 M, pH 8.5). The mixture of 3 and ethanolamine was used in the immobilization step in order to reduce the amount of 3 that was immobilized.

Figure 13a shows the sensorgrams obtained when solutions of CA were passed over the dextran matrix immobilized with 3. The binding observed in all cases was greater than 80% reversible. Values of \(k_{on}\) and \(k_{off}\) obtained using methods similar to those for the mixed SAMs were \(k_{on} = 0.0052 \, \text{s}^{-1}\), \(k_{off} = 7600 \, \text{M}^{-1} \, \text{s}^{-1}\), and \(K_d = 0.68 \, \mu M\). Figure 13b shows the sensorgrams obtained when solutions of CA containing 4 were passed over the substrate; binding of CA to the substrate was inhibited in the presence of 4. These experiments show that binding of CA to 3 immobilized in the carboxymethyl dextran matrix is biospecific; we estimate that $K_d$ for the interaction between CA and 4 is \(~0.65 \, \mu M\).
The interaction of vancomycin with model surfaces presenting DADA groups in the bacterial cell wall. To test this hypothesis and others related to di- or multivalent binding, the development of surfaces that are reversible, offer the convenience of coupling of bioactive peptides, especially at higher mole fractions of carboxylic acid groups is undesirable, then the mixed SAM should be used instead of the common intermediate approach. The issue of coupling yield has its counterpart in the formation of mixed SAMs, where incorporation of each thiol into the monolayer does not usually occur statistically. Therefore, regardless of the method, it is necessary to quantify the composi-

Figure 14. (a) Sensorgrams obtained for the binding of vancomycin (0.2, 0.5, 1, 2, and 5 μM in 10 mM sodium phosphate, pH 7.4) to a mixed SAM presenting N-α-Ac-KoAoA groups. The mixed SAMs were prepared by NHS/EDC-mediated coupling of 6 (2 mg mL⁻¹ in 25 mM phosphate buffer, pH 8.0) to a mixed SAM (χ(2) = 0.15). The inset shows a Scatchard plot (ΔΘeq/Var) versus ΔΘeq) used to determine the Kd for the interaction of vancomycin with immobilized N-α-Ac-KoAoA. (b) Sensorgrams obtained when vancomycin (0.5, 1, 2, and 5 μM in 10 mM phosphate buffer, pH 7.4) was passed over a dextran matrix to which 6 had been covalently immobilized. The inset shows a Scatchard plot (ΔΘeq/Var) versus ΔΘeq) used to determine the Kd for the interaction of vancomycin with immobilized N-α-Ac-KoAoA.

Interaction of N₂-Ac-KoAoA Immobilized on a Mixed SAM with Vancomycin. The interaction of vancomycin at model surfaces presenting DADA groups is presently of great interest. Williams et al. have proposed that noncovalent divalency is important in the binding of vancomycin to dADA groups in the bacterial cell wall. To test this hypothesis and others related to di- or multivalent binding, the development of surfaces that are well defined, such as those consisting of mixed SAMs made from 1 and 2, offer the convenience of coupling of bioactive peptides, and have the flexibility of control of surface ligand density, could be useful.

We immobilized N-α-Ac-KoAoA to a mixed SAM with χ(2) = 0.15. Figure 14a shows the sensorgrams obtained when solutions of vancomycin were passed over this surface. The interaction was more than 80% reversible, and only small amounts of nonspecific adsorption were observed. The dissociation constants were determined by Scatchard analysis (eq 8) of the steady-state binding levels (ΔΘeq) (Figures 14a); we estimate Kd = 0.37 μM. These values are similar to the value determined previously in solution (Kd = 1.1 μM). We attempted to determine Koff by fitting the dissociation phase of the sensorgrams in Figure 14 to a single-exponential decay (eq 12) and also by linear fitting of the transformed data (eq 11). The experimental data fit poorly to both of these equations, and the values of Koff obtained depended on the concentration of vancomycin. These results suggested that mass transport of vancomycin to and from the surface of the mixed SAM limited the rates of interaction. The influence of the rate of mass transport on the kinetics of the interaction of vancomycin with immobilized N-α-Ac-KoAoA was verified by increasing the rate of flow of the vancomycin solution—at the higher rates of flow, the rates of association and rates of dissociation were faster, but the values of ΔΘeq were unaffected. The influence of the rate of mass transport was also detected by adding soluble N-α-Ac-K(N-v-Ac)-DADA (7) to the buffer during the dissociation of vancomycin from immobilized N-α-Ac-KoAoA—soluble N-α-Ac-K(N-v-Ac)-oAoA increased the rate of dissociation to a rate that was faster than that could be measured by the instrument. Our inability to eliminate the effects of mass transport effects in our system is not surprising given the reported kinetic constants (koff = 2.8 × 10⁷ M⁻¹ s⁻¹; koff = 31 s⁻¹).

