Maleimide-Functionalized Self-Assembled Monolayers for the Preparation of Peptide and Carbohydrate Biochips†

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This paper reports a convenient method for immobilizing biologically active ligands to self-assembled monolayers of alkanethiolates on gold (SAMs). This methodology is based on monolayers that present maleimide and penta(ethylene glycol) groups. The maleimide groups react efficiently with thiol-terminated ligands, whereas the penta(ethylene glycol) groups prevent the nonspecific adsorption of protein to the substrate. The rate and selectivity of the immobilization of a ferrocene-thiol conjugate were characterized using cyclic voltammetry. This paper presents three examples of biochips prepared using this methodology. In the first example, four carbohydrate-thiol conjugates were immobilized to monolayers and the lectin-binding properties of the substrates were examined using fluorescence and surface plasmon resonance spectroscopy. The second biochip was used to study the enzymatic phosphorylation of the immobilized peptide IYGEFKKKC by the tyrosine kinase c-*src*. Monolayers presenting this peptide were then used to study the inhibition of the enzyme in an array format. The final class of substrates, which presents the tripeptide Arg-Gly-Asp, was used for studies of integrin-mediated cell adhesion. The immobilization methodology described here, which combines the structural order and inert properties of SAMs with the efficient reaction between soluble thiol and surface-bound maleimide groups, will be useful for preparing substrates for a wide range of applications in basic science and biotechnology.

Introduction

The development of strategies to immobilize groups of biopolymers to substrates has given rise to the field of biochips and has dramatically increased the rate and scope of discoveries in basic and applied science. Examples include the development of DNA chips for genome analysis,[1,2] the preparation of protein chips for evaluation of protein−substrate interactions,[3−6] and the construction of peptide[7−10] and carbohydrate chips[11−14] for the evaluation of ligand−receptor interactions and enzymatic activities. A key challenge in biochip technology has been the development of reliable and reproducible chemistries for the immobilization of ligands to a single substrate. In this paper, we describe a class of maleimide-terminated self-assembled monolayers for the immobilization of ligands and demonstrate this methodology with the preparation of peptide and carbohydrate chips.

To be broadly useful for the preparation of a wide variety of biochips, an immobilization reaction should have several characteristics. First, the reaction should occur rapidly and therefore allow the use of low concentrations of reagents for immobilization. Second, the chemistry should require little, if any, postsynthetic modification of ligands before immobilization to maximize the number of compounds that can be generated by solution or solid-phase synthesis and minimize the cost of these reagents. Third, the immobilization process should occur selectively in the presence of common functional groups, including amines, thiols, carboxylic acids, and alcohols, to ensure that ligands are immobilized in an oriented and homogeneous manner. Finally, the reaction should have well-behaved kinetics and be easily monitored with conventional spectroscopic methods to control the density of ligands on the chip.

Several groups have reported immobilization chemistries that possess one or more of the above features. Examples include the reaction of carboxylic acids or aldehydes with amines[15−17] and the reaction of aldehydes or ketones with aminooxy groups.[9,18,19] A larger number of immobilization methodologies have taken advantage of the reactivity of sulfhydryl groups at neutral pH. Schreiber and co-workers, for example, used the reaction between thiols and surface-bound maleimide groups to immobilize several small molecules, including biotin, FK-

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506 and tetramethylrhodamine, to glass slides.[20] More recently, Lam and co-workers prepared peptide arrays on glass slides by the reaction of N-terminal cysteine residues with immobilized aldehyde groups.[9] In a related report, Corn and co-workers used thiol-terminated self-assembled monolayers as a platform for the immobilization of maleimide-terminated nucleic acids.[21] Other groups have used thiol chemistry to prepare substrates for cell adhesion. Sigrist and co-workers, for example, derivatized titanium surfaces with maleimide groups for the immobilization of laminin and fibronectin fragments.[22,23] Zull and co-workers used a similar approach to immobilize laminin peptides to titanium surfaces presenting alkyl halides, benzyl halides, and α-haloacetyl groups.[24] Although useful, each of these methods uses substrates that are not strictly inert toward nonspecific interactions with proteins or radioisotopes in solution, making quantitative analysis of protein binding interactions difficult. Furthermore, these methods do not offer good control over the density of maleimide in the film and therefore are not well suited for defining the density of ligands on the substrate.

Our group has used the Diels−Alder reaction between ligand-cyclopentadiene conjugates and quinone groups immobilized on self-assembled monolayers for the preparation of peptide and carbohydrate chips.[7,11] This methodology has several attractive features, including well-behaved reaction kinetics, compatibility with a wide range of functional groups, and the ability to monitor the reaction using cyclic voltammetry.[25−27] The combination of this immobilization chemistry with inert monolayers presenting oligo(ethylene glycol) groups enabled the quantitative analysis of ligand binding and enzymatic modification in an array format.[7,11] The principle disadvantage of the Diels−Alder reaction is that each ligand-cyclopentadiene conjugate must be synthesized before immobilization. This requirement may limit the size and complexity of biochips that can be prepared using this chemistry.

The use of maleimide-terminated self-assembled monolayers for the immobilization of thiol-terminated ligands has many of the advantages of Diels−Alder-mediated immobilization, including inert surfaces, well-behaved reactivity, and the capability of quantitative analysis on the chip, but does not require the postsynthetic modification of ligands. Here we report the use of this immobilization reaction for attaching ligands to SAMs. We characterize this reaction using several techniques, including electrochemistry, matrix-assisted laser desorption ionization-time of flight (MALDI-ToF) mass spectrometry, surface plasmon resonance spectroscopy (SPR), and radioisotopic labeling. We then demonstrate the utility of this model system with the preparation of peptide and carbohydrate arrays.

Materials and Methods

Reagents. All reagents for chemical synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI). Tetrahydrofuran (THF) was distilled from sodium/benzophenone, and dichloromethane was distilled from calcium hydride. Anhydrous dimethylformamide (DMF) was purchased from Aldrich and used without further purification. Flash chromatography was performed with EM Science Kiselgel 60 (230−400 mesh). Dulbecco's phosphate-buffered saline, Dulbecco's modified Eagle's medium, fetal calf serum, penicillin, and streptomycin were purchased from Gibco Life Sciences (Gaithersburg, MD). Lectins were purhased from EY Laboratories (San Mateo, CA). ScintiVerse scintillation fluid and glass coverslips (no. 2, Corning) were obtained from Fisher Scientific. Quercetin dihydrate, mineral oil (PCR grade), and rhodamine-labeled concanavalin A were purchased from Sigma (Milwaukee, WI). C-src kinase was purchased from Calbiochem (La Jolla, CA). Goat anti-phosphotyrosine IgG was purchased from BD Biosciences (Location). [γ-^{32}P]ATP was obtained from ICN (Costa Mesa, CA). PP1 was purchased from New England BioLabs. Peptides were synthesized on Fmoc-Rink MBHA resin as described previously.[28] Ferrocene thiol **6** and *O*-allyl glycoside peracetates **7a**−**10a** were synthesized according to refs 29 and 30, respectively. The trifluoroacetate salt of 2-aminoethylmaleimide was synthesized according to ref 31.

Preparation of Substrates. Titanium (5 nm) and then gold (15 nm) were evaporated onto glass coverslips. Self-assembled monolayers were prepared by immersing the coverslips in a methanolic solution containing maleimide-terminated disulfide **1** and penta(ethylene glycol) disulfide **2** (20−50 μM in **1**, 1 mM in total disulfide). After 12 h the substrates were rinsed with methanol and dried under a stream of nitrogen.

