Anal. Chem. **1996,** *68,* 490−497

A Self-Assembled Monolayer for the Binding and Study of Histidine-Tagged Proteins by Surface Plasmon Resonance

George B. Sigal,[†] Cynthia Bamdad,[§] Alcide Barberis,[‡] Jack Strominger,[‡] and George M. Whitesides*[,†]

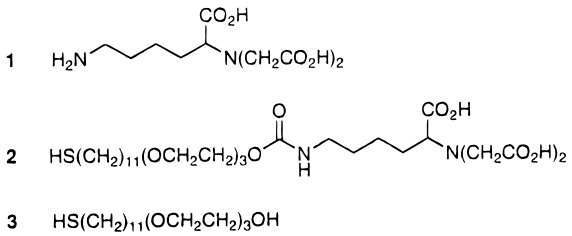
Departments of Chemistry and Biochemistry and the Committee for Higher Degrees in Biophysics, Harvard University, Cambridge, Massachusetts 02138

This paper reports the generation of a self-assembled monolayer (SAM) that selectively binds proteins whose primary sequence terminates with a His-tag: a stretch of six histidines commonly incorporated in recombinant proteins to simplify purification. The SAM was prepared by the adsorption onto a gold surface of a mixture of two alkanethiols: one thiol that terminated with a nitrilotriacetic acid (NTA) group, a group that forms a tetravalent chelate with Ni(II), and a second thiol that terminated with a tri(ethylene glycol) group, a group that resists protein adsorption. His-tagged proteins bound to the SAM by interaction of the histidines with the two vacant sites on Ni(II) ions chelated to the surface NTA groups. Studies with model proteins showed the binding was specific for His-tagged proteins and required the presence of Ni(II) on the surface. Immobilized His-tagged proteins were kinetically stable in buffered saline at pH 7.2 but could be desorbed by treatment with 200 mM imidazole. Surface plasmon resonance studies for two model systems showed that His-tagged proteins adsorbed on the NTA-SAM retained a greater ability to participate in binding interactions with proteins in solution than proteins immobilized in a thin dextran gel layer by covalent coupling.

Surface plasmon resonance (SPR) is a useful technique for measuring the kinetics of association and dissociation of ligands from proteins in aqueous solution.[1−3] It is particularly useful for processes occurring at or near interfaces. The active sensing element is a thin (40−50 nm) gold or silver film deposited on a glass substrate. Monochromatic, p-polarized light is reflected from the glass−gold interface from the back side. A plot of reflected intensity as a function of the angle of incidence (Θ) shows a minimum (Θ_m) corresponding to the excitation of surface plasmons at the gold−solution interface.[4] The value of Θ_m shifts with changes in the refractive index of the interfacial region near the surface of the gold (within approximately a wavelength of the incident light). For thin organic films (<100 nm) and light with a wavelength of 760 nm, the shift in Θ_m is roughly proportional to the thickness of the film.[5] Changes in the concentration of a molecule of interest (for example, a protein) in this interfacial region occurring by adsorption to the interface or association with an immobilized ligand can be monitored as a function of time by measuring Θ_m.

A variety of techniques have been used to immobilize proteins on silver or gold films for studies using SPR: the two major classes are (i) physical adsorption of the protein either on the metal surface[6] or on a hydrophobic film spin-coated on the metal surface[7] and (ii) covalent attachment of the protein to thin functionalized dextran gel layers[3] or to layers of silica deposited on silver and modified with (aminopropyl)trimethoxysilane.[2]

We and others have developed procedures to modify the surface of gold films by formation of self-assembled monolayers (SAMs) of alkanethiolates.[8,9] One class of SAMs useful for immobilizing proteins uses alkanethiolates terminally functionalized with a biotin moiety; the biotin binds streptavidin, which in turn permits the immobilization of biotin-labeled proteins.[10] This system is successful and widely used, but shares the disadvantages of most immobilization schemes requiring chemical modification of a protein: (i) chemical modification may lead to denaturation or loss of activity and (ii) the presence of multiple sites on the protein available for modification results in loss of control over the orientation of the protein after immobilization. Here we describe the generation of a SAM functionalized with the nitrilotriacetic (NTA) group **1**; this group, when complexed with Ni-



(II), selectively binds proteins whose sequence terminates with a stretch of six histidines (His-tag).[11,12] His-tags are commonly

[†] Department of Chemistry.
[‡] Department of Biochemistry.
[§] Committee for Higher Degrees in Biophysics.

(1) Liedberg, B.; Nylander, C.; Lundström, I. *Sens. Actuators* **1983**, *4*, 299−304.
(2) Daniels, P. B.; Deacon, J. K.; Eddowes, M. J.; Pedley, D. G. *Sens. Actuators* **1988**, *16*, 11−18.
(3) Löfås, S.; Johnsson, B. *Chem. Commun.* **1990**, 1526−1528.
(4) Raether, H. *Phys. Thin Films* **1977**, *9*, 145−261.
(5) Stenberg, E.; Persson, B.; Roos, H.; Urbaniczky, C. J. *J. Colloid Interface Sci.* **1991**, *143*, 513−526.
(6) Flanagan, M. T.; Pantell, R. H. *Electron. Lett.* **1984**, *20*, 968−970.
(7) Davies, J.; Roberts, C. J.; Dawkes, A. C.; Sefton, J.; Edwards, J. C.; Glasbey, T. O.; Haymes, A. G.; Davies, M. C.; Jackson, D. E.; Lomas, M.; Shakesheff, K. M.; Tendler, S. J. B.; Wilkins, M. J.; Williams, P. M. *Langmuir* **1994**, *10*, 2654−2661.
(8) Dubois, L. H.; Nuzzo, R. G. *Annu. Rev. Phys. Chem.* **1992**, *43*, 437−463.
(9) Whitesides, G. M.; Gorman, C. G. In *Handbook of Surface Imaging and Visualization*; Hubbard, A. T., Ed.; CRC Press: Boca Raton, FL, 1995; pp 713−733.
(10) Spinke, J.; Liley, M.; Guder, H.-J.; Angermaier, L.; Knoll, W. *Langmuir* **1993**, *9*, 1821−1825.

0003-2700/96/0368-0490$12.00/0

incorporated into the primary sequence of recombinant proteins to facilitate purification. The objective of this work was to develop a method of immobilization of proteins that would (i) control the orientation of the immobilized protein such that the active site would be accessible to molecules in solution, (ii) create a surface that would specifically immobilize a protein of interest while resisting nonspecific binding of other proteins, and (iii) avoid the requirement for nonspecific, covalent modification of the protein.

EXPERIMENTAL SECTION

Reagents. Hepes-buffered saline (HBS) is 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 150 mM NaCl in water adjusted to pH 7.2 with NaOH. Lysozyme (EC 3.2.1.17, grade III from chicken egg white, Sigma) was used as received. The preparation of the His-tag containing model proteins and their protein ligands is described elsewhere.[13,14] The Gal 4 construct used (Gal 4 1−147 + AH) comprised the Gal 4 DNA binding and dimerization domains attached to a short activating peptide (AH, amphipathic helix) and a His-tag. The His-tagged Gal 11 construct used (mini-Gal 11) was a shortened form of Gal 11 that contained a His-tag and a myc epitope near the carboxy terminus.