Interaction of N-α-Ac-KoAoA Immobilized in the Dextran Matrix with Vancomycin. We immobilized N-α-Ac-KoAoA to the carboxymethyl dextran substrates using a procedure identical to that used for 3. Figure 14b shows the sensorgrams obtained when solutions of vancomycin were passed over these substrates. As in the case of the SAM bearing immobilized N-α-Ac-KoAoA, the dissociation of vancomycin from immobilized N-α-Ac-KoAoA on dextran was more than 80% reversible. The value of Kd (obtained by Scatchard analysis) was 0.38 μM; values of Koff and koff were not determined because the rates of interaction were limited by mass transport.

Comparisons among Methods Based on Immobilizing a Ligand Using a Common Intermediate, Mixed SAMs, and a Dextran Matrix. Comparisons between Ligands Immobilized Using a Common Intermediate Method and Mixed SAMs. The results obtained using a reactive common intermediate for the interaction of CA with surface-bound benzanesulfonamide are similar to those obtained previously using SAMs formed directly from 1 and 5 (Table 2). In addition to the close agreement between the derived kinetic constants, both procedures gave SAMs that showed largely reversible (>80%) binding.

Aside from the consideration of kinetic and thermodynamic constants, there are several issues concerning the use of the common intermediate approach. (1) Yields are not quantitative, especially at higher mole fractions of 2 in the SAM. Thus, SAMs formed by coupling 3 to mixed SAMs comprising 1 and 2 contain at least three thiolates (1, 2, 5); SAMs made using 1 and 5 only contain the two corresponding thiolates. If the presence of residual carboxylic acid groups is undesirable, then the mixed SAM approach should be used instead of the common intermediate approach. The issue of coupling yield has its counterpart in the formation of mixed SAMs, where incorporation of each thiol into the monolayer does not usually occur statistically. Therefore, regardless of the method, it is necessary to quantify the composi-


tion of each mixed SAM by direct methods, if this information is important. (ii) The common intermediate approach minimizes organic synthesis: a single set of mixed SAMs and a common procedure serve for a variety of ligands (low-molecular-weight molecules and proteins). In contrast, the synthesis of a ligand linked to an alkanethiol requires several chemical steps for low-molecular-weight ligands and may be impractical for chemically sensitive or expensive ligands. (iii) Since SAMs prepared by the common intermediate approach all derive from one common SAM, the distribution of ligands across the surface should be relatively independent of the structure of the ligand. In contrast, mixed SAMs prepared from a solution containing two different thiols may yield different spatial distributions of ligands in the SAM for different headgroups. Since the chemical structures of 1 and 2 are similar, they are unlikely to phase separate. On the basis of these considerations, we believe that the common intermediate approach for the functionalization of mixed SAMs is more convenient and versatile than that based on formation of mixed SAMs from a solution of two structurally complex thiols.