Substrates presenting a single ligand were prepared by inverting a maleimide-terminated monolayer onto a piece of Parafilm presenting an aqueous solution of the appropriate thiol conjugate (30 μL, 2 mM in H_2O or buffer). The substrates were kept in a humidified chamber at 37 °C for 4 h, washed with water and methanol, and dried under a stream of nitrogen. For the preparation of arrays, aliquots (0.5−1 μL) of each thiol conjugate were spotted onto discrete regions of the chip. The array was kept in a humidified chamber at 37 °C for 4 h, washed with water and methanol, and dried under a stream of nitrogen.

Surface Plasmon Resonance Spectroscopy. SPR measurements were performed using a BIACore 1000 instrument. Monolayer substrates (prepared as described above using glass coverslips coated with 10 nm Ti and 50 nm Au) were incorporated into the BIACore cassettes by removing the manufacturer's substrate and gluing the chip into the cassettes using a two-part epoxy (Devcon) as described previously.[32] Measurements were performed using Dulbecco's phosphate-buffered saline (DPBS, pH 7.4) as the running buffer and are reported in changes in resonance angle ($\Delta\theta$), where 1° = 10 000 RU. The background response of the instrument to each solution of protein was measured by injection of the solution over a monolayer presenting penta(ethylene glycol) groups alone.

Electrochemistry. Electrochemical measurements were performed using a Bioanalytical Systems CV-50W potentiostat. Electrochemistry on SAMs was performed in a 1:1 mixture of THF/ PBS (pH 7.4), which served as the electrolyte. The gold substrate (12 nm Ti, 100 nm Au on a ⟨100⟩ Si wafer) served as the working electrode, a platinum wire as the counter electrode, and an Ag/AgCl/ KCl electrode as the reference electrode. All experiments were performed at a scan rate of 25 mV/s in the cyclic voltammetry mode.

Protein-Binding Assay. Monolayers presenting the carbohydrate array were inverted onto a solution of a rhodamine-lectin conjugate (100 μL of a 200 μg/mL solution in DPBS) on Parafilm. After 30 min, the substrates were gently washed with DPBS (2 × 2 min) and analyzed using an array scanner. For reproducible results, it is important that the substrates not dry during the analysis.

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Kinase Assays. Protein kinase assays were performed according to the manufacturer's protocol for c-*src*. In brief, reaction mixtures containing ATP mix [10 μL of an aqueous solution containing 150 μM ATP, [γ-^{32}P]ATP (1.25 μL, 3.75 μCi/μL), and 30 mM $MgCl_2$] and kinase (0.5 unit in 0.1 mg/mL BSA, 0.2% β-mercaptoethanol, 10 μL total volume) were added to substrates in a humidified chamber, and after a specified amount of time at 37 °C each substrate was washed with SDS (10% aqueous solution, 3 × 5 min) and water (3 × 5 min). Monolayers presenting radioactive phosphate were then exposed to a phosphor screen for 12 h or immersed in 5 mL of scintillation fluid. Analysis was performed using a Molecular Dynamics phosphorimager or a Beckmann scintillation counter, respectively.

For array experiments, substrates were covered with a layer of mineral oil and reaction mixtures (0.25 μL) containing [γ-^{32}P]-ATP (1.25 μL, 3.75 μCi/μL) were pipetted onto monolayers. Reactions were incubated at 37 °C for 4 h, and the mineral oil layer was removed by washing with hexanes. The surface was then rinsed with SDS (10%, 3 × 5 min) and water (3 × 5 min). Phosphorylation was detected by exposure to a phosphor screen for 12 h and analyzed using ImageQuant. For inhibition experiments, reaction mixtures containing a series of concentrations of inhibitors were prepared in 96-well microtiter plates. Each reaction mixture contained a final concentration of 2% dimethyl sulfoxide (DMSO). Reaction mixtures (0.25 μL) containing [γ-^{32}P]ATP (1.25 μL, 3.75 μCi/μL) were spotted onto monolayers overlaid with mineral oil, and after incubation for 4 h at 37 °C, the substrate was washed and analyzed as described above.

Cell Culture and Microcontact Printing. Swiss Albino 3T3 fibroblasts (ATCC) were cultured in DMEM, supplemented with 10% fetal bovine serum, 200 units/mL penicillin, and 200 μg/mL streptomycin (complete medium). Cultures were maintained at 37 °C in a humidified atmosphere containing 10% CO_2. Near confluent monolayers of cells were passaged by treatment with a solution of 0.05% trypsin/0.53 mM EDTA (Gibco). Cells were used between passages 3 and 10.

Microcontact printing was used to pattern hexadecanethiol [$HS(CH_2)_{15}CH_3$] onto gold-coated substrates.[33,34] The substrate was then immersed for 12 h in an ethanolic solution containing the disulfides **1** and **2** (10 μM in **1**, 1 mM in total disulfide) to install the monolayer in the remaining regions of gold. The substrates were rinsed thoroughly with absolute ethanol and dried under a stream of nitrogen. Substrates were then immersed in a solution of fibronectin (100 μg/mL in DPBS, pH 7.4) for 4 h to adsorb protein to the regions of hexadecanethiolate. Swiss 3T3 fibroblasts were removed from tissue culture substrates by treatment with a solution of 0.05% trypsin/0.53 mM EDTA and resuspended at a concentration of 60 000 cells/mL in serum-free culture medium. The cells were plated onto a patterned monolayer precoated with fibronectin and allowed to attach for 4 h. Microscopy was performed on a Zeiss Axiovert 135 microscope, and photomicrographs were captured at 5 X magnification using a Sony black and white CCD camera.

Synthesis of (Pyridin-2-yl)disulfanyl-11-undecyl-penta(ethylene glycol) 4. To a solution of 11-mercaptoundecyl penta(ethylene glycol) **3**[35] (525 mg, 1.23 mmol) in methanol (10 mL) were added aldrithiol-2 (528 mg, 2.4 mmol) and triethylamine (0.37 mL, 2.4 mmol). After 12 h at room temperature, the reaction mixture was concentrated in vacuo to afford a yellow oil. The crude product was purified by silica gel chromatography to afford pyridyl disulfide **4** as a clear oil (429 mg, 67%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (d, 1H, J = 4.8), 7.71 (d, 1H, J = 8.1), 7.64−7.60 (m, 1H), 7.06−7.03 (m, 1H), 3.69 (t, 2H, J = 4.7), 3.66−3.54 (m, 16H), 3.41 (t, 2H, J = 6.8), 2.76 (t, 2H, J = 7.4), 1.68−1.62 (m, 2H), 1.57−1.51 (m, 2H), 1.35−1.31 (m, 2H), 1.27−1.21 (m, 14H); ^{13}C NMR ($CDCl_3$) δ 149.30, 138.21, 136.80, 120.27, 119.33, 72.46, 71.33, 70.39, 70.36, 70.33, 70.29, 70.05, 69.79, 61.47, 38.81, 29.39, 29.33, 29.27, 29.24, 28.95, 28.71, 28.27, 25.86.

Synthesis of Acid Disulfide 5. To a solution of **4** (400 mg, 0.75 mmol) in THF (5 mL) were added 11-mercaptoundecyl-hexa(ethylene glycol) acetic acid[15] (473 mg, 0.9 mmol) and 4-dimethylaminopyridine (DMAP) (110 mg, 0.9 mmol). After 8 h at room temperature, the reaction mixture was concentrated and chromatographed on silica gel (eluent 88:10:2 CH_2Cl_2/MeOH/AcOH) to afford the title compound as a clear oil (622 mg, 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.06 (s, 2H), 3.68−3.52 (m, 44H), 3.39 (t, 4H, J = 6.8), 2.61 (t, 4H, J = 7.5), 1.62−1.58 (m, 4H), 1.52−1.48 (m, 4H), 1.32−1.20 (m, 30H); ^{13}C NMR ($CDCl_3$) δ 173.06, 72.35, 71.34, 70.36, 70.30, 70.24, 70.16, 70.11, 70.07, 70.01, 69.80, 69.77, 61.40, 38.92, 29.39, 29.34, 29.30, 29.04, 29.01, 28.32, 25.87.