Preparation of Gold Substrates. Gold substrates were prepared by evaporating 1 nm of Ti, followed by 40 nm of Au, onto either silicon wafers (Silicon Sense) for ellipsometric measurements or No. 2 glass coverslips (Corning Glass) for SPR measurements. Substrates were coated with metals by electron beam evaporation at pressures of less than 5×10^{-7} Torr and evaporation rates of 0.2 nm/s. Gold substrates were broken into smaller pieces after scribing with a diamond stylus.

Preparation of SAMs. Stock solutions of thiols **2** and **3** in (1 mM in 95% ethanol) were combined in glass scintillation vials to give mixtures with a total thiol concentration of 1 mM. Gold substrates were incubated between 12 and 20 h in the solutions of thiols, rinsed with 95% ethanol, and dried in a stream of nitrogen. SAMs used in SPR measurements were prepared from a solution of thiols **2** and **3** containing a mole fraction of thiol **2,** $\chi^2_{soln} = 0.1$.

X-ray Photoelectron Spectroscopy (XPS). XPS spectra were obtained using an SSX-100 spectrometer (Surface Sciences Instruments). The spectra were accumulated at a take-off angle of 35° relative to the surface and at pressures less than 1×10^{-8} Torr. Peaks were fitted and integrated using software from Surface Science Instruments.

Ellipsometry. Ellipsometric measurements were made with a Rudolf Research Type 43603-200E manual thin-film ellipsometer using a He−Ne laser (632.8 nm) at an angle of incidence of 70°. The PCSA (polarizer−compensator−sample−analyzer) configuration was used with the compensator set to −45°. Ellipsometric constants for surfaces were measured before and after adsorption of thiols or proteins. The thickness of adsorbed layers were calculated using a planar, three-layer, isotropic model[15] with assumed refractive indices of 1.00 for air and 1.45 for both protein and SAM.[16]

Protein adsorption experiments were carried out on SAMs incubated sequentially in 1 mM aqueous. NaOH for 5 min, and 40 mM aqueous nickel sulfate for 1 h to adsorb Ni(II) to the surface NTA groups. The samples were washed with ∼1 mL of HBS, followed by ∼5 mL of water, and dried in a stream of nitrogen. Proteins were adsorbed from solutions in HBS. To determine binding in the absence of Ni(II) or other heavy metals on the surface, the step involving incubation with the solution of nickel sulfate was eliminated, and 5 mM of EDTA was added to the protein solutions.

Surface Plasmon Resonance Measurements. SPR measurements were made with a BIACore instrument (Pharmacia Biosensor).[5] Plastic cassettes holding a gold-coated glass substrate derivatized with a thin carboxydextran gel layer were purchased from Pharmacia Biosensor. For experiments using mixed monolayers of thiols **2** and **3**, the glass substrates supporting the carboxydextran films were removed with a razor blade. SAMs were prepared on gold films evaporated on No. 2 coverslips. The coverslips were cut to size and glued in place on the plastic cassettes using a two-part epoxy (5 Minute Epoxy, Devco Corp.).

SPR experiments were conducted with a constant 5 μL/min flow of solution over the surfaces. Protein adsorption on the NTA-SAM was carried out by sequential injections of 25−35 μL of a 40 mM aqueous solution of nickel sulfate and then 35 μL of the protein solution diluted in HBS. The surface was washed with HBS after each injection. Protein adsorption resulted in a shift in the resonance angle that was reported in resonance units (RU; 10 000 RU = 1.0°). To determine binding of proteins in the absence of Ni(II) or other heavy metals on the surface, the nickel sulfate injection was omitted and 5 mM EDTA was included in the protein solution. Covalent attachment of proteins to the carboxydextran-derivatized surface after activation with *N*-ethyl-*N*′-[(dimethylamino)propyl]carbodiimide hydrochloride in the presence of *N*-hydroxysuccinimide (EDC−NHS) was carried out according to established procedures.[3]

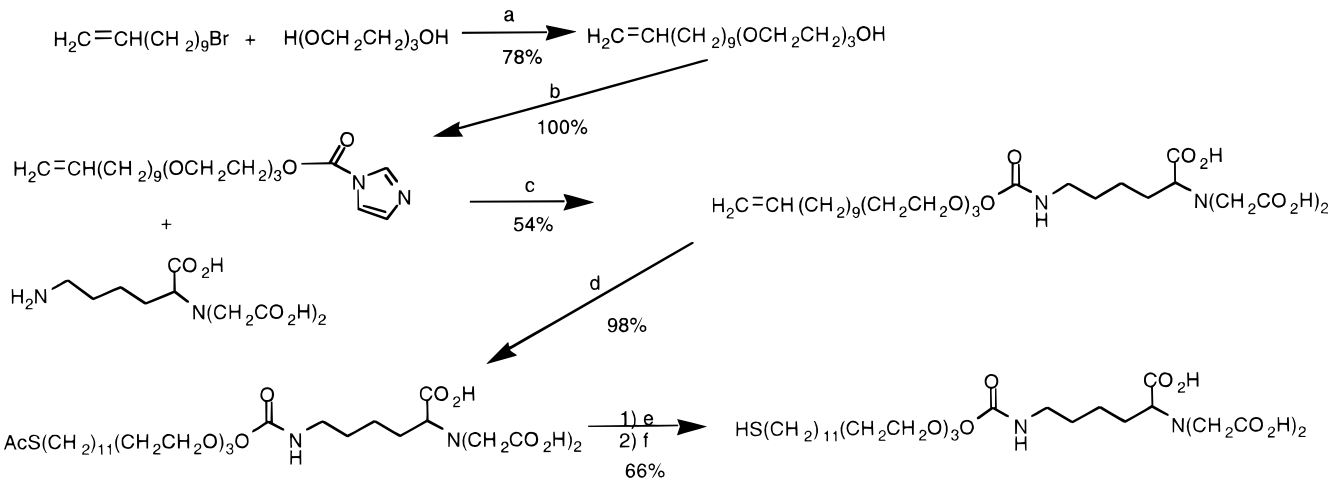
Synthesis of the NTA-Terminated Thiol 2. *N*-[5-[[[(3,6,9-Trioxaeicos-19-en-1-yl)oxy]carbonyl]amino]-1-carboxypentyl]iminodiacetic Acid (4). Carbonyldiimidazole (3.8 g, 2 equiv) was added while stirring to 3.5 g (11 mmol) of undec-1-en-11-yltri(ethylene glycol) (prepared according to ref 19) dissolved in 35 mL of methylene chloride. After stirring for 2 h, the solution was applied to a column containing 300 g of silica gel 60 (230−400 mesh, E. Merck) equilibrated with ethyl acetate and the imidazole carbamate eluted with 1 L of ethyl acetate. Evaporation of the solvent under reduced pressure gave 4.6 g (100%) of the imidazole carbamate as an oil.

