Surface Plasmon Resonance Permits *in Situ* Measurement of Protein Adsorption on Self-Assembled Monolayers of Alkanethiolates on Gold

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This paper demonstrates that surface plasmon resonance (SPR) spectroscopy can be used to measure the nonspecific adsorption of proteins to self-assembled monolayers (SAMs) of alkanethiolates on gold *in situ* and in real time. Mixed SAMs comprising hexa(ethylene glycol) and methyl groups that have values of $\chi_{Me} < 0.5$ resisted the adsorption of four test proteins: RNase A, lysozyme, fibrinogen, and pyruvate kinase. These four proteins adsorbed irreversibly to surfaces having values of $\chi_{Me} > 0.5$: the amount of adsorbed protein correlated with χ_{Me}. The *initial* rate for adsorption of fibrinogen to a methyl-terminated SAM ($\chi_{Me} = 1.0$) followed first-order kinetics. The combination of SAMs and SPR described here is particularly well suited for investigations of the interactions of proteins with structurally well-defined organic surfaces.

The adsorption of proteins to surfaces is a central concern in systems in which man-made materials contact biological fluids: examples include prostheses,[1] supports for anchorage-dependent cell culture,[2] in-dwelling sensors,[3] and catheters.[4] While much has been learned about the mechanisms of protein adsorption,[5] experimental studies have been limited by a lack of well-defined surfaces capable of presenting a wide range of groups. Self-assembled monolayers (SAMs) of alkanethiolates on gold are an excellent model system for studying the effects of surface properties on protein adsorption.[6-8] In previous work we have shown that SAMs terminated in oligo(ethylene glycol) groups resist the nonspecific adsorption of protein as measured by *ex situ* ellipsometry;[7] however, it was unclear whether this *ex situ* measurement accurately reflected the partitioning of protein to the surface in solution. There is a need for a broadly and conveniently applicable analytical technique that permits the *in situ* measurement of protein adsorption in this system. Surface plasmon resonance (SPR) spectroscopy has been used extensively to measure the binding of proteins to surfaces that are less well-defined than SAMs,[9] but much of this work has employed home-built instruments that are not readily available. Here we demonstrate that a commercial SPR instrument[10] has the characteristics required for use with SAMs on gold as a technique for examining protein adsorption:[11] it has excellent sensitivity and is capable of detecting adsorbed proteins in sub-monolayer quantities with excellent signal-to-noise; it provides both thermodynamic and kinetic information about adsorption; it requires only modest quantities of protein; it does not require the protein to be modified to introduce a chromophore. This work demonstrates the effectiveness of oligo(ethylene glycol)-terminated SAMs to resist the nonspecific adsorption of protein *in situ*.

Experimental Design. In the SPR experiments described here, a glass slide with a thin layer of gold (40 nm) supporting a SAM was mounted as part of a flow cell.[12] p-Polarized light that is directed at the glass surface causes excitation of plasmons at the metal–solution interface.[13] The excitation of surface plasmons is detected as a minimum in the intensity of reflected light at the resonance angle (θ_m) (Figure 1A). The value of θ_m depends on the dielectric constant of the interfacial region that is in contact with the gold and depends (*inter alia*) on the thickness of an adsorbed layer of protein. Our protocol for measuring the adsorption of protein to these SAMs consisted of allowing a solution of buffer (10 mM phosphate, 150 mM sodium chloride, pH = 7.2, T = 25 °C) to flow through the cell for 7 min, substituting a flow of a solution of protein (1 mg/mL) in the same buffer for 7 min,

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(1) Frentzen, M.; Oxborn, J. F.; Nolden, R. *Dtsch. Zahnaerztl. Z.* **1988**, *43*, 719–727.

(2) Schakenraad, J. M.; Busscher, H. J. *Colloids Surf.* **1989**, *42*, 331–339. Kibira, S.; Dudek, R.; Narayan, K. S.; Bing, R. *J. Mol. Cell. Biochem.* **1991**, *108*, 75–84.

(3) Ishihara, K.; Ohta, S.; Yoshikawa, T.; Nakabayashi, N. *J. Polym. Sci., Part A: Polym. Chem.* **1992**, *30*, 929–932. Cha, G. S.; Liu, D.; Meyerhoff, M. E.; Cantor, H. C.; Midgley, A. R.; Goldberg, H. D.; Brown, R. B. *Anal. Chem.* **1991**, *63*, 1666–1672.

(4) Goodman, S. L.; Simmons, S. R.; Cooper, S. L.; Albrecht, R. M. *J. Colloid Interface Sci.* **1990**, *139*, 561–570.