Synthesis of Maleimide Disulfide 1. To a solution of acid **5** (568 mg, 0.6 mmol) in THF (5 mL) were added isobutyl chloroformate (66 μL, 0.72 mmol) and triethylamine (0.17 mL, 0.72 mmol). After 20 min, the trifluoroacetate salt of 2-aminoethylmaleimide[31] (200 mg, 0.8 mmol) was added, followed by another portion of triethylamine (0.35 mL, 2.5 mmol). The reaction mixture was allowed to stir for 12 h, concentrated, and chromatographed (eluent 10:1 CH_2Cl_2/MeOH) to afford disulfide **1** as a clear oil (456 mg, 71%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28 (br s, 1H), 6.67 (s, 2H), 3.93 (s, 2H), 3.69−3.54 (m, 44H), 3.51−3.48 (m, 2H), 3.47−3.39 (m, 4H), 2.65 (t, 4H, J = 7.4), 1.62−1.58 (m, 4H), 1.52−1.48 (m, 4H), 1.32−1.20 (m, 30H); ^{13}C NMR ($CDCl_3$) δ 170.69, 170.65, 134.07, 72.47, 71.39, 70.73, 70.45, 70.40, 70.36, 70.34, 70.30, 70.13, 70.09, 70.05, 69.89, 69.86, 61.53, 38.98, 37.63, 37.48, 29.47, 29.42, 29.38, 29.35, 29.34, 29.09, 29.06, 28.37, 25.93. MS (MALDI) calculated for $C_{52}H_{98}O_{16}N_2S_2$ (M+Na) 1104.5, found 1105.4.

Synthesis of Penta(ethylene glycol) disulfide 2. To a solution of 11-mercaptoundecyl penta(ethylene glycol) **3**[35] (425 mg, 1 mmol) in methanol (10 mL) was added iodine (127 mg, 0.5 mmol). After 2 h, the reaction mixture was concentrated and redissolved in EtOAc (25 mL). The solution was washed with NaS_2O_4 (2 × 10 mL) and brine (10 mL), dried over sodium sulfate, filtered, and concentrated *in vacuo*. The product was purified by silica gel chromatography (9:1 CH_2Cl_2/MeOH) to provide disulfide **2** as a clear oil (550 mg, 63%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.72−3.70 (m, 2H), 3.65−3.55 (m, 36H), 3.45−3.41 (m, 4H), 2.68−2.64 (m, 4H), 1.66−1.63 (m, 4H), 1.56−1.54 (m, 4H), 1.35−1.25 (m, 28H); ^{13}C NMR ($CDCl_3$) δ 72.51, 71.40, 70.45, 70.42, 70.39, 036, 70.12, 69.86, 61.55, 39.02, 29.46, 29.43, 29.39, 29.35, 29.09, 29.07, 28.39, 25.93.

Synthesis of 3-Thioacetylpropyl glycosides 7b−10b. To a solution of olefin **7a**−**10a**[30] (5 mmol) in dry THF (25 mL) was added thiolacetic acid (12.5 mmol) and 2,2′-azobisisobutyronitrile (AIBN, 1 mmol). The reaction mixture was irradiated in a photochemical reactor (Rayonet reactor lamp, Southern New England Ultraviolet Company, Model RPR-100) for 5 h under an atmosphere of nitrogen. Concentration of the reaction mixture, followed by flash chromatography (2:1 Hex/EtOAc) provided compounds **7b**−**10b** as clear oils.

3-Thioacetyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside 7b. 215 mg (93%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.32−5.21 (m, 4H), 4.77 (s, 1H, J = 1.5), 4.27−4.23 (m, 1H), 4.10−4.06 (m, 1H), 3.96−3.92 (m, 1H), 3.75−3.70 (m, 1H), 3.50−3.45 (m, 1H), 2.93 (t, 2H, J = 7.0), 2.31 (s, 3H), 2.13 (s, 3H), 2.09 (s, 3H), 2.02 (s, 3H), 1.96 (s, 3H), 1.92−1.84 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 190.59, 170.00, 169.82, 169.67, 97.59, 69.47, 68.96, 68.52, 66.69, 66.04, 62.39, 30.56, 29.16, 25.76, 20.84, 20.69, 20.63.

3-Thioacetyl-2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranoside 8b. 211 mg (91%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 (m, 1H), 5.26−5.23 (m, 1H), 5.04−5.00 (m, 2H), 4.13−4.10 (m, 1H), 4.01−3.99 (m, 2H), 3.69−3.64 (m, 1H), 3.40−3.35 (m, 1H), 2.87 (t, 2H, J = 7.0), 2.42 (s, 3H), 2.05 (s, 3H), 1.99 (s, 3H), 1.96 (s, 3H), 1.89 (s, 3H), 1.83−1.78 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 195.29, 170.19, 170.02, 169.80, 96.06, 67.84, 67.35, 66.56, 66.12, 61.56, 30.39, 28.96, 25.50, 20.55, 20.48, 20.42.

3-Thioacetyl-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside 9b. 207 mg (89%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.28 (t, 1H, J = 10.2), 4.89−4.84 (m, 2H), 4.71−4.67 (m, 1H), 4.10−4.07 (m, 1H), 3.93 (d, 1H, J = 10.2), 3.90−3.82 (m, 1H), 3.62−3.57 (m, 1H), 3.33−3.28 (m, 1H), 2.80 (t, 2H, J = 6.96), 2.16 (s, 3H), 1.91 (s, 3H), 1.86 (s, 3H), 1.84 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 194.99, 170.18, 169.77, 169.70, 169.21, 95.53, 70.41, 69.79, 68.23, 67.05, 66.50, 61.60, 30.29, 28.86, 25.38, 20.38, 20.34, 20.28.

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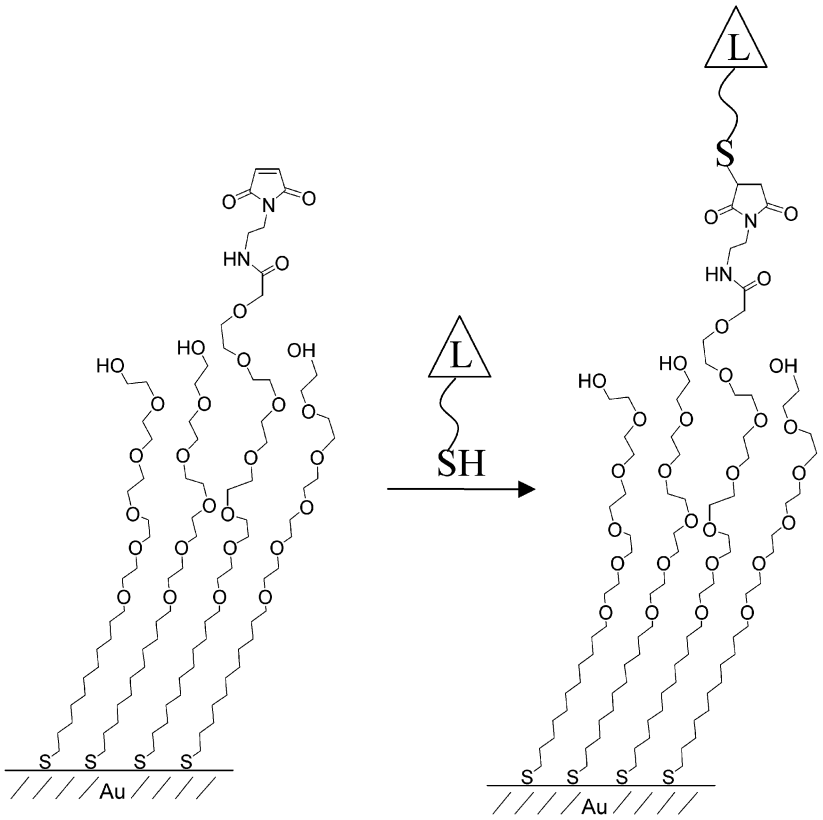

Figure 1. Structure of a self-assembled monolayer used to immobilized thiol-terminated ligands. The maleimide groups react selectively with thiol groups in a contacting solution while the penta(ethylene glycol) groups prevent the nonspecific adsorption of protein to the monolayer.