Amine **1** (8.0 g, 31 mmol), prepared according to ref 11, was dissolved in 100 mL of water. The solution was titrated to pH 10.2 with 12 N NaOH, and 130 mL of dimethylformamide was added. The imidazole carbamate (4.5 g, 11 mmol) in 10 mL of dimethylformamide was added dropwise to the amine while stirring. After 12 h, the solution was added to 500 mL of water and washed three times with 250 mL portions of ethyl acetate (using gentle stirring to avoid the formation of an emulsion). The aqueous phase was acidified with 6 N HCl to pH 1.5 and extracted with ethyl acetate (4 × 250 mL). The combined extracts were

(11) Hochuli, E.; Döbeli, H.; Schacher, A. J. *Chromatogr.* **1987**, *411*, 177−184.
(12) Gentz, R.; Chen, C.-H.; Rosen, C. A. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 821−824.
(13) Chung, S.; Wucherfennig, K.; Freidman, S.; Hafler, D.; Strominger, J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *91*, 12654−12658.
(14) Barberis, A.; Pearlberg, J.; Simkovich, N.; Farell, S.; Reinagel, P.; Bamdad, C.; Sigal, G.; Ptashne, M. *Cell* **1995**, *81*, 359−368.
(15) McCrackin, F. L.; Passaglia, E.; Stromberg, R. R.; Steinberg, H. L. *J. Res. Natl. Bur. Stand., Sect. A* **1963**, *67*, 363−377.

(16) The refractive indices of proteins usually fall between 1.35 and 1.55. The effect of error in the assumed refrective index on the calculated thickness is described in detail in ref 18.

Scheme 1. Synthesis of the NTA-Terminated Thiol **2**[a]



[a] (a) Aqueous NaOH, 100 °C; (b) CDI, CH_2Cl_2; (c) water−DME, pH 10.2; (d) CH_3COSH, AIBN, THF, *hν*; (e) NaOH, O_2, water−DME; PEt_3.

washed with saturated NaCl and dried over $MgSO_4$. The solvent was removed under reduced pressure to give 3.6 g (54%) of olefin **5** as a hydroscopic white solid: ^{1}H NMR (CD_3OD, 400 MHz) δ 5.80 (m, 1H), 5.00 (dd, 1H), 4.92 (dd, 1H), 4.13 (t, 2H), 3.60 (br m, 14H), 3.47 (m, 3H), 3.08 (t, 2H), 2.02 (m, 2H), 1.78 (m, 1H), 1.65 (m, 1H), 1.52 (br m, 4H), 1.28 (br m, 14H).

***N*-[5-[[[[20-(Acetylthio)-3,6,9-trioxaeicos-1-yl]oxo]carbonyl]amino]-1-carboxypentyl]iminodiacetic Acid (5).** The olefin **4** (3.6 g, 6.1 mmol) was dissolved in 15 mL of distilled tetrahydrofuran, together with 1.8 mL of thiolacetic acid (4 equiv) and 300 mg of azobis(isobutyronitrile) (AIBN). The solution was irradiated for 4 h under a 450 W medium-pressure mercury lamp (Ace Glass). The solvent was removed under reduced pressure, and the crude product was triturated with hexane. Recrystalization from ethyl acetate−hexane gave 4.0 g (98%, calculated as a pure compound) of thioacetate as a hydroscopic tan solid **5**: ^{1}H NMR (CD_3OD, 400 MHz) δ 4.14 (t, 2H), 3.63 (br m, 14H), 3.44 (m, 3H), 3.09 (t, 2H), 2.85 (t, 2H), 2.29 (s, 3H), 1.78 (m, 1H), 1.66 (m, 1H), 1.54 (br m, 8H), 1.29 (br m, 14H).

***N*-[5-[[[(20-Mercapto-3,6,9-trioxaeicos-1-yl)oxo]carbonyl]amino]-1-carboxypentyl]iminodiacetic Acid (2).** To thioacetate **5** (4.0 g, 6.0 mmol) in 20 mL of dimethoxyethane was added 17 mL of water followed by 20 mg of I_2. After the addition of 3 mL of 2 N NaOH, the solution was stirred for 4 h while O_2 was bubbled through it. Addition of 100 mL of water and acidification to pH 1.5 with 6 N HCl led to precipitation of the product as the disulfide. The disulfide was filtered, washed with water and dried, under vacuum to give 2.9 g (78%) of a white powder.

The disulfide was reduced to the thiol **2** with triethylphosphine.[17] To the disulfide (1.0 g, 0.80 mmol) in 36 mL of methanol containing 4 mL of water under an atmosphere of nitrogen was added 1.0 g (10 equiv) of triethylphosphine. The solution was stirred for 5 h and concentrated to an oil under reduced pressure. The residue was dissolved in 40 mL of degassed water and acidified to pH 1.5 with 6 N HCl. The product was extracted three times with 20 mL portions of ethyl acetate. The combined organic phases were washed with saturated NaCl, dried over $MgSO_4$, and concentrated under reduced pressure to give thiol **2** as a tan hydroscopic solid (0.84 g, 84%). Purification was achieved by elution from a column of silica gel 60 (230−400 mesh, E. Merck) with a gradient of 20−80% (v/v) 2-propanol in hexane plus 2% (v/v) acetic acid, followed by a gradient of 0−8% (v/v) water in 80% (v/v) 2-propanol, 2% (v/v) acetic acid in hexane: ^{1}H NMR (DMSO, 400 MHz) δ 7.18 (t, 1H), 4.01 (t, 2H), 3.49 (br m, 14H), 3.34 (m, 3H), 2.91 (q, 2H), 2.44 (q, 2H), 2.21 (t, 1H), 1.51 (br m, 5H), 1.22 (br m, 17H); ^{13}C NMR (DMSO, 400 MHz) δ 173.91, 173.14, 156.10, 70.31, 69.78, 69.71, 69.46, 68.89, 64.21, 62.96, 53.22, 40.05, 33.39, 29.28, 29.20, 29.14, 29.00, 28.86, 28.50, 27.74, 25.64, 23.75, 22.97; HRMS-FAB $[M + Na]^+$ Calcd for $C_{28}H_{51}N_2O_{11}S$ 647.3190. Found 647.3163.

RESULTS AND DISCUSSION

The approach used in this work to generate a surface appropriate for immobilizing His-tagged proteins for study by SPR was to prepare a surface analogous to that used for nickel-affinity chromatography, commonly used to purify His-tagged proteins.[11,12] This technique utilized the NTA derivative **1** to coordinate Ni(II), leaving two vacant coordination sites on the nickel ion for chelation by His-tag imidazole side chains. In nickel-affinity chromatography, chelate **1** is attached to chromatography beads through the primary amine.[11] For SPR, chelate **1** was attached to gold surfaces by formation of mixed monolayers from two alkanethiols: the first, **2,** was terminally functionalized with the nickel ligand; the second, **3**, was terminated with tri(ethylene glycol)—a functional group that resists nonspecific adsorption of protein.[18]

The synthesis of thiol **2** is outlined in Scheme 1. The synthesis is analogous to the previously described synthesis of the tri(ethylene glycol)-terminated alkane thiol **3**,[19] with the addition of a carbonyldiimidazole coupling step to link the NTA derivative **1** to the terminal hydroxyl group. In order to make purification simpler, the product from deacetylation of the thioacetate was isolated in the form of a disulfide and reduced to the thiol in a separate step.