(5) For reviews, see: Andrade, J. D.; Hlady, V. *Adv. Polym. Sci.* **1986**, *79*, 1–63. Sadana, A. *Chem. Rev.* **1992**, *92*, 1799–1818. Wahlgren, M.; Arnebrant, T. *Trends Biotechnol.* **1991**, *9*, 201–208.

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

(7) Prime, K. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1993**, *115*, 10714–10721.

(8) DiMilla, P. A.; Folkers, J. P.; Biebuyck, H. A.; Harter, R.; Lopez, G. P.; Whitesides, G. M. *J. Am. Chem. Soc.* **1994**, *116*, 2225–2226.

(9) Lofas, S.; Malmqvist, M.; Ronnberg, I.; Stenberg, E.; Liedberg, B.; Lundstrom, I. *Sensors Actuators B* **1991**, *5*, 79–84. Davies, J.; Roberts, C. J.; Dawkes, A. C.; Sefton, J.; Edwards, J. C.; Glasbey, T. O.; Haynes, A. G.; Davies, M. C.; Jackson, D. E.; Lomas, M.; Shakesheff, K. M.; Tendler, S. J. B.; Wilkens, M. J.; Williams, P. M. *Langmuir* **1994**, *10*, 2654–2661.

(10) Lofas, S.; Johnsson, B. *J. Chem. Soc., Chem. Commun.* **1990**, 1526–1528. Stenberg, E.; Persson, B.; Roos, H.; Urbaniczky, C. *J. Colloid Interface Sci.* **1991**, *143*, 513–526. Liedberg, B.; Lundstrom, I.; Stenberg, E. *Sensors Actuators B* **1993**, *11*, 63–72.

(11) For examples of using SPR to measure interactions of proteins with SAMs, see: Spinke, J.; Liley, M.; Guder, H.-J.; Angermaier, L.; Knoll, W. *Langmuir* **1993**, *9*, 1821–1825. Spinke, J.; Yang, J.; Wolf, H.; Liley, M.; Ringsdorf, H.; Knoll, W. *Biophys. J.* **1992**, *63*, 1667–1671. Kooyman, R. P. H.; van den Heuvel, D. J.; Drijfhout, J. W.; Welling, G. W. *Thin Solid Films* **1994**, *244*, 913–916.

(12) We used the Biacore instrument (Pharmacia) for all studies described here. The substrates that are sold by the manufacturer contain a layer of carboxylated dextran for immobilization of proteins and minimization of nonspecific adsorption and were not useful for our studies. We fabricated sensor chips by evaporation of an adhesion layer of titanium (1 nm) and a layer of gold (40 nm) onto glass cover slips (0.20 mm, No. 2, Corning). The gold-coated glass slides were immersed in solutions containing mixtures of **1** and **2** (2 mM in ethanol) for 24 h to give SAMs with different compositions of these two alkanethiolates. The mole fraction of **2** in each SAM was determined by X-ray photoelectron spectroscopy as described previously.[6] The glass slides were cut and glued into cartridges using 5 Minute Epoxy (Devcon) for use in the Biacore instrument.

(13) Raether, H. In *Physics of Thin Films*; Hass, G., Francombe, M., Hoffman, R., Eds.; Academic Press: New York, 1977; pp 145–261.

0743-7463/95/2411-4383$09.00/0

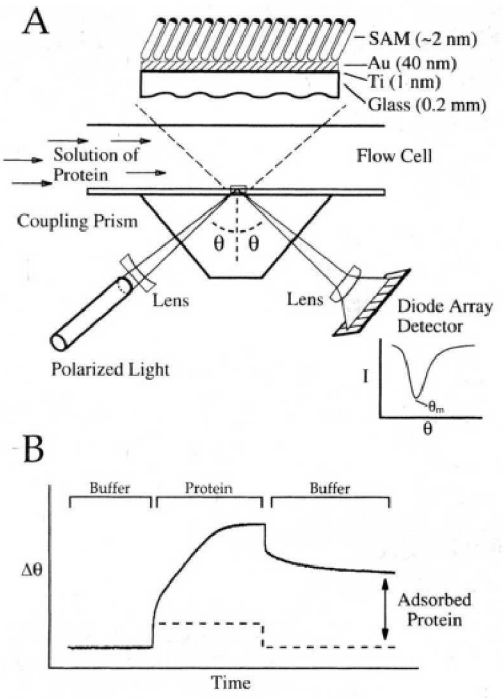

Figure 1. Surface plasmon resonance spectroscopy can be used to measure the adsorption of proteins to self-assembled monolayers *in situ* and in real time. (A) p-Polarized light is incident on the substrate, and the angle at which the reflected light shows a minimum in intensity is recorded. This angle (θ_m) is characteristic of the optical properties of the interface and depends on the average thickness of a protein adlayer. (B) For the experiments described here, a solution of buffer was flowed through the cell, substituted with a solution of protein, and then replaced with buffer. The SPR experiment records $\Delta\theta$ ($\theta_m - \theta_m°$) versus time (solid curve). The change in the index of refraction of the protein-containing solution also causes a change in θ_m; this contribution to the measured signal is indicated by the dashed curve. The *difference* between the two curves measures the amount of protein adsorbed to the SAM.