3-Thioacetyl-2-deoxy-2-acetamido-3,4,6-tri-*O*-acetyl-α-D-glucopyranoside 10b. 219 mg (95%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.06 (d, 1H, J = 9.5), 5.15 (t, 1H, J = 10), 5.03 (t, 1H, J = 9.8), 4.73 (d, 1H, J = 3.56), 4.32−4.26 (m, 1H), 4.18−4.14 (m, 1H), 4.03−3.99 (m, 1H), 3.89−3.84 (m, 1H), 3.71−3.65 (m, 1H), 3.41−3.36 (m, 1H), 3.04−2.97 (m, 1H), 2.90−2.84 (m, 1H), 2.38 (s, 3H), 2.02 (s, 3H), 1.95 (s, 3H), 1.95 (s, 3H), 1.94 (s, 3H), 1.91 (s, 3H), 1.84−1.81 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 195.48, 171.04, 170.46, 170.13, 169.14, 97.07, 71.14, 68.00, 67.63, 65.82, 61.79, 51.46, 30.46, 29.06, 25.27, 22.88, 20.55, 20.43.

Synthesis of 3-mercaptopropyl glycosides 7c−10c. Argon was bubbled through a solution of each 3-thioacetylpropyl glycoside (**7b**−**10b**, 3 mmol) in methanol (10 mL) for 30 min. Sodium methoxide (18 mmol) was added, and the reaction mixture was allowed to stir at room temperature for 4 h. The reaction mixture was acidified to a pH of 7 with 5 M methanolic HCl, and the solution was concentrated to an oil. Purification by silica gel chromatography (eluent 4:1 CH_2Cl_2/MeOH) afforded compounds **7c**−**10c**. MS (ESI) **7c** calculated for $C_9H_{18}O_6S$ (M+Na) 277.8, found 277.4; MS (ESI) **8c** calculated for $C_9H_{18}O_6S$ (M+Na) 277.8, found 278.1; MS (ESI) **9c** calculated for $C_9H_{18}O_6S$ (M+Na) 277.8, found 277.6; MS (ESI) **10c** Calcd for $C_{11}H_{19}O_6NS$ (M+Na) 318.7; found, 319.0.

Results

Approach. We used self-assembled monolayers on gold as a platform for immobilizing several classes of thiol-containing ligands. The monolayers were prepared by immersing gold-coated glass slides in a mixture of two disulfides, one presenting a terminal maleimide group and the other presenting terminal penta(ethylene glycol) groups (Figure 1). The relative concentration of the two compounds was adjusted to give monolayers having maleimide groups at a density of 1% relative to total alkanethiolate. The maleimide group reacts selectively and efficiently with thiol-containing molecules, whereas the penta(ethylene glycol) groups prevent the nonspecific adsorption of protein to the substrate and ensure that only specific interactions between soluble proteins and immobilized ligands occur. The excellent control over unwanted adsorption with monolayers presenting oligo(ethylene glycol) groups has been validated in several studies.[6,7,11,13−15,25−28]

Synthesis of Maleimide-Terminated Disulfide. Disulfide **1** was prepared in three steps from 11-mercaptoundecyl-penta(ethylene glycol) **3**[35] (Figure 2). This compound was converted into pyridyl disulfide **4** by treatment with aldrithiol-2. Displacement of the thiopyridyl group with 11-mercaptoundecyl-hexa(ethylene glycol)acetic acid[15] generated disulfide **5**. The carboxylic acid group of **5** was activated with isobutyl chloroformate and coupled with 2-aminoethylmaleimide[31] to afford compound **1** in 71% yield. Symmetric penta(ethylene glycol) disulfide **2** was prepared in 63% yield by oxidation of 11-mercaptoundecyl-penta(ethylene glycol)[35] with iodine.

Characterization of Reaction Rate and Scope. Before preparing chips presenting biologically active ligands, it was necessary to characterize the rate and selectivity of the immobilization reaction. We studied the immobilization of ferrocene thiol **6**[29] because the ferrocene group is electroactive and therefore allows the use of cyclic voltammetry (CV) to monitor the accumulation of the immobilized species. This technique is also convenient because the underlying gold substrate of the monolayer can be used as a working electrode to monitor the reaction in real time. SAMs presenting maleimide groups at a density of 2.5% (relative to total alkanethiolate) were immersed in a solution containing compound **6** (0.5 mM in 1:1 THF/PBS), and the surface was scanned in situ using CV at a rate of 25 mV/s. The ferrocene redox peak was centered at −190 mV and grew in intensity until the reaction reached completion at 3 h (Figure 3A). We

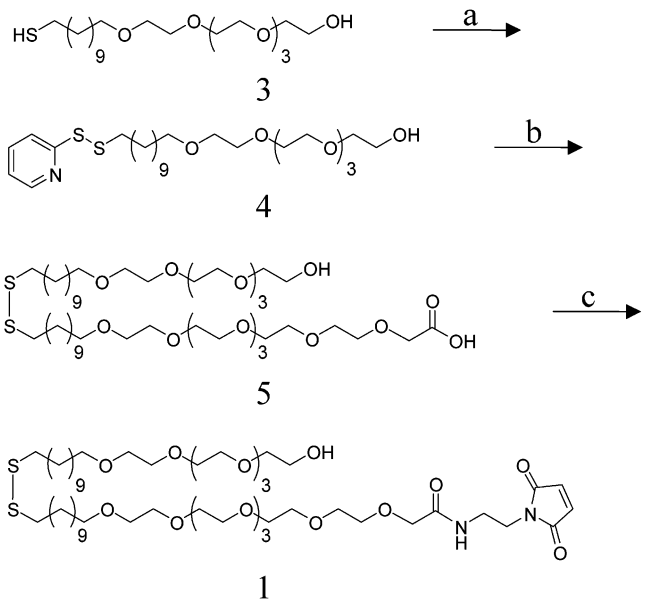

Figure 2. Synthesis of disulfide **1** used for the preparation of surfaces presenting maleimide groups. Reagents: (a) Aldrithiol-2, MeOH; (b) $HS(CH_2)_{11}O(CH_2CH_2O)_6CH_2CO_2H$, DMAP, THF; (c) *i.* ^{i}BuOCOCl, Et_3N, DMF, *ii.* β-aminoethylmaleimide.

integrated the area under the redox peak to calculate the total charge and therefore the number of redox active molecules on the surface. The value of 1.6×10^{-11} mol/cm^2 determined by this method corresponds to a monolayer having maleimide groups at a density of 2.5%.

To characterize the specificity of the immobilization reaction, maleimide-terminated monolayers were treated for 3 h with solutions containing ferrocene thiol **6** (0.5 mM in 1:1 THF/PBS, pH 7.4), β-mercaptoethanol (2 mM in PBS, pH 7.4), or dimethylamine (1 M in 1:1 THF/H_2O, pH 7.4). The monolayers were then incubated with ferrocene thiol **6** for 3 h to determine the number of maleimide groups still remaining on the monolayer. Figure 3B shows that treatment of the monolayer with compound **6** gave 1.0 μA of current. Pretreatment of a monolayer with β-mercaptoethanol, however, completely prevented subsequent immobilization of compound **5** to the monolayer. Pretreatment with dimethylamine had no influence on the immobilization of thiol **6**, demonstrating that amino groups do not react with the immobilized maleimide groups.