To determine the optimal concentration of ligand on the surface for protein binding, mixed monolayers[20] of thiols **2** and **3** were prepared on gold films by adsorption from solutions containing a mixture of the two thiols (at a total thiol concentration of 1.0 mM) in 95% ethanol. To bind Ni(II) to the NTA groups, the SAMs were

(17) Overman, L. E.; Smoot, J.; Overman, J. D. *Synthesis* **1974**, 59−60.
(18) Prime, K. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1993**, *115*, 10714−10721.
(19) Pale-Grosdemange, C.; Simon, E. S.; Prime, K. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1991**, *113*, 12−20.
(20) Laibinis, P. E.; Nuzzo, R. G.; Whitesides, G. M. *J. Phys. Chem.* **1992**, *96*, 5097−5105.

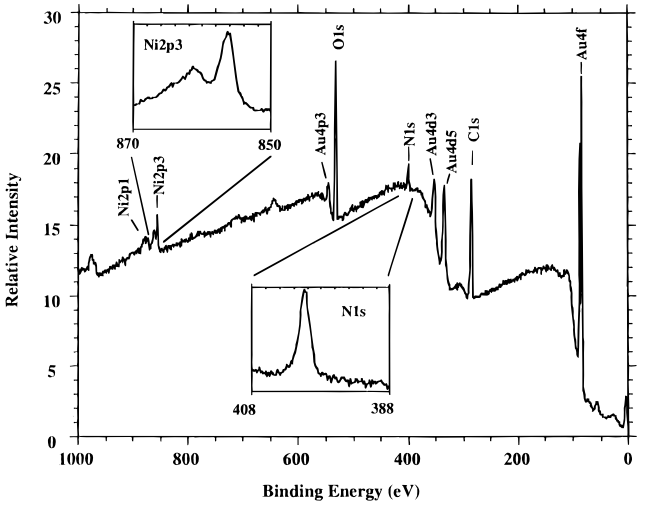


Figure 1. XPS analysis of SAM prepared from thiol **2**. SAM was treated sequentially with 1 mM aqueous NaOH, 40 mM aqueous $Ni^{II}SO_4$, 150 mM aqueous NaCl, and water and dried before analysis.

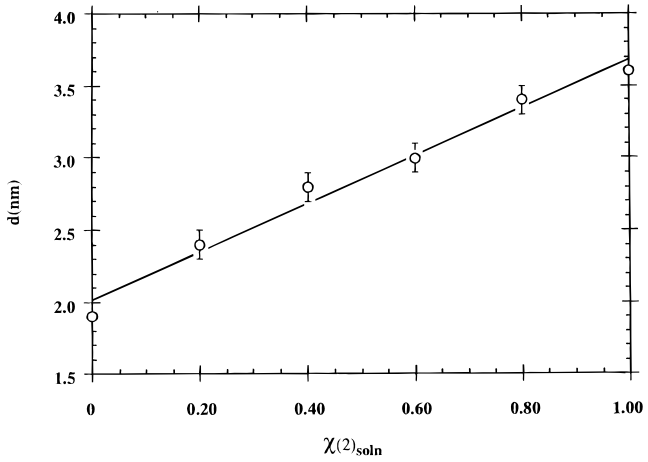


Figure 2. Ellipsometric thickness of mixed monolayers of thiols **2** and **3** on gold. $\chi(\mathbf{2})_{soln}$ is the mole fraction of thiol **2** in the solution of thiols **2** and **3** used to prepare the mixed monolayer.

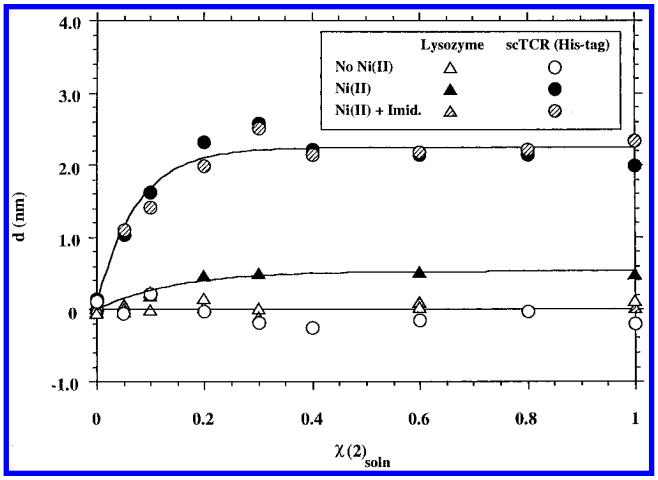


Figure 3. Ellipsometric thickness of adsorbed protein layers on mixed monolayers of thiols **2** and **3** as a function of the mole fraction of thiol **2**. Lysozyme and the His-tag-containing protein scTCR were adsorbed to SAMs for 1 h from 0.3 mg/mL solutions in HBS. "No Ni(II)" refers to surfaces without chelated Ni(II), "Ni(II)" refers to surfaces that were pretreated with a 40 mM aqueous solution of $Ni^{II}SO_4$, and "Ni(II) + Imid." refers to protein solutions containing 5 mM imidazole that were adsorbed onto surfaces pretreated with $Ni^{II}SO_4$.

treated with 1.0 mM NaOH, followed by 40 mM $Ni^{II}SO_4$ in water, and then washed with 150 mM NaCl and water. The SAMs were characterized by XPS and ellipsometry. Figure 1 shows an XPS spectrum of a SAM containing only the thiolate from **2**, after treatment with Ni(II) and washing. Peaks are present for both nitrogen and nickel. The integrated areas of the Ni 2p3 and N 1s peaks, after correcting for the different photoelectric cross sections of the orbitals,[21] are consistent with the expected 2:1 stochiometry of nitrogen to nickel, indicating that the Ni(II)-binding sites on the surface were active. Incubation of this surface in HBS solution for 1 h led to no observable loss of nickel (as measured by XPS after washing the sample). Measurement of the thickness of mixed monolayers of **2** and **3** by ellipsometry demonstrated an approximately linear increase with the mole fraction of **2** in solution [$\chi(\mathbf{2})_{soln}$] and established that the ratio of **1**:**2** on the surface corresponded roughly to that in solution (Figure 2).[22]

Ellipsometry was used to measure the average thickness of the protein layer that resulted from adsorption of proteins on the mixed monolayers and to test for the selectivity of these surfaces. As a model for a His-tagged protein, we used a three-domain single-chain T-cell receptor construct (ABC scTCR) with a $(His)_6$ tag incorporated near the C-terminus, and a molecular mass of 42 kDa;[13] this protein was available from other studies. To test for nonspecific binding of proteins to the negatively charged SAMs by an ion-exchange mechanism, we measured the adsorption of a highly positively charged model protein, lysozyme (pI ~11). Figure 3 shows the results of these binding studies. Surfaces that were not pretreated with Ni(II) did not bind either protein as long as the buffer included EDTA to scavenge metal ion impurities that could complex with the NTA group. The lack of nonspecific adsorption of lysozyme indicated that adsorption of even strongly positively charged proteins to the negatively charged surface through charge−charge interactions was not significant under these conditions. Pretreatment of the surfaces with aqueous Ni(II) led to strong binding of the scTCR. The binding increased with increasing surface concentration of the NTA group until a maximal value was reached at $\chi(\mathbf{2})_{soln} = 0.2$ (presumably from formation of a complete monolayer of protein). The binding was concentration dependent. Half-maximal binding required an scTCR concentration of ~1.5 μM (Figure 4). Figure 3 shows some binding of lysozyme was also observed when Ni(II) was present, through histidines on the surface of the protein,[23] but the thickness of this layer was much less than observed for the scTCR. The addition of low concentrations of imidazole to solutions of protein enhanced the selectivity of the surfaces for His-tagged proteins: 5 mM imidazole eliminated nonspecific binding of lysozyme while having little or no effect on the binding of the scTCR. Immobiliza-

(21) Corrections were applied by the Surface Science Instruments software according to: Scofield, J. H. *J. Electron Spectrosc.* **1976**, *8*, 129−137.

