Biospecific Adsorption of Carbonic Anhydrase to Self-Assembled Monolayers of Alkanethiolates That Present Benzenesulfonamide Groups on Gold

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The biospecific adsorption of proteins at interfaces that present appropriate ligands is important for anchorage-dependent cell culture,[1] screening of combinatorial libraries,[2] biosensors,[3] and biocompatible surfaces.[4] Fundamental studies of molecular recognition at surfaces by proteins have been limited by a lack of an experimental system having the requisite characteristics:[5] (i) a method to prepare surfaces with excellent control—at the molecular scale—over the density and environment of ligands; (ii) an "inert surface" that resists *nonspecific* adsorption; (iii) a convenient and readily available analytical technique that can measure the adsorption of protein at sub-monolayer coverage *in situ* and in real time. Here we demonstrate that the *combination* of (a) mixed self-assembled monolayers (SAMs) of alkanethiolates on gold presenting oligo(ethylene glycol) moieties and ligands and (b) surface plasmon resonance (SPR) spectroscopy meets these requirements. We demonstrate the characteristics of this system using a model system comprising immobilized benzenesulfonamide groups interacting with bovine carbonic anhydrase (EC 4.2.1.1) (Figure 1).

We used bovine carbonic anhydrase (CA) in this work because it is a well-characterized monomeric protein (MW = 30 000) that binds para-substituted benzenesulfonamide ligands with equilibrium dissociation constants (K_d) of approximately 10^{-6}−10^{-9} M.[6] We prepared mixed SAMs comprising different mole fractions (χ) of an alkanethiolate terminated in a benzenesulfonamide ligand (**2**);[7,8] the corresponding ligand **3** binds CA with a value of K_d of 5×10^{-8} M in solution.[9] The tri(ethylene glycol)-terminated alkanethiol **1** was used as the major component of the SAMs because it resists the nonspecific adsorption of protein (Figure 1).[10] We measured binding of carbonic anhydrase to these SAMs using surface plasmon resonance (using a Pharmacia Biacore instrument) as described previously.[11] In the SPR experiment, p-polarized light is incident on the back side of a gold-coated glass slide supporting a SAM, and the angle (θ) at which the reflected light shows a minimum in intensity is measured.[12] The value of this resonance angle depends linearly on the amount of protein adsorbed to the surface and is recorded as a function of time. For all experiments described here, a solution of buffer (10 mM phosphate, 150 mM sodium chloride, pH = 7.2, $T \approx 25$ °C) was allowed to flow through the cell for 4 min, replaced with a solution of CA in the same buffer for 6 min, and then returned to the original buffer for 20 min.

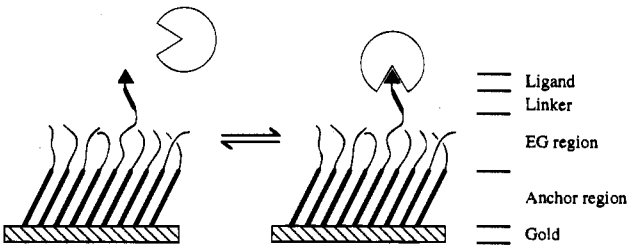

Figure 1. Cartoon showing reversible adsorption of CA to mixed SAMs presenting ligands.

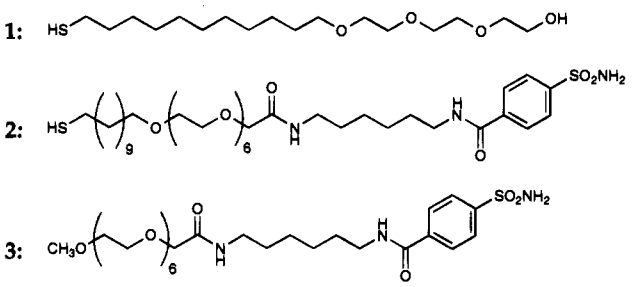

Figure 2 shows SPR response curves for adsorption of CA (0.3 mg/mL, 10 μM) to mixed SAMs having values of χ_2 ranging from 0 to 0.08: the amount of CA that adsorbed to the SAMs increased with the mole fraction of **2** in the mixed monolayer (χ_2) (Figure 2). Assuming values for the cross-sectional areas of an alkanethiolate and CA of 21 and 1800 Å^2, respectively, a mixed SAM having a value of $\chi_2 \approx 0.01$ would support adsorption of a complete monolayer of CA.[13] The approximate value of $\chi_2 = 0.08$ that we observed to be limiting is not inconsistent with this model, since mixed SAMs comprising two alkanethiolates are not randomly mixed and may even contain phase-separated domains of each SAM.[14] For all SAMs