Immobilization of Carbohydrates for Biospecific Recognition. To demonstrate the utility of this chemistry for the immobilization of carbohydrates, we treated a monolayer presenting maleimide groups at a density of 1% with a 2 mM aqueous solution of mannose conjugate **7c** (prepared according to Figure 4). After 2 h at room temperature, the resulting substrate was washed with distilled water and examined using MALDI-ToF mass spectrometry (Figure 5A). The mass spectrum showed peaks corresponding to the immobilized mannose conjugate (*m*/*z* 1349.8) and the sodium adduct of penta(ethylene glycol) disulfide **2** (*m*/*z* 871.1). The absence of a peak corresponding to maleimide disulfide **1** provides further evidence that the reaction had gone to completion.

To demonstrate that the immobilized mannose groups were competent for interacting with proteins in a contacting solution, we examined the binding of the lectin concanavalin A[36] (5 μM in DPBS, pH 7.4) to the chip using surface plasmon resonance spectroscopy.[37,38] Figure 5B shows that substrates presenting mannose bound the lectin, whereas unmodified substrates or substrates presenting galactose conjugate **8c** did not interact with the protein.

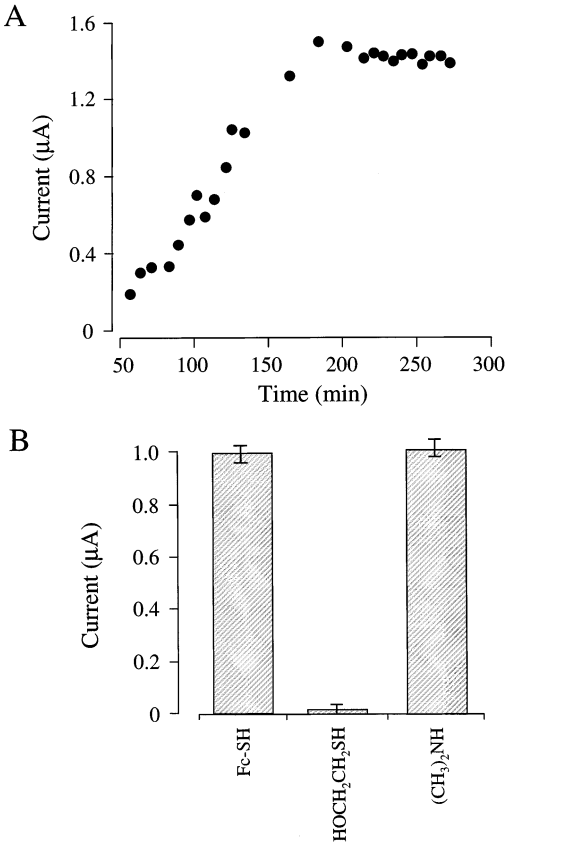

Figure 3. Characterization of the reaction between soluble thiol and immobilized maleimide groups. (A) Rate of the immobilization reaction. SAMs presenting the maleimide group at a density of 2.5% were treated with a solution of ferrocene thiol **6** (0.5 mM in 1:1 THF/PBS) for 4 h in an electrochemistry cell (1:1 THF/PBS, pH 7.4, scan rate = 25 mV/s). The intensity of the oxidation peak of ferrocene at −190 mV was measured in situ. A plot of peak intensity with respect to time shows that the reaction reaches completion in 3 h, resulting in a surface density of ferrocene of 1.6×10^{-11} mol/cm^2. (B) Selectivity of the immobilization reaction. SAMs presenting the maleimide group at a density of 2.5% were treated for 2 h with solutions containing various reactive molecules. The substrates were then incubated with ferrocene thiol **6** (2 mM in 1:1 THF/PBS, pH 7.4) for 3 h and the resulting substrates were characterized by cyclic voltammetry in situ. Preincubation with compound **6** resulted in 1.0 μA of current, whereas preincubation with β-mercaptoethanol (2 mM in PBS, pH 7.4) prevented the subsequent immobilization of compound **6**. Preincubation with dimethylamine (1 M in 1:1 THF/H_2O, pH 7.4), did not influence the incorporation of ferrocene into the monolayers, showing the specificity of the reaction for thiols.

Preparation and Characterization of Carbohydrate Arrays. We next prepared an array presenting four monosaccharide conjugates and characterized the binding of several lectin proteins to the array. The arrays were prepared by applying a drop of each carbohydrate conjugate (**7c**−**10c**; 1 μL of a 2 mM solution in H_2O) to discrete locations on a single monolayer presenting maleimide groups (Figure 6A). The substrates were kept in a humidified chamber at 37 °C for 2 h, washed with water and dried under a stream of nitrogen. These conditions permitted near quantitative immobilization

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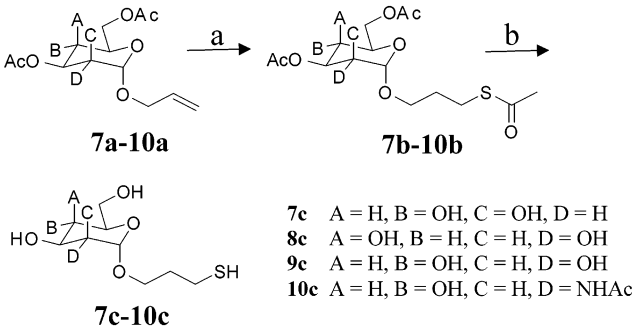

Figure 4. Synthesis of carbohydrate-terminated thiols **7c--10c** from the peracetyl glycosides of mannose (**7a**; A = H, B = OAc, C = OAc, D = H), galactose (**8a**; A = OAc, B = H, C = OAc, D = H), glucose (**9a**; A = H, B = OAc, C = H, D = OAc), and *N*-acetylglucosamine (**10a**; A = H; B = OAc, C = H, D = NHAc). Reagents: (a) AcSH, *hν*, AIBN, THF; (b) NaOMe, MeOH.

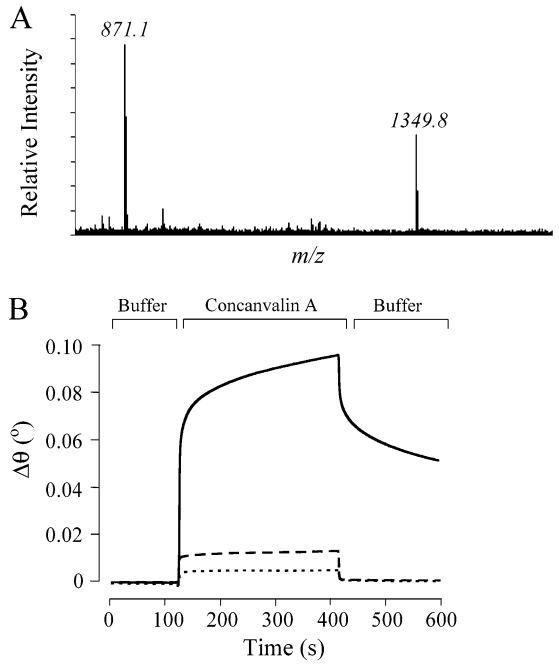

Figure 5. Characterization of monolayers presenting immobilized carbohydrates. (A) Maleimide-terminated SAMs were treated with 3-mercaptopropylmannose **7c** (2 mM in PBS, pH 7.4) for 2 h and characterized by MALDI-ToF mass spectrometry. The spectrum shows a penta(ethylene glycol) disulfide peak (*m*/*z* 871.1) and a mannose-maleimide disulfide peak at *m*/*z* 1349.8. (B) Surface plasmon resonance spectroscopy was used to characterize the interaction between the lectin concanavalin A (5 μM in DPBS, pH 7.4) and monolayers presenting immobilized 3-mercaptopropylmannose **7c** or 3-mercaptopropylgalactose **8c**. Substrates modified with mannose strongly interacted with the lectin concanavalin A (solid curve), whereas substrates presenting no carbohydrate (dotted curve) or galactose (dashed curve) did not interact with the lectin.

using minimal quantities of carbohydrate conjugates. The remaining maleimide groups on the substrate were inactivated by treatment of the monolayer with β-mercaptoethanol (10 mM in PBS, pH 7.4).