(22) Since ellipsometry measures an average thickness over a macroscopic area (~1 mm^2), the data do not rule out the possibility that the two thiols are not homogeneously mixed on the surface. Recently, Stranick et al. have shown, by scanning tunneling microscopy, that mixed monolayers formed from $CH_3(CH_2)_{15}SH$ and $CH_3O_2C(CH_2)_{15}SH$ phase segregate into microdomains with length scales of approximately 1−10 nm: Stranick, S. J.; Parikh, A. N.; Tao, Y.-T.; Allara, D. L.; Weiss, P. S. *J. Phys. Chem.* **1994**, *98*, 7636−7646. Disorder in the tri(ethylene glycol) chains attached to thiols **2** and **3** could lead to better mixing of these thiols; however, the process of phase separation in SAMs is not yet understood well enough to make predictions with confidence.

(23) Zhao, Y.; Sulkowski, E.; Porath, J. *Eur. J. Biochem.* **1991**, *202*, 1115−1119.

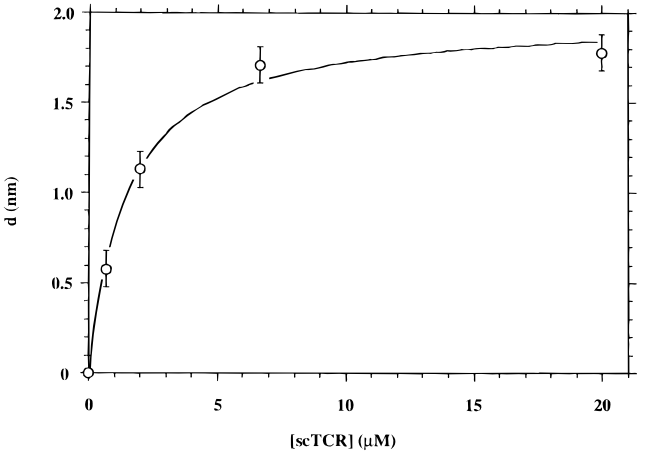


Figure 4. Ellipsometric thickness of His-tag-containing scTCR adsorbed on Ni(II)−NTA-SAM as a function of the protein concentration. Solutions of the scTCR in HBS were adsorbed for 1 h on a mixed monolayer of thiols **2** and **3** [$\chi(\mathbf{2})_{soln} = 0.1$] that had been pretreated with a 40 mM aqueous solution of $Ni^{II}SO_4$.

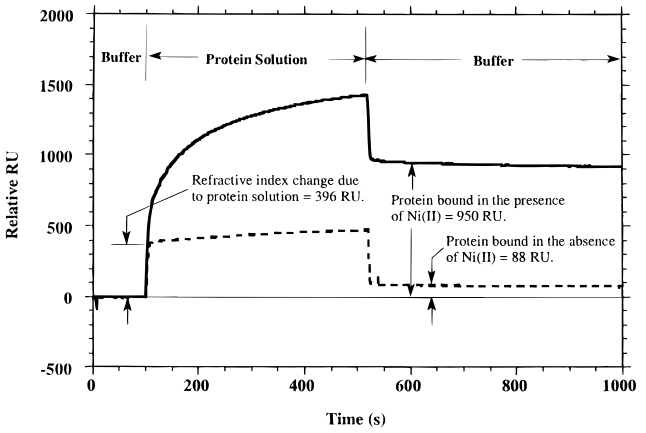


Figure 5. SPR signal on injection of the scTCR His-tag protein over a mixed SAM comprising NTA (**2**) and $(EG)_3OH$ (**3**) groups. The solid line is the signal after pretreatment with 25 μL of 40 mM aqueous $Ni^{II}SO_4$ before injection of the protein. The dashed line is the signal in the absence of Ni(II) on the surface.

tion of the scTCR was reversed by high concentrations of imidazole: treatment of the adsorbed protein layer with 200 mM imidazole in HBS, pH 7.2, for 30 min led to complete dissociation of the bound protein from the surface.

For SPR studies, we used SAMs prepared with a low mole fraction of thiol **2** [$\chi(\mathbf{2})_{soln} = 0.1$] to ensure that adsorbed proteins would not be densely packed on the surface.[24] Figure 5 shows that the adsorption of the scTCR can be followed by SPR under conditions of continuous flow and that the adsorption requires Ni(II) to be present on the surface. Solutions of the scTCR were passed over two surfaces: one had been pretreated with a solution of $Ni^{II}SO_4$; the other had no metal ions bound to the NTA groups. Upon injection of protein-containing solution over each surface, there was a rapid response due to differences in the refractive indices of the buffer and protein solution (1000 RU is a change in resonance angle of 0.1° and corresponds to a change in the index

(24) Adsorbed proteins should not be densely packed as long as the NTA-terminated thiolates are randomly distributed or phase segregated into protein size microdomains (see ref 22). Another possibility that we cannot rule out is that the thiolates phase segregated into domains much larger than a protein. This possibility could lead to islands of densely packed proteins surrounded by areas free of protein.