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(7) Alkanethiol **1** was synthesized as described: Pale-Grosdemange, C.; Simon, E. S.; Prime, K. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1991**, *113*, 12−20. Alkanethiol **2** was synthesized in eight steps; details will be described in a subsequent report.

(8) Substrates were prepared by evaporating an adhesion layer of titanium (1 nm) and gold (40 nm) onto glass cover slips (0.20 mm, No. 2, Corning). The metalized substrates were immersed in solutions containing mixtures of **1** and **2** for 12 h to give mixed SAMs. The values of χ_2 were determined by X-ray photoelectron spectroscopy as described previously;[7] we report estimates of χ_2 for SAMs having values of $\chi_2 < 0.05$, due to the limited sensitivity of the XPS measurement.

(9) Bird, R. E.; Mrksich, M.; Gao, J.; Whitesides, G. M. Unpublished.

(10) Mrksich, M.; Whitesides, G. M. *Trends Biotechnol.* **1995**, *13*, 228−235.

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(13) The value for the alkanethiolate was calculated assuming a well-ordered SAM where the alkanethiolates coordinate to the hollow 3-fold sites of a gold (111) surface. The thiolates are arranged in a hexagonal symmetry with a S−S spacing of 4.97 Å; Strong, L.; Whitesides, G. M. *Langmuir* **1988**, *4*, 546−550. The value for CA was calculated from the dimensions of CA obtained from the crystral structure: Eriksson, A. E.; Jones, T. A.; Liljas, A. *Proteins: Struct., Funct., Genet.* **1988**, *4*, 274−282.

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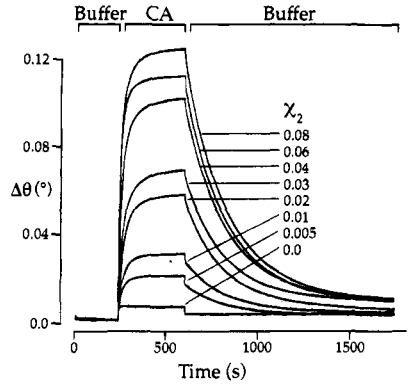

Figure 2. SPR response curves for binding of CA to mixed SAMs having different values of χ_2; nominal values of χ_2 are indicated on the plot. The change in resonance angle ($\Delta\theta$) is plotted against time; the curves were adjusted vertically to facilitate comparison of the data. There is a background contribution to the signal due to the increased refractive index of the protein-containing solution; this contribution is most apparent for the SAM having a value of $\chi_2 = 0$. The time over which the solution of CA (5 μM) was allowed to flow through the cell is indicated at the top of the plot.

having values of $\chi_2 < 0.1$, the adsorption of CA was more than 90% *reversible*; the SPR response curves indicated, however, that there was a small amount of protein (<10%) that remained irreversibly adsorbed to the mixed SAMs. The amount of CA that adsorbed irreversibly increased with χ_2; CA did not adsorb to a SAM containing only **1**; CA adsorbed irreversibly to a SAM containing only **2**. This set of observations suggests that the irreversible adsorption was due to phase-separated domains of **2** present in the mixed SAMs.