To investigate the lectin-binding properties of the chip, identical arrays were treated separately with each of three rhodamine-labeled lectins (2 μM in DPBS, pH 7.4) for 30 min, rinsed with DPBS, and then imaged with a confocal array scanner (Figure 6B). The resulting images show that essentially no fluorescence exists in regions of the substrate that present no carbohydrate (signal-to-noise > 25:1) and that each of the lectins binds to its known ligands. The carbohydrate array treated with concanavalin A, for example, showed significant fluorescence intensity in the regions presenting α-mannose, α-glucose, and α-GlcNAc. Experiments with each of the two other lectins also gave the expected results: *E. cristagalli* bound only to regions presenting α-Gal, whereas *G. nivalis* bound to the region presenting α-mannose.

Characterization of Kinase Activity. We next demonstrate the utility of these substrates for characterizing the phosphorylation of the peptide IYGEFKKKC by the nonreceptor tyrosine kinase c-*src*.[7] To immobilize the peptide, substrates were treated with a solution of the peptide AcIYGEFKKKC−NH_2 (2 mM in PBS, pH 7.4) for 4 h. The substrate was then rinsed with water and dried under a stream of nitrogen. To characterize the enzymatic activity, a solution continaing the kinase and [γ-^{32}P]ATP was placed onto the substrate and incubated for 4 h at 37 °C. The substrates were then rinsed and examined using a scintillation counter. Figure 7A shows that substrates presenting the tyrosine peptide incorporated nearly 500 cpm/cm^2 of radiolabel, whereas those presenting the control peptide AcIFGEFKKKC−NH_2 or no peptide incorporated only background levels of radioactive phosphate.

To demonstrate the presence of phosphotyrosine after the enzymatic modification of the substrate, we examined the binding of an antibody to phosphotyrosine to the monolayers using SPR. Figure 7B shows that the antibody interacts with monolayers presenting the tyrosine peptide only after enzymatic modification (solid line). Control experiments using an immobilized phophotyrosine peptide (dashed line) show that a similar amount of antibody binds to the independently synthesized phophotyrosine group. The observed difference in binding is likely due to experimental variation in preparing the monolayers and performing the SPR experiments.

Quantitative Inhibition Assays with Peptide Chips. We next used these substrates to characterize the activity of c-*src* in the presence of the noncompetitive inhibitor PP1.[39] Mixtures containing the kinase, [γ-^{32}P]-ATP, and each inhibitor (at concentrations ranging from 1 nM to 2 μM) were arrayed onto monolayers under a layer of mineral oil (Figure 8A). After 4 h at 37 °C, the substrates were washed and quantified by phosphorimager analysis. Figure 8B shows concentration-dependent inhibition of enzyme activity by PP1. The inhibition data were fit to a 1:1 association model and analyzed to obtain a K_i value of 31 nM. This value is in good agreement with those obtained in solution phase assays (K_i = 25 nM) and in monolayer-based assays where the same peptide was immobilized using the Diels−Alder reaction (K_i = 39 nM).[7]

Immobilization of Peptide Ligands for Cell Adhesion. To demonstrate the suitability of this immobilization chemistry for preparing substrates for mammalian cell culture, we immobilized the peptide CGGRGDS−NH_2 to monolayers presenting the maleimide group. This peptide is a known ligand for cellular integrin receptors,[40] and previous work has demonstrated that monolayers presenting the Arg-Gly-Asp peptide support cell adhesion and spreading.[25,27,41] To demonstrate the specificity of the interaction between cells and immobilized peptides, we prepared a substrate presenting two monolayers. One-half of the substrate consisted of hydrophobic hexadecanethiolate groups coated with the cell adhesion protein fibronectin,[42] whereas the other half presented penta(ethylene glycol) groups and maleimide-RGD conjugates (Figure 9A). 3T3 fibroblasts attached efficiently to both

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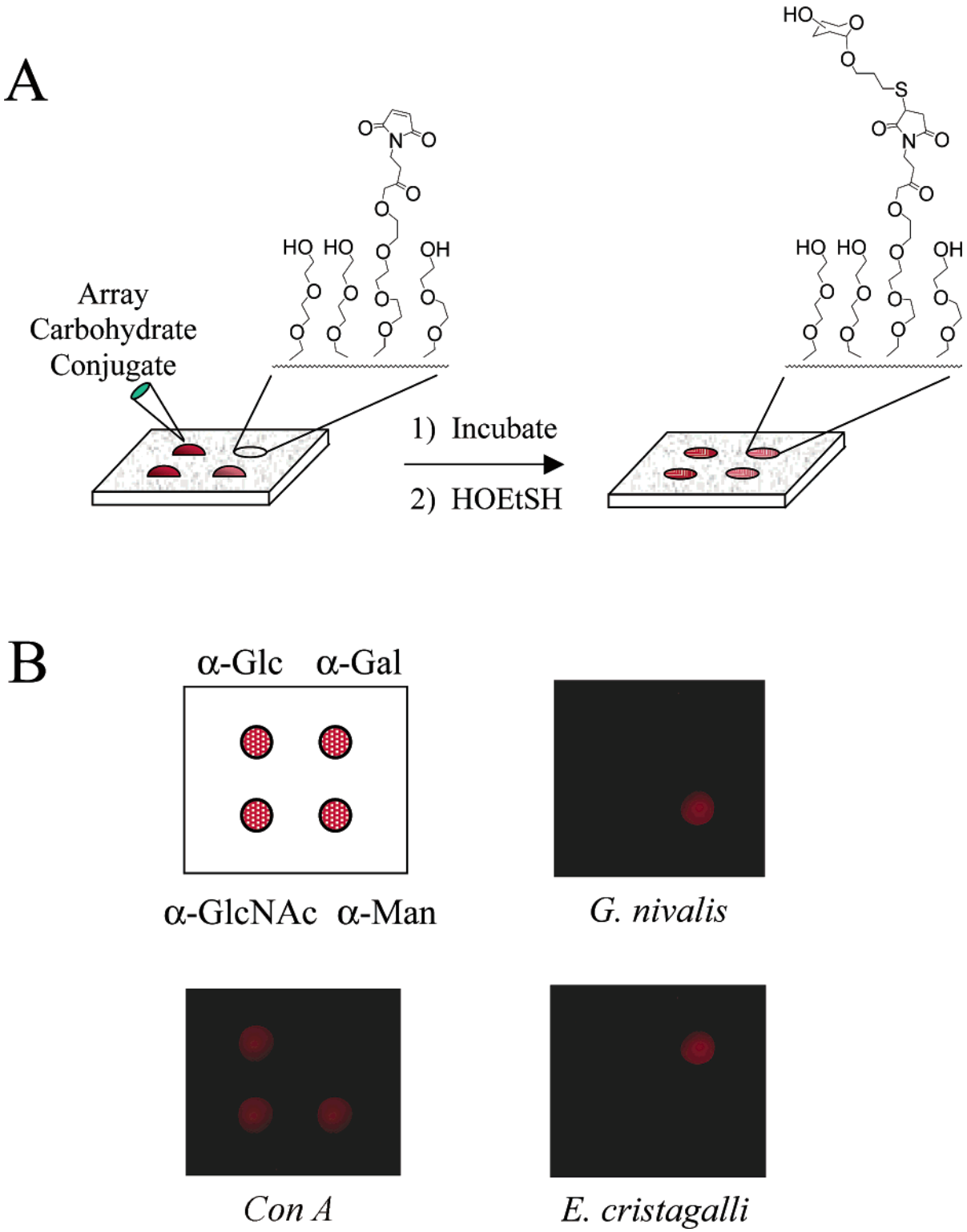

Figure 6. Preparation and characterization of a carbohydrate array. (A) Substrates were prepared by applying solutions of carbohydrate thiols **7c**−**10c** onto discrete regions of a monolayer presenting maleimide groups. After 4 h, the slide was rinsed with water and unreacted maleimide groups were inactivated by incubation with β-mercaptoethanol (10 mM in PBS, pH 7.4) for 30 min. (B) Identical carbohydrate chips were separately incubated with each of five rhodamine-labeled lectins (2 μM in DPBS) for 30 min, gently rinsed, and evaluated by confocal fluorescence microscopy. Fluorescent images of the resulting arrays are shown for each lectin. These images reveal that each of the proteins associates specifically with its known carbohydrate ligands on the array.

regions of the substrate (Figure 9B). Addition of soluble GRGDS−NH_2 peptide (2 mM in PBS, pH 7.4) to the culture medium resulted in detachment of cells only from the region of the substrate presenting the immobilized peptides, demonstrating the specificity of the cell−peptide interaction (Figure 9C).