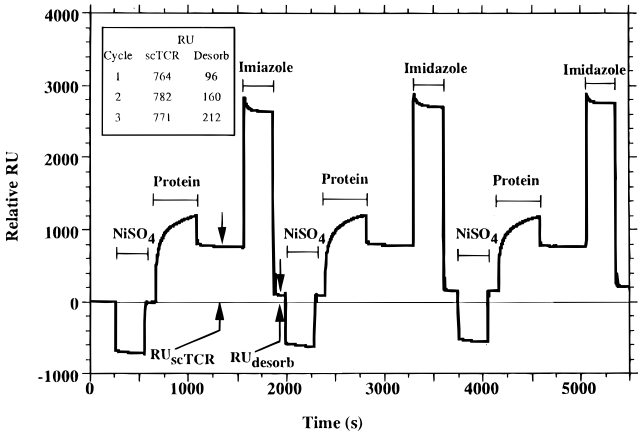


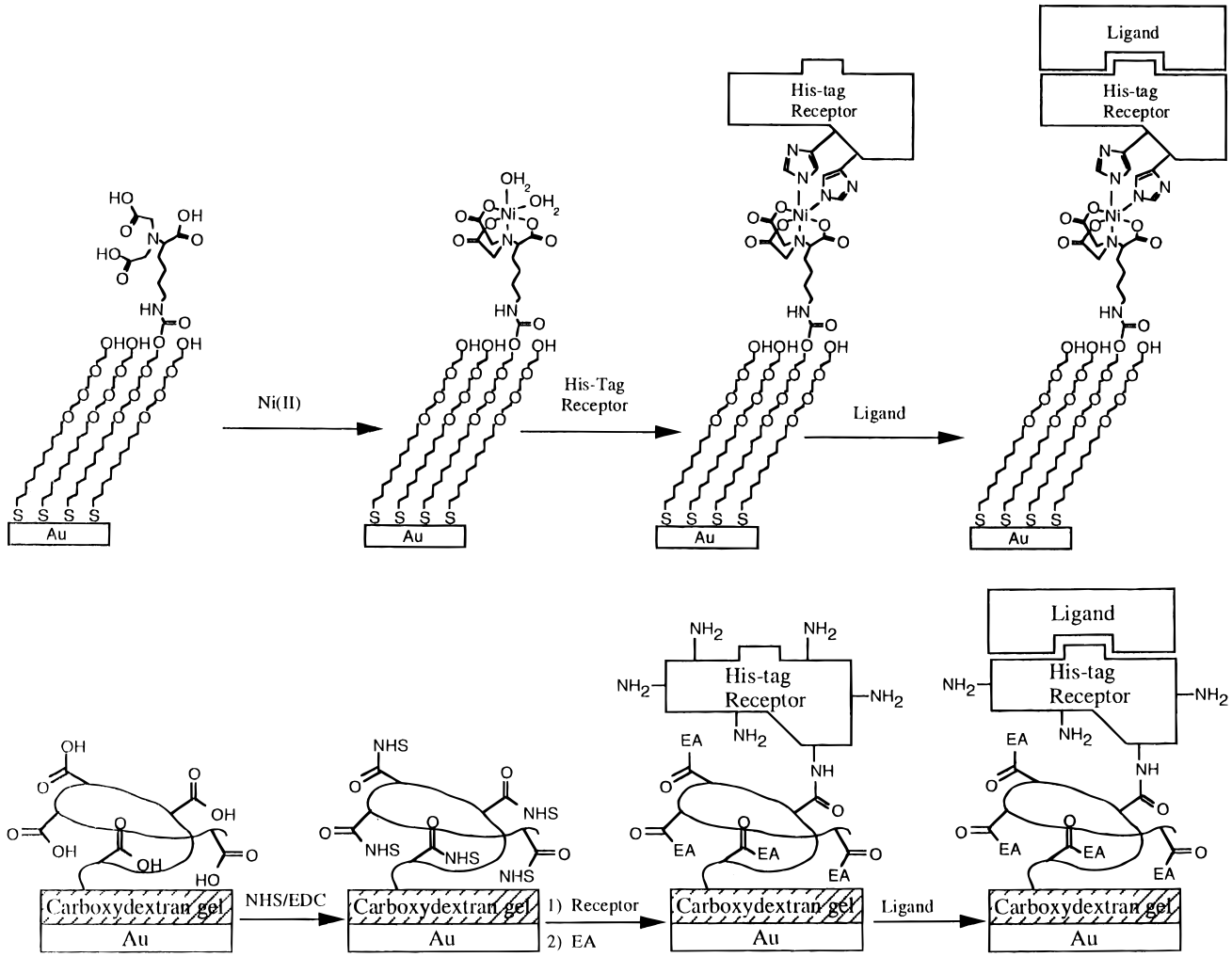
Figure 6. SPR signal on adsorption of the His-tagged scTCR followed by desorption with imidazole. The surface was treated sequentially with 25 μL of a 40 mM aqueous solution of $Ni^{II}SO_4$, 35 μL of a 0.1 mg/mL solution of the scTCR, and 25 μL of a 200 mM solution of imidazole in HBS, pH 7.2. The surface was washed with HBS after each injection. This cycle was repeated three times.

of refraction in solution of 0.0011), followed by a slower increase as the His-tag-modified protein bound to the surface (the formation of a protein film with a refractive index of 1.45 will give an SPR response of 700 RU/nm of film thickness).[25] On reinjection of buffer into the flow cell, there was an immediate drop in the SPR signal due to the change in refractive index, but the signal remained elevated compared to the original baseline because of the bound scTCR. The mass of adsorbed protein is proportional to the difference between the SPR signal before injection of the protein solution and the SPR signal after reinjection of buffer.[5] The surface presenting Ni(II) ions bound more protein than one that was free of Ni(II) by more than a factor of 10; this observation confirms the requirement for Ni(II) on the surface for protein binding.

In order to use SPR to study the interaction of bound His-tagged proteins with molecules in solution, the rate of dissociation of His-tagged proteins from the surface must be slow. There should, however, also be a way to desorb His-tagged proteins quickly from the surfaces so that they can be used in multiple experiments. Adsorbed layers of the scTCR bound to the NTA−Ni(II) surface were kinetically stable in HBS. After flowing HBS over adsorbed scTCR for 1 h, the SPR signal indicated that greater than 95% of the protein remained on the surface. Despite the stability of the adsorbed protein layers in HBS, the NTA surface was easily regenerated by treatment with 200 mM imidazole, pH 7.2, for 5 min. Three cycles of adsorption of the scTCR followed by desorption with imidazole are shown in Figure 6. About 90% of the adsorbed protein was dissociated from the surface during the first treatment with imidazole. In subsequent cycles, each treatment with the scTCR left the same total mass of protein on the surface, but the SPR signal due to protein remaining on the

(25) The theoretical SPR response to changes in the index of refraction of the bulk liquid and to deposition of thin protein films was determined by calculating the reflection of p-polarized light from a stratified, planar, isotropic structure, as described by: Azzam, R. M. A.; Bashara, N. M. *Ellipsometry and Polarized Light*; North-Holland: New York, 1977. The model used consisted of three layers with finite thicknesses (gold, SAM, and protein) between two semiinfinite media (glass and solution). The indices of refraction for the gold (0.17 + 4.93I), glass (1.511), and water (1.329) layers were taken from ref 5. We used an index of refraction of 1.45 for both the SAM and protein layers (see ref 16).

Scheme 2. Procedures for Immobilizing Proteins on Surfaces To Test for the Ability of Immobilized Proteins To Interact with Ligands in Solution[a]



[a] (a, top) Receptors are immobilized through the interaction of a His-tag group with Ni(II) chelated to an NTA-SAM. (b, bottom) Receptors are immobilized by covalent coupling to a carboxylated dextran gel layer. In this procedure, treatment of the surface with EDC in the presence of NHS leads to formation of NHS-activated esters on the surface. Proteins are immobilized on this surface by reaction of protein amino groups with the activated esters. Ethanolamine (EA) is added to quench unreacted NHS esters.

surface after each treatment with imidazole gradually increased. We have not yet identified the origin of this apparent irreversibility and heterogeneity in binding.[26] It may be possible to reduce the amount of residual protein further by increasing the concentration of imidazole or by increasing the time of treatment; we have not, however, optimized this procedure.