Comparisons between the Common Intermediate Method and Dextran Substrates. The kinetic and thermodynamic constants for the two interactions that we studied in this paper—CA-3 and vancomycin—were effectively indistinguishable for the SAMs prepared by the common intermediate method and the dextran substrates (Table 2). The reversibilities of the interactions were also comparable. Thus, in terms of kinetic and thermodynamic parameters and reversibility, these two approaches are indistinguishable in these systems. There are, however, several advantages (and a few disadvantages) to using the common intermediate approach. (i) SAMs are more well-defined structurally than a functionalized gel layer, and the character of the interface can be easily tailored by changing the chemical properties of the headgroups on the thiols. In addition, the mole fraction of each thiolate in the SAM can be controlled by changing the mole fractions of the thiols in solution. (ii) Mass transport phenomena are better defined for a SAM than for a hydrogel matrix. In particular, mass transport into the gels can pose a serious limitation for high-molecular-weight proteins. (iii) The ligands in a SAM are positioned at a uniform distance from the gold layer, rather than being distributed throughout a thick gel layer. The sensitivity of SPR to changes in refractive index decreases as the protein is farther from the gold layer; proteins that are bound at the edge of the hydrogel matrix, therefore, will make a smaller contribution to \( \Delta \theta \) than those nearer the gold film. (v) The cost is significantly lower.

There is, nonetheless, at least one advantage in the use of the available dextran-based substrates: they are commercially available and several methods for immobilization have been published. Similar coupling methods might be adapted for use on SAMs, although we have only demonstrated the applicability of the NHS/EDC-mediated peptide coupling reactions (see ref 17 for a SAM that immobilizes His-tagged proteins).

CONCLUSIONS

We have developed a strategy for the generation of interfaces presenting ligands that relies on the activation of carboxylic acid groups to NHS esters in a mixed SAM and the formation of an amide bond by reaction with an amine. This method combines many of the features of established methods using mixed SAMs and carboxymethyl dextran—well-defined interfacial structure, an interface that effectively resists the nonspecific adsorption of protein, control over ligand density, applicability to the immobilization of proteins and to a wide range of ligands without the need for organic synthesis, and low cost. This combination is attractive; the savings in terms of synthetic effort can be substantial since a potential ligand can be immobilized and evaluated in 1 day with the common intermediate method, whereas the solution-phase synthesis of complicated thiol molecules can take weeks. In addition, the common intermediate approach should produce a constant distribution of ligands on the SAM that would allow better comparisons between ligands than the approach based on mixed SAMs. Although these studies were performed using SPR, the common intermediate strategy for the generation of biospecific surfaces should also be useful in conjunction with other analytical techniques.

Abbreviations and Terminology Used. CA, bovine carbonic anhydrase II; CM5, the commercially available carboxymethyl dextran substrates available from BIACore; EDC, 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride; IgG, immunoglobulin G; K_d, dissociation constant; k_{off}, dissociation rate constant; K_a, association rate constant; \( \kappa_s \), the negative slope of the dinate/\( \Delta \theta \) plot; L, a ligand; MW, molecular weight; NHS, N-hydroxysuccinimide; \( \Delta \theta_{\text{rep}} \), the steady-state change in resonance angle; \( \Delta \theta_{\text{exp}} \), the experimentally determined amount of protein immobilized; \( \Delta \theta_{\text{SB}} \), change in resonance angle due to tightly and loosely bound nonspecifically adsorbed protein; \( \Delta \theta_{\text{ri}} \), change in resonance angle due to differences in refractive index; \( \Theta_m \), the surface plasmon resonance angle; \( \Delta \theta_{\text{immob}} \), a theoretical estimate of the amount of protein that can be immobilized; \( \Delta \theta_{\text{immob}} \), the observed amount of immobilization of protein; P, a protein; PBS, phosphate-buffered saline (10 mM phosphate, 138 mM NaCl, 2.8 mM KCl, pH = 7.4); pl, isoelectric point; \( \Delta \theta_{\text{frac.cover}} \), the fraction of a monolayer containing immobilized protein; RU, resonance units (10 000 RU = 1°); SAMs, self-assembled monolayers; SDS, sodium dodecyl sulfate; SPR, surface plasmon resonance; Van, vancomycin; *, species (protein or ligand) bound to a surface.

ACKNOWLEDGMENT

Financial support from the National Institutes of Health (Grant GM 30367 and a postdoctoral fellowship to L.I.) and DARPA/SPAWAR is gratefully acknowledged. We thank Professor Paul Laibinis (MIT) for access to his IR spectrometer, Laura Goetting for help with the IR, and Bartosz Grzybowski for helpful discussions.

Received for review August 26, 1998. Accepted November 18, 1998.

AC980959T