Discussion

This paper uses the reaction between thiol and maleimide groups to immobilize ligands to self-assembled monolayers of alkanethiolates on gold. This reaction is simple, selective, and has been used for the preparation

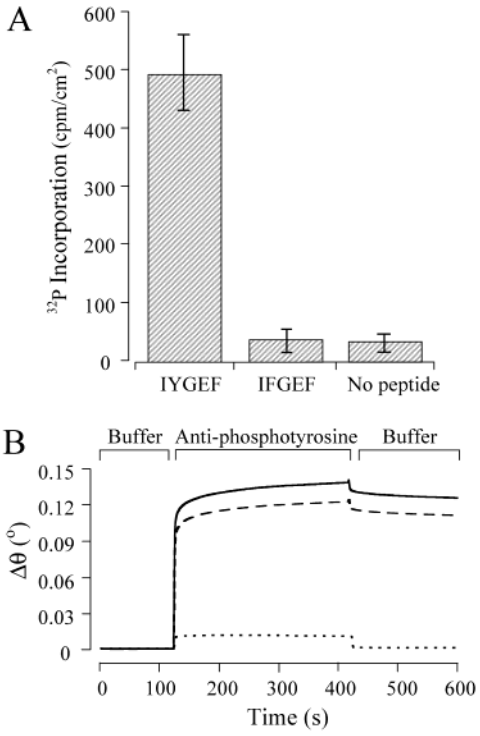

Figure 7. Characterization of the enzymatic phosphorylation of immobilized peptides. Monolayers presenting maleimide groups at a density of 1% were treated with AcI**X**GEFKKKC−NH_2 (2 mM in PBS, pH 7.4; **X** = Y or F) for 30 min at room temperature. (A) The resulting substrates were treated with c-*src* and [γ-^{32}P]ATP for 4 h at 37 °C, washed, and analyzed in a scintillation counter. Substrates presenting the tyrosine peptide incorporated 500 cpm/cm^2 of radioisotope, whereas substrates presenting no peptide or the phenylalanine peptide incorporated background levels of radioactivity. (B) Binding of an antibody to phosphotyrosine to enzymatically modified substrates was analyzed using SPR. The antibody interacted strongly with monolayers presenting AcIYGEFKKKC−NH_2 after enzymatic modification (solid curve). The magnitude of binding was similar to monolayers presenting the phosphotyrosine substrate AcIY(PO_3)GEFKKKC−NH_2 (dashed curve). Little protein binding was observed on substrates not treated with the kinase (dotted curve).

of a wide range of protein, peptide, carbohydrate, and lipid conjugates.[20−24] Here we use this coupling chemistry to immobilize several biologically active ligands to inert surfaces and characterize the substrates using fluorescence spectroscopy, SPR, MALDI-ToF mass spectrometry, electrochemistry, and cell culture. This approach has several features that make it useful for the preparation of a wide variety of biochips. First, the maleimide reaction gives high yields and excellent selectivity for immobilization (Figure 3). This feature minimizes the need for postsynthetic modification of ligands and permits the immobilization of thiol-containing ligands in the presence of other reactive functional groups. Second, because the density of immobilized ligands is determined by the density of maleimide groups in the film, and not on the particular structure of ligand, the number of ligands in each particular region of the substrate can be controlled and kept constant. Third, ligands immobilized using this method can participate in binding interactions with protein partners and can serve as substrates for enzymes (Figures 5−9). Finally, monolayers that present penta(ethylene glycol) groups prevent nonspecific adsorption of proteins and other components from solution, eliminating the need for blocking procedures and the use of detergents. The last two characteristics permit quantitative analysis of protein binding and enzymatic modification of the chips with minimal quantities of reagents.

The monolayers used in this work were prepared using alkyl disulfides rather than alkanethiols because the maleimide group would react with the terminal sulfhydryl group of an alkanethiol. Previous work has suggested that monolayers prepared from alkyl disulfides are structurally distinct from those prepared from alkanethiols.[43,44] This paper and two other studies[6,45] have demonstrated that oligo(ethylene glycol)-terminated monolayers prepared from asymmetric alkyl disulfides are inert to the nonspecific adsorption of protein. Furthermore, specific interactions between proteins in solution and ligands presented at the monolayer−solution interface remain intact. These results suggest that any differences between monolayers prepared from alkyl disulfides and alkanethiols do not influence the inert properties of oligo(ethylene glycol)-terminated SAMs or protein binding to immobilized ligands.

The methodology described here is distinguished from other immobilization chemistries in that it relies on a sophisticated application of surface engineering. Several groups have developed selective and efficient chemistries for the immobilization of ligands,[15−24] but the majority of these studies used routine glass or plastic substrates as the solid support. Although these chips have proven very important in biology and drug discovery, they still do not compete with quantitative performance and reproducibility of conventional solution-based assays. This limitation stems largely from two factors. First, most glass or plastic supports present immobilized ligands in a heterogeneous environment and therefore display different activities toward proteins in solution.[46] Second, these substrates are often not very effective at blocking unwanted interactions, which can lead to high background levels and false-positive interactions. The use of blocking proteins or detergents can optimize the performance of an assay, but introduces further heterogeneities that compromise the quantitative character of the chip.[47] The work described here avoids these limitations by immobilizing ligands in a homogeneous manner onto inert substrates.

The utility of this approach, particularly for substrates that present many distinct ligands, depends ultimately on the detection methods that can be adapted for reading the results of a chip-based assay. The substrates described here have the advantage that they are compatible with all of the principal techniques used for analyzing chips. In this paper, we used fluorescence microscopy to quantitate the binding of lectins to carbohydrate arrays (Figure 6B). We also used SPR spectroscopy to measure the real-time interaction between immobilized peptides and carbohydrates with proteins in solution (Figures 5B and 7B). SPR is probably the best-suited method for measuring low-affinity ligand−receptor interactions because it does not require specially labeled reagents and does not require that the slide be rinsed before imaging. SPR has not yet