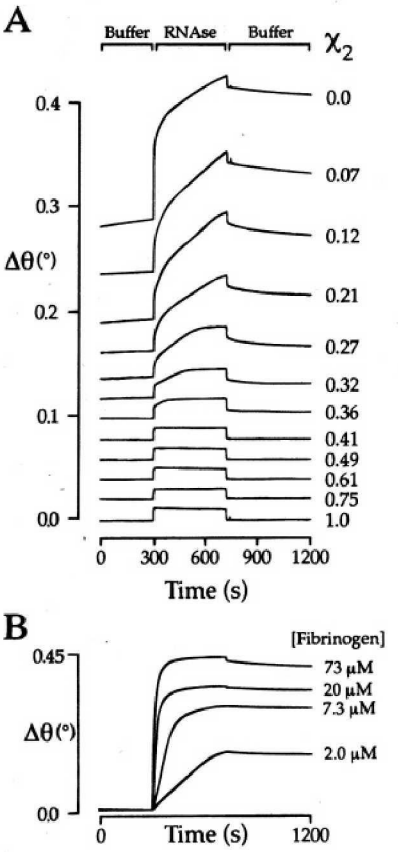

Figure 2. (A) Data for the adsorption of RNase A to mixed SAMs comprising **1** and **2** (1 mg/mL protein in 10 mM phosphate, 150 mM sodium chloride, pH = 7.2, T = 25 °C). The *relative* change in the resonance angle (θ_m) is plotted versus time for each mixed SAM. The region of time during which protein was present in the buffer is indicated above the plot. The curves are offset vertically for clarity. The mole fraction of **2** in each SAM is indicated on the right side of the plot. (B) Effect of concentration of fibrinogen in the buffer on the amount of protein adsorbed to SAMs terminated in methyl groups ($\chi_1 = 1.0$). The curves were adjusted vertically to have the same value of θ_m at the time when protein was introduced into the flow cell. The concentration of fibrinogen for each response curve is indicated on the right side.

and then replacing with the original buffer for 8 min.[14] The change in θ_m from the initial value ($\Delta\theta = \theta_m - \theta_m°$) was recorded versus time (Figure 1B).

Adsorption of Protein to SAMs. We examined the adsorption of four proteins—RNase A, lysozyme, fibrinogen, and pyruvate kinase[15] —to mixed SAMs that contained different mole fractions of the two alkanethiolates $S(CH_2)_{10}CH_3$ (**1**) and $S(CH_2)_{11}(OCH_2CH_2)_6OH$ (**2**).[7] Figure 2A shows data for the adsorption of RNase A to several mixed SAMs; the data for adsorption of lysozyme, fibrinogen, and pyruvate kinase to these SAMs were qualitatively similar. SAMs with values of χ_1 ranging from 0 to 0.5 resisted almost entirely the adsorption of protein. SPR reliably measured the low level of adsorption of protein on these SAMs (~2% of a full monolayer); we believe that this adsorption is due to defects in the SAM. The resistance of these surfaces to protein adsorption is consistent with our previous work using *ex situ* ellipsometry.[7] The ellipsometric experiments left unresolved the possibility that a weakly bound layer of protein was removed from the surface when the substrate was rinsed and dried prior to the measurement. The data in Figure 2A demonstrate that these SAMs do indeed resist the *in situ* adsorption of protein. Protein adsorbed to SAMs having values of χ_1 ranging from 0.6 to 1.0. Although a small amount of protein (~5%) dissociated from the SAMs in the first minute after the solution of protein was replaced with buffer, the adsorption of protein on these SAMs was essentially irreversible in the absence of detergents or other proteins; only the amount of adsorbed protein changed with the value of χ_1 in the mixed SAMs. We believe that these data are most consistent with mixed SAMs that present domains of each alkanethiolate, rather than a random arrangement of the two alkanethiolates in the monolayer.

Measurement of Kinetics of Protein Adsorption. Figure 2B demonstrates that the amount of fibrinogen that adsorbed to a SAM comprising **1** depended on the concentration of protein in solution. A plot of the initial rates of adsorption (measured as $\Delta\theta$/s) versus the concentration of fibrinogen gave a straight line (correlation of $R = 0.998$) with a slope of 2448 deg M^{-1} s^{-1}; this first-order rate constant is consistent with a model for irreversible and noncooperative adsorption of protein to the SAM.[16] We are unable to report an accurate rate constant because the density of protein on the surface at saturation

(14) The buffer was caused to flow through a rectangular cell (500 μm wide × 50 μm high) at a rate of 5 μL/min. The corresponding Reynold's number of approximately 10 suggests that the flow is laminar and not turbulent.