To ensure that His-tagged proteins other than the scTCR bound well to nickel on the NTA-SAM, we tested four other His-tagged proteins that were available from other studies. These included human TATA box binding protein (huTBP), the transcriptional activator Gal 4, and two components of the yeast RNA polymerase II holoenzyme—TFIIB and Gal 11.[14] Gal 4 without a His-tag group was also available and was tested as a control to ensure that binding of Gal 4 was through the His-tag. Table 1 lists the results of the binding experiments. All the His-tag labeled proteins bound well. The Gal 4 construct without a His-tag did not bind.

Table 1. SPR Signal from Adsorption of Proteins to Ni(II) on NTA-SAM[a]

protein	His-tag	conc (mg/mL)	ΔRU
ABC scTCR	yes	0.30	1928
Gal 11	yes	0.05	673
hu TBP	yes	0.23	2265
TFIIB	yes	0.23	1344
Gal 4	yes	0.25	2685
Gal 4	no	0.50	47

[a] $\chi(\mathbf{2})_{soln} = 0.10$. Surface was pretreated with a 40 mM aqueous solution of $NiSO_4$ for 7 min and then with protein solution in HBS for 7 min.

We used SPR to compare the effect of the immobilization technique on the ability of immobilized proteins to interact with proteins in solution. As shown in Scheme 2, His-tagged proteins were immobilized either by adsorption to Ni(II) on an NTA-SAM or by chemically coupling to a carboxylated dextran surface after activation with EDC in the presence of NHS.[27] We tested

(26) Some possible explanations for the heterogeneity in the dissociation of adsorbed scTCR by imidazole include the following: (i) some fraction of adsorbed protein may have bound to multiple Ni(II) ions and been less sensitive to treatment with imidazole, (ii) there may have been a strongly adsorbing impurity in the scTCR preparation, or (iii) some denaturation of protein may have occurred on the surface.

(27) This procedure is commonly used to immobilize proteins on the carboxydextran-modified substrates sold by Pharmacia for use with the BIACore SPR instrument (see ref 3 for details).

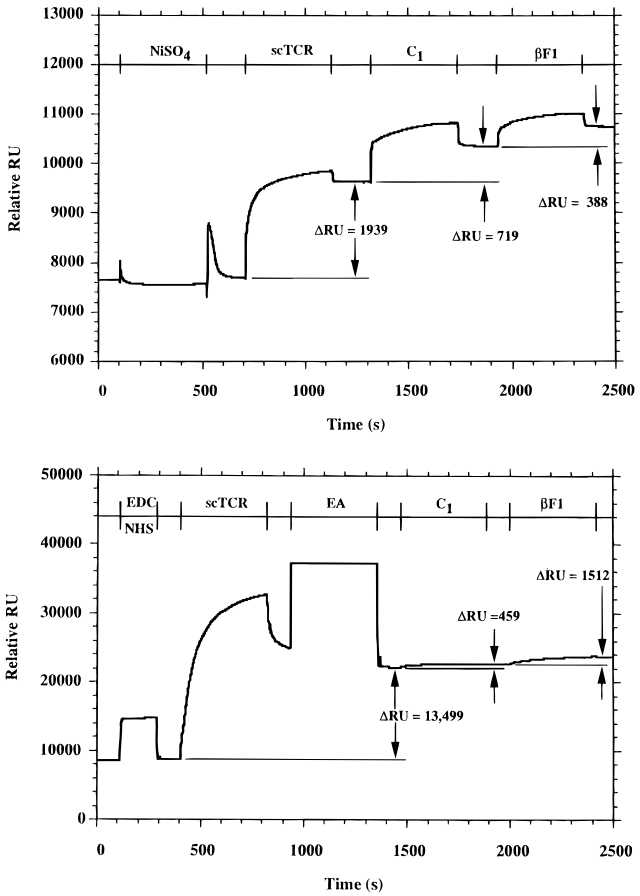


Figure 7. SPR-detected enhanced binding to the His-tagged scTCR, by two monoclonal antibodies (C_1 and βF1) recognizing different epitopes, when the scTCR was immobilized on a Ni(II)−NTA-SAM compared to when the scTCR was immobilized by covalent coupling to a carboxydextran gel layer. (a, top) Plot shows four sequential injections of solutions over the NTA-SAM: $Ni^{II}SO_4$ (40 mM aqueous solution, 35 μL), scTCR (0.3 mg/mL, 35 μL), C_1 (0.2 mg/mL 35 μL), and βF1 (0.2 mg/mL, 35 μL). The surface was washed with HBS after each injection. The proteins were diluted in HBS containing 20 mM imidazole. (b, top) Plot shows five sequential injections over the carboxydextran surface: an aqueous solution containing 200 mM EDC and 50 mM NHS (15 μL), scTCR (0.3 mg/mL, 35 μL), ethanolamine (1 M, 35 μL), C_1 (0.2 mg/mL, 35 μL), and βF1 (0.2 mg/mL, 35 μL). The proteins were diluted in HBS.

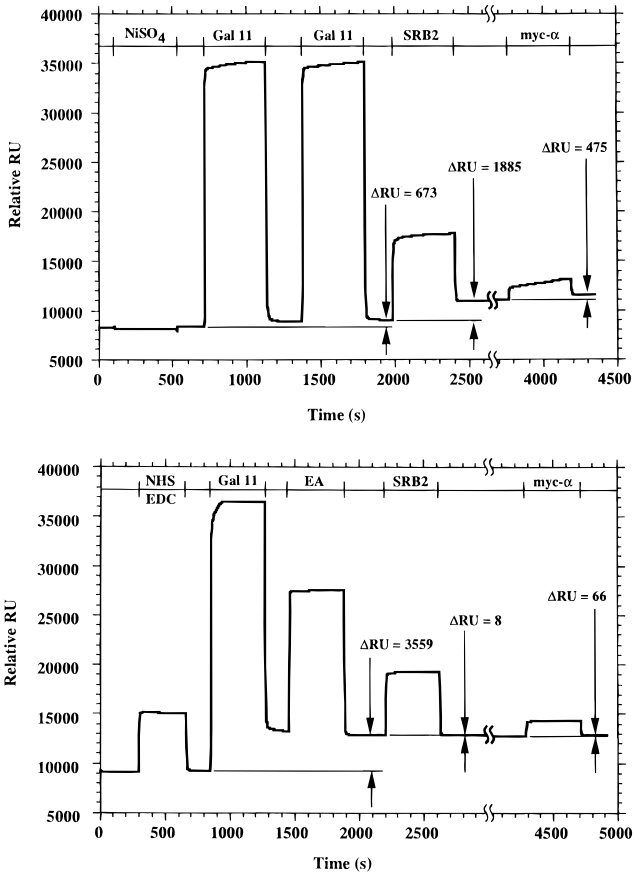


Figure 8. Interaction of mini-Gal 11 with SRB2 and a monoclonal antibody (myc-α) detected by SPR when mini-Gal 11 was immobilized by metal affinity on a Ni(II)-containing NTA-SAM but not detected when Gal 11 was covalently immobilized on a carboxydextran gel layer. (a, top) His-tag mini-Gal 11 (0.05 mg/mL) was immobilized on the Ni(II)−NTA-SAM by two consecutive injections because of the low protein concentration. SRB2 (0.25 mg/mL) and myc-α bound to the immobilized protein as evidenced by significant increases in the SPR signal. (b, bottom) Mini gal 11 was covalently attached to the carboxydextran surface as described in Figure 6. Although more protein was immobilized in the dextran gel layer than on the SAM, the protein immobilized on the SAM was not recognized by SRB2 or myc-α. The large refractive index changes during the protein injections are due to the presence of glycerol in the stock protein solutions.