We measured the binding of CA—at concentrations ranging from 60 μM to 18 nM—to a mixed SAM having a value of $\chi_2 \approx 0.03$. Analysis of the rates of binding gave a value of $k_{on} = 1.9 \times 10^4$ M^{-1} s^{-1}.[15] The region of the response curve representing dissociation of protein was fitted to an exponential decay function to give a value of $k_{off} = 5.4 \times 10^{-3}$ s^{-1}. The ratio of these two rate constants (k_{off}/k_{on}) provides a value for the equilibrium dissociation constant, K_d, of 2.8×10^{-7} M for binding of CA to the SAM. This value indicates that binding of CA to analogous ligands on the surface and in solution (**3**) occurs with tighter binding in solution ($K_d = 5 \times 10^{-8}$ M). This difference may reflect unfavorable steric interactions between the adsorbed protein and the surface, or an entropic repulsion between the bound protein and the tri(ethylene glycol)-terminated SAM.

The binding of CA (5 μM) to a mixed SAM having a value of $\chi_2 \approx 0.03$ was inhibited by the addition of the inhibitor 4-carboxybenzenesulfonamide (**4**) to the CA-containing solution; the level of inhibition increased with the concentration of soluble ligand (Figure 3A). The SAMs also resisted the nonspecific adsorption of protein from a solution containing a mixture of nine proteins (Figure 3B). When CA (5 μM) was added to this sample, however, SPR measured adsorption of protein to the SAM. This high level of specificity is due to the effectiveness of the tri(ethylene glycol) group at preventing nonspecific adsorption of protein.[10,11]

This work provides a structurally well defined, synthetically flexible model system with which to investigate biomolecular recognition at surfaces. SAMs offer several advantages over traditional materials (for example, gel layers[12]) as an inert surface for conjugation of proteins or other ligands: SAMs permit reliable control over the density of an immobilized ligand, or several different ligands; a variety of methods for immobilizing proteins to SAMs are available; the environment of the ligand can be controlled by changing the linker and molecular identity of the surface; SAMs do not suffer from problems associated with mass transport of proteins through gel layers. Although we employed SPR as the analytical technique for measuring protein adsorption in this work, SAMs on gold are compatible with a range of analytical techniques, including those based on the quartz crystal microbalance,[16] surface acoustic wave,[16] and acoustic plate mode sensors,[17] and electrochemiluminescence-based devices.[18] We believe that SAMs of alkanethiolates on gold are the best model systems currently available with which to investigate fundamental aspects of biointerfacial science, and for many related applications.

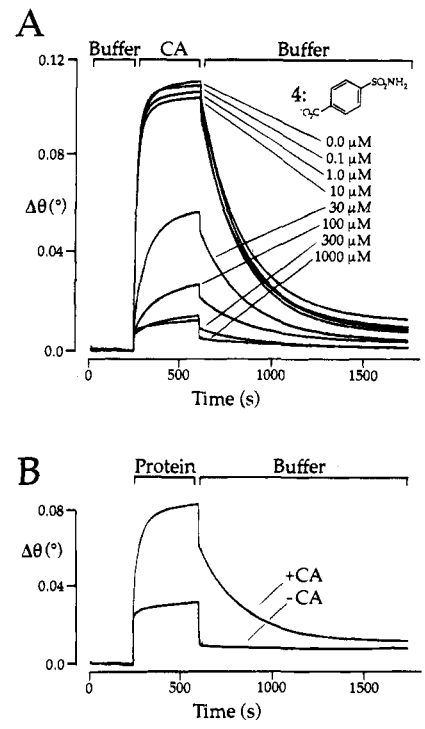

Figure 3. (A) The binding of CA (5 μM) to a mixed SAM having a value of $\chi_2 \approx 0.06$ was inhibited by the addition of 4-carboxybenzenesulfonamide (**4**) to the protein; concentrations of **4** are indicated on the plot. (B) The mixed SAM (having a value of $\chi_2 \approx 0.03$) resists the nonspecific adsorption of protein from a solution containing β-casein, myoglobin, alcohol dehydrogenase, trypsin inhibitor, acylase I, α-lactalbumin, cytochrome *c*, fibrinogen, and RNase A (0.2 mg/mL each; these proteins were chosen arbitrarily from those readily available and were intended only to test specificity) (bottom curve). When CA (5 μM) was present in this sample, however, SPR measured adsorption of the protein (upper curve).

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