(15) RNase A (R-5125), lysozyme (L-6876), pyruvate kinase (P-9136), and fibrinogen (F-4883; 94% clottability) were purchased from Sigma (catalog numbers are indicated). Solutions of proteins in PBS buffer were prepared and filtered through 0.22 μm filter units.

(16) For a review of investigations of the kinetics of protein adsorption, see: Ramsden, J. J. *Q. Rev. Biophys.* **1994**, *27*, 41–105.

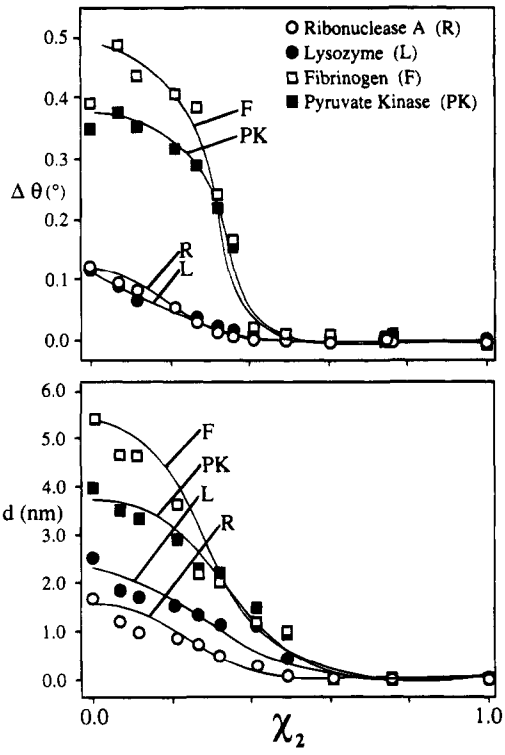

Figure 3. Quantitation of the adsorption of protein to SAMs by *in situ* SPR and *ex situ* ellipsometry (conditions described in text). (Top) The net change in resonance angle 10 s after protein was removed from the flow cell is plotted versus χ_2 for each protein: RNase A (○), lysozyme (●), fibrinogen (□), and pyruvate kinase (■). (Bottom) The average thickness of the layer of adsorbed protein on each SAM measured using *ex situ* ellipsometry.[7,17] The curves serve only to organize the data visually and do not represent a physical model.

was not constant but depended on the concentration of protein in the flowing buffer (Figure 2B).

Comparison of *in Situ* SPR and *ex Situ* Ellipsometry. Figure 3A shows the amount of protein that remained adsorbed to each mixed SAM 10 s after the flow cell was flushed with protein-free buffer. The response curves for *binding* of lysozyme, fibrinogen, and pyruvate kinase to each mixed SAM reached a constant value during the time that the surfaces were exposed to protein; this behavior indicated that the strong adsorption sites on the surface were all occupied. By contrast, the response curves for adsorption of RNase A did not reach a constant value, and the data do not measure the amount of adsorbed protein at saturation. Figure 3B shows the amount of protein that adsorbed to each mixed SAM as measured by ellipsometry.[17] Since the conditions (flow rate and adsorption time) for the SPR and ellipsometric experiments were different, a direct comparison of the two methods is not possible; there is, nonetheless, a good qualitative agreement between the two sets of data.

The combination of SAMs and SPR described here is especially well suited for studies of adsorption of proteins at surfaces: both nonspecific, irreversible adsorption of the type examined here and biospecific adsorption to surfaces presenting ligands can be examined.[18,19] Studies of these systems demand a sensitive analytical technique capable of measuring the thermodynamic and kinetic parameters of interactions of biomolecules with substrates *in situ* and in real time.

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(17) For measurements of adsorbed protein by ellipsometry, SAMs were placed in buffer (8 mL; 10 mM phosphate, 150 mM sodium chloride, pH = 7.2) and a solution of protein in the same buffer (2 mL, 5 mg/mL) was added. After 30 min, the protein-containing buffers were exchanged several times with water without allowing the substrates to pass through the liquid–air interface. The SAMs were removed from solution under a flow of water, dried with a stream of nitrogen, and characterized using ellipsometry as described previously.[7]

(18) Sigal, G. B.; Bamdad, C.; Barberis, A.; Strominger, J.; Whitesides, G. M. *Anal. Chem.*, submitted for publication.

(19) Mrksich, M.; Grunwell, J. R.; Whitesides, G. M. *J. Am. Chem. Soc.*, submitted for publication.