antibodies against two epitopes on the scTCR for their ability to bind to scTCR immobilized by the two techniques (Figure 7). Monoclonal antibody C_1 is specific for an epitope of the $V_{\beta 17}$ TCR gene segment present in the scTCR, while the monoclonal antibody βF1 recognizes an epitope present in the constant region of the β-chain (called C_β).[13] Comparison of the SPR signal (ΔRU_{scTCR}) on immobilization of the scTCR to the Ni(II)−NTA and carboxylated dextran surfaces indicates that much more scTCR immobilized to the dextran layer. The dextran surface exhibited a higher immobilization capacity because the thick (~100 nm) hydrogel layer can incorporate the weight equivalent of many monolayers of protein,[5] while the Ni(II)−NTA-SAM can adsorb a maximum of one full monolayer. Despite the larger immobilization capacity of the dextran surface, scTCR adsorbed on the Ni(II)−NTA-surface was better able to bind antibodies in solution (as indicated by the ratio $\Delta RU_{Ab}/\Delta RU_{scTCR}$, which is proportional to the percentage of immobilized scTCR molecules recognized by the antibodies). Figure 7a shows a large portion of the scTCR immobilized to Ni(II) on the surface was reactive to both C_1 and βF1 added sequentially ($\Delta RU_{C1}/\Delta RU_{scTCR} = 0.37$, $\Delta RU_{\beta F1}/\Delta RU_{scTCR} = 0.20$). In contrast, Figure 7b shows that when the scTCR was chemically coupled to a carboxylated dextran surface only a small fraction of the protein attached to the surface could bind to either C_1 or βF1 ($\Delta RU_{C1}/\Delta RU_{scTCR} = 0.03$, $\Delta RU_{\beta F1}/\Delta RU_{scTCR} = 0.11$). Either the dextran matrix or the transformations involved in covalent coupling appeared to mask the epitopes recognized by these antibodies. A negative control antibody (myc-α) did not bind to the scTCR on either surface (data not shown). This observation confirmed that the measured interactions were specific. Possible explanations for the differences in behavior for the two systems include the following: (i) the covalent immobilization procedure may have led to chemical modification of the antibody epitopes or to changes in the three-dimensional structure of the scTCR, (ii) the scTCR may have been linked to the dextran matrix in an orientation that made the epitopes inaccessible to the antibodies, or (iii) the dextran matrix itself may have interfered with the ability of the antibodies to access the scTCR.

Similarly, mini-Gal 11,[14] produced with a His-tag at the amino terminus, was immobilized either through Ni(II) to the NTA-SAM or by EDC−NHS coupling to the carboxylated dextran surface (Figure 8a,b, respectively). SRB2, another component of the yeast RNA polymerase II holoenzyme,[28] and myc-α, an antibody to an epitope included in the mini- Gal 11 construct,[14] bound to Gal 11 immobilized through Ni(II) to the NTA-SAM ($\Delta RU_{SRB2}/\Delta RU_{Gal\ 11} = 2.80$, $\Delta RU_{myc\text{-}\alpha}/\Delta RU_{Gal\ 11} = 0.71$) but not to Gal 11 chemically immobilized to the carboxylated dextran surface ($\Delta RU_{SRB2}/\Delta RU_{Gal\ 11} = 0.00$, $\Delta RU_{myc\text{-}\alpha}/\Delta RU_{Gal\ 11} = 0.02$).

CONCLUSIONS

Many proteins of biological interest are prepared by overexpression in cells. We have taken advantage of a modification commonly included in the constructs encoding these proteins to aid in their purification (His-tag) to develop a method to immobilize them on the surface of gold for study by SPR. This method offers advantages over methods that have historically been used for immobilizing proteins on metal surfaces, including covalent attachment to a carboxylated dextran layer. The surface of the gold modified by inclusion of thiolate **1** allows immobilization of protein containing His-tag with a higher percentage of protein recognizable by antibodies and other proteins than can be achieved by covalent modification.[29]

This method of immobilization may also be useful for other bioanalytical techniques that require or are compatible with proteins immobilized on metal surfaces, for example: interferometry,[30] surface acoustic wave sensing, [31] ellipsometry,[32] amperometric detection,[33] and electrochemiluminescence.[34]

ACKNOWLEDGMENT

This study was supported by NIH (GM 30367, CA 47554, GM 22526) and ONR/ARPA (N00014-93-1-0498). C.B. was supported by a training program in Molecular Biophysics (MRSA 2T32GM08313-06). We thank Prof. Don Wiley (Dept. of Biochemistry and Molecular Biology, Harvard University) for the use of his Biacore instrument, Dr. Steve Friedman (Dept. of Medicine, Cornell University Medical College) for providing the C_1 monoclonal antibody, Luisella Barberis-Maino (Dept. of Bochemistry and Molecular Biology, Harvard University) for technical assistance, and Prof. Mark Ptashne (Dept. of Biochemistry and Molecular Biology, Harvard University) for helpful discussions.

NOTE ADDED IN PROOF

Subsequent to the submission of this paper, Gershon and Khilko published a report describing the preparation of a carboxydextran film with immobilized NTA groups for use in SPR studies.[35]

Received for review April 26, 1995. Accepted October 16, 1995.[⊗]

AC9504023

[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1995.

(28) Koleske, A. J.; Young, R. A. *Nature* **1994**, *368*, 466−469.

(29) The Ni−NTA surface we describe here has already been applied in probing the interactions between an array of defined proteins and protein fragments involved in gene regulation in yeast (ref 14). Without exception, a strong correlation was found between the strengths of the interactions observed in vitro and the known biological activity of the molecules. These data indicate that, at least within the range of the interaction strengths tested (10^{-6}−10^{-7} M), the surface can be used to quantitate certain protein−protein interactions accurately. More experience with the method is, however, required to evaluate its generality.

(30) Schlatter, D.; Barner, R.; Fattinger, Ch.; Huber, W.; Hubscher, J.; Hurst, J.; Koller, H.; Mangold, C.; Muller, F. *Biosens. Bioelectron.* **1993**, *8*, 109−116.

(31) Dahint, R.; Grunze, M.; Josse, F. *Anal. Chem.* **1994**, *66*, 2888−2892.

(32) Steibel, Ch.; Brecht, A.; Gauglitz, G. *Biosens. Bioelectron.* **1994**, *9*, 139−146.

(33) Janata, J.; Bezegh, A. *Anal. Chem.* **1988**, *60*, 62−74.

(34) Gudibande, S. R.; Kenten, J. H.; Link, J.; Friedman, K.; Massey, R. J. *Mol. Cell. Probes* **1992**, *6*, 495−503.

(35) Gershon, P. D.; Khilko, S. *J. Immunol. Methods* **1995**, *183*, 65−76.