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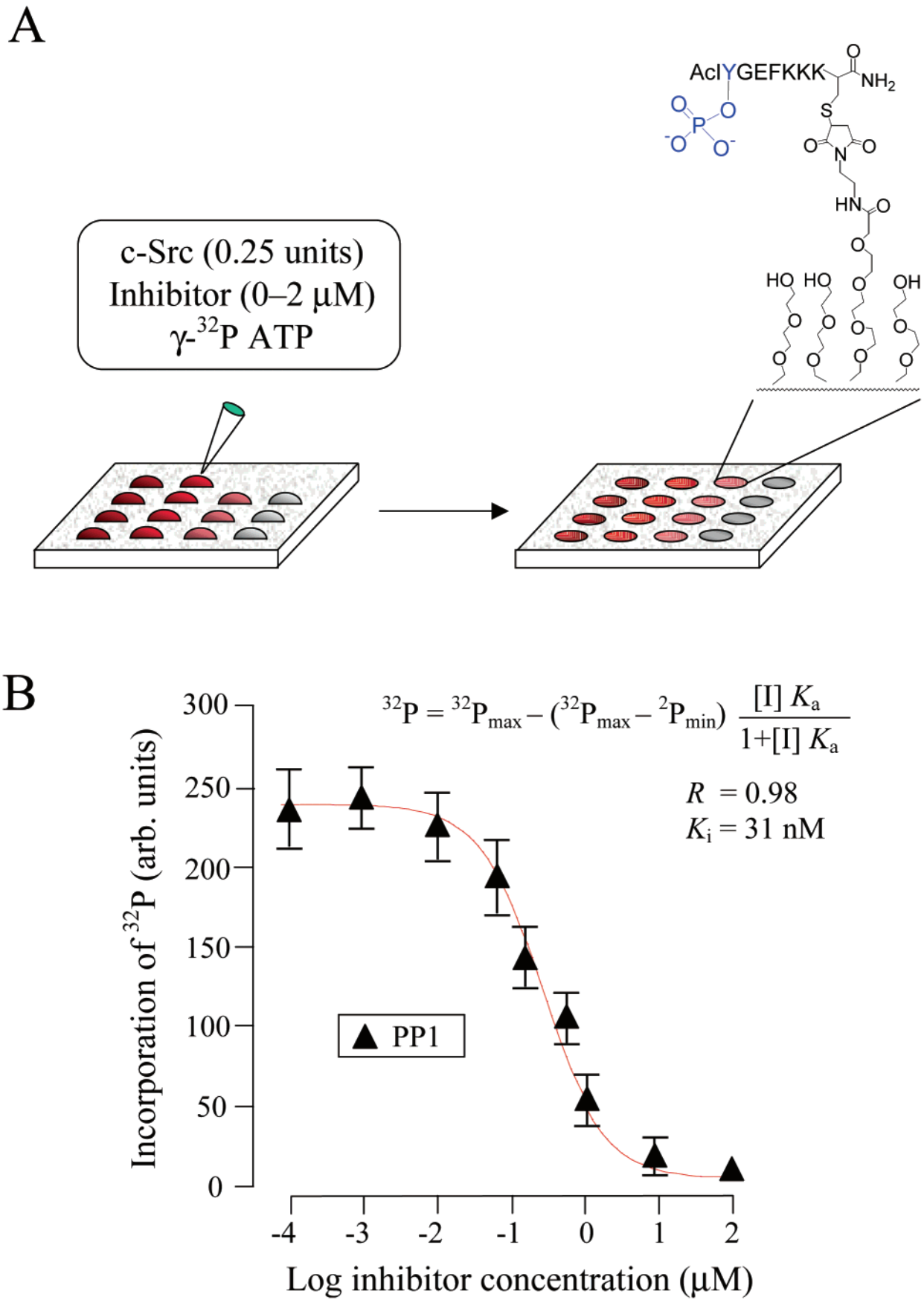

Figure 8. Use of the maleimide-terminated monolayer for quantitative assays of tyrosine kinase activity. (A) Substrates presenting the peptide AcIYGEFKKKC−NH_2 were treated with solutions containing c-*src*, [γ-^{32}P]ATP, and a series of concentrations of the kinase inhibitor PP1. (B) The substrates were analyzed by phosphorimaging, and the data were fitted to a 1:1 model to afford an inhibition constant (K_i) of 31 nM.

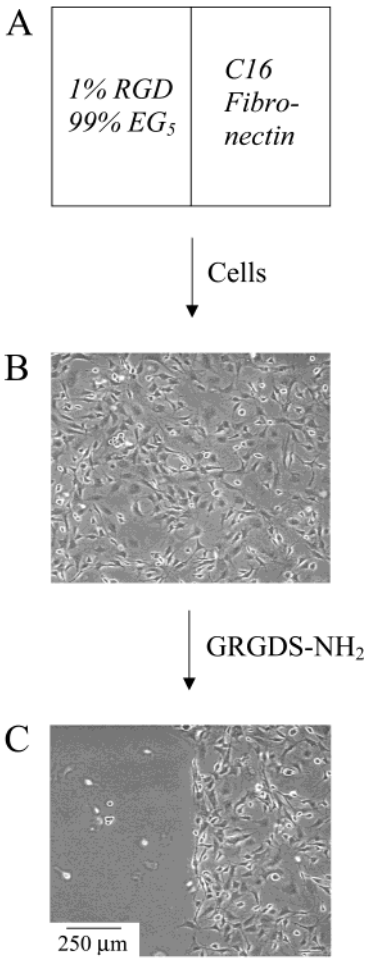

Figure 9. Use of maleimide-terminated monolayers to pattern the attachment of mammalian cells. (A) Substrates presenting hexadecanethiolate and maleimide groups were prepared by patterning one-half of a gold substrate with hexadecanethiol (2 mM in EtOH) and derivatizing the remaining bare regions of gold with a monolayer presenting maleimide and penta(ethylene glycol) groups. The peptide GRGDS was immobilized to the maleimide groups by treating the substrate with CGGRGDS−NH_2 (2 mM in PBS) for 4 h. The substrate was then treated with fibronectin (100 μg/mL in PBS, pH 7.4) for 4 h to immobilize the protein to the hexadecanethiolate monolayer. (B) Swiss 3T3 fibroblasts were seeded onto the monolayers and allowed to incubate at 37 °C for 4 h. Fibroblasts attached to regions of the substrate presenting the RGD peptide and fibronectin. (C) When the culture media was exchanged with media containing soluble AcGRGDS−NH_2 (2 mM in PBS), cells on regions of the substrate presenting the RGD peptide detached from the substrate, whereas cells attached to regions presenting fibronectin did not. This result demonstrates the specificity of the cell−peptide interaction.

been widely used for imaging arrays, but the recent introduction of two-dimensional SPR technology is likely to make this an important technique in the next several years.[20,48] We also demonstrate here that radiolabel assays and MALDI-ToF mass spectrometry can be used to characterize the immobilization reaction (Figure 5A). The latter technique can identify unanticipated enzymatic activities and has the additional advantage that it does not require antibodies or radioisotopes for analysis.[49,50]

We believe that the immobilization chemistry described here has a special relevance to applications in glycobiology because this class of biopolymers does not contain native thiol functionality. This feature, combined with advances in solid-phase carbohydrate synthesis,[51,52] will permit the preparation of complex and diverse carbohydrate chips with control over the density and environment of multiple ligands on a single array. These biochips will permit the analysis of carbohydrate−protein interactions, such as those found in bacterial and viral adhesion,[53,54] and enzymatic activities, such as the substrate specificity of glycosyltransferases and sulfotransferases.[55,56] Experiments to evaluate these opportunities are currently underway.

Conclusions

The approach we describe here—which relies on the immobilization of thiols to self-assembled monolayers presenting maleimide groups—has several characteristics that make it well suited for the preparation of a wide range of biochips. First, the immobilized ligands participate in biospecific interactions with proteins and enzymes, whereas the penta(ethylene glycol) groups on the monolayers provide essentially complete resistance to unwanted protein adsorption and other nonspecific interactions at the surface. Second, the use of the maleimide−thiol reaction for immobilization ensures that the density of ligand is well controlled and constant across the entire array. Third, the structural order of the monolayer substrates ensures that the ligands are presented in a uniform microenvironment and therefore have equal activity toward soluble proteins, making possible quantitative assays. Fourth, arrays can be prepared using large numbers of thiols generated in solid-phase schemes, thereby extending significantly the complexity and diversity of arrays. Finally, the monolayers are compatible with robotic tools for preparing arrays and with a range of detection technologies for interrogating chips. We believe that this approach for preparing biochips will have broad utility for applications in drug discovery and diagnostics.

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