Patterning Ligands on Reactive SAMs by Microcontact Printing

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This report describes a method for patterning ligands onto mixed SAMs of alkanethiolates on gold by microcontact printing (μCP). The mixed SAMs were made from thiols presenting terminal tri(ethylene glycol) groups ($HS(CH_2)_{11}(OCH_2CH_2)_3OH$, **1**) and terminal hexa(ethylene glycol)−CH_2CO_2H groups ($HS(CH_2)_{11}(OCH_2CH_2)_6OCH_2CO_2H$, **2**). Ligands were printed using a two-step procedure. The carboxylic acid groups of **2** were first converted to reactive pentafluorophenyl esters. A freshly oxidized PDMS stamp, inked with a ligand derivatized with a primary amine, was then brought into contact with the activated SAM; in the areas of contact, the amine reacted with the activated ester and formed an amide. Two ligands, biotin and benzenesulfonamide, were printed onto these SAMs. The formation of patterned SAMs presenting biotin ligands was detected by fluorescence microscopy of substrates that were incubated with a solution of fluorescently labeled antibiotin antibody. The formation of patterned biotin was also detected using a sandwich experiment; in this experiment, the SAM was incubated sequentially in solutions of streptavidin, protein G-biotin conjugate, and fluorescently labeled goat antirabbit IgG. The smallest features resolved in images obtained by these methods were squares with a 5 μm side. Using surface plasmon resonance (SPR) to detect binding of antibiotin antibody to SAMs presenting biotin groups, the yield of coupling by μCP was estimated to be ∼90% of that obtained by immersion. Printing of the benzenesulfonamide ligand was detected by binding of carbonic anhydrase (CA) to the sulfonamide-derivatized SAMs; the yield of coupling, as estimated by SPR, was ∼ 75% of that obtained by immersion. For both ligands, oxidation of the PDMS stamp before inking was found to be critical for good coupling yields.

Introduction

This report describes the use of microcontact printing (μCP) on reactive, mixed self-assembled monolayers (SAMs) of alkanethiolates on gold to pattern ligands. The resulting SAMs present both the patterned ligand and oligo(ethylene glycol) groups (Scheme 1). We chose mixed SAMs consisting of **1** and **2**, because SAMs presenting tri(ethylene glycol) ($(EG)_3$) groups resist the adsorption of proteins and cells,[1−4] and because ligands can be conjugated in high yield to the carboxylic acid groups of **2** via an intermediate active ester. The carboxylic acid groups were activated to pentafluorophenyl ester groups; these esters have a reactivity that is ∼10 times greater than *N*-hydroxysuccinimidyl esters.[5]

There are several applications that require the ability to pattern biological ligands onto surfaces. The use of living cells in biosensors can be assisted by patterning the surface of the sensor with ligands that cause adhesion of cells or of protein adhesion factors recognized by cells.[6,7] Techniques for the miniaturization of DNA analyses also require the ability to pattern surfaces with ligands.[8−10] Immunoassays often involve antibodies or antigens patterned on surfaces.[11−13] Although many techniques exist for patterning ligands onto surfaces, they often involve photolithographic procedures that require expensive equipment or processing conditions that damage many types of ligands. Microcontact printing is a convenient and inexpensive method to pattern biologically relevant ligands on SAMs.[14−16]

We have recently described the use of μCP to pattern amines on SAMs terminating in interchain anhydride groups.[15] This technique and the one described here offer several advantages over conventional μCP for fabrication of patterned interfaces for studies in biochemistry. (i) They both minimize organic synthesis: the only structural requirement for coupling is, in principle, that the ligand to be coupled contain at least one nucleophilic amino group. (ii) They allow the patterning of several ligands (at least two are straightforward) on the same surface. (iii) The

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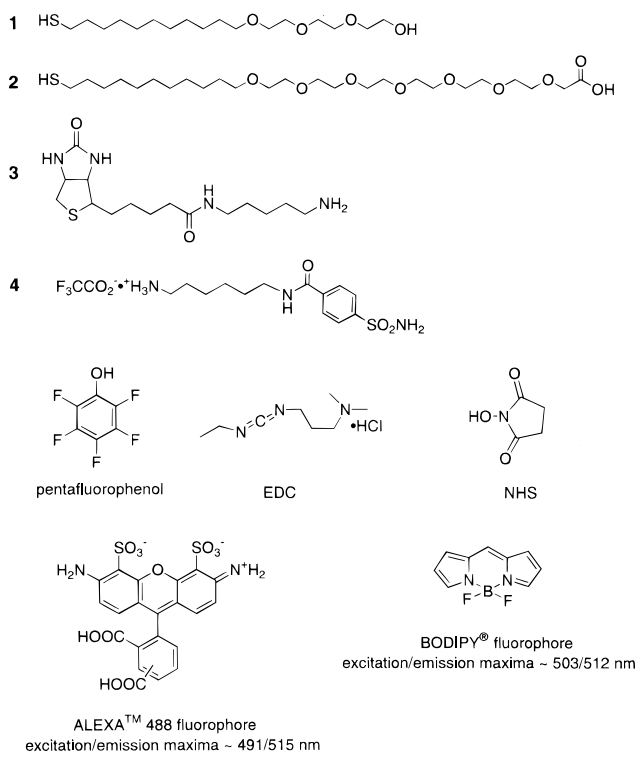
$(EG)_3OH$ groups make the unpatterned surface highly resistant to nonspecific adsorption of protein.

The interchain anhydride method has the advantage that it uses a thiol (16-mercaptohexadecanoic acid) that can be easily synthesized in two steps from commercially available compounds (the analogous 11-mercaptoundecanoic acid is commercially available)[17] and a straightforward procedure for activation and μCP. The interchain anhydride method results in a mixed SAM in which at least 50% of the terminal groups are carboxylic acids. The method offers a convenient route for the generation of patterned surfaces for applications in which precise control of the surface ligand density is not critical and in which a high concentration of CO_2H/CO_2^- groups on the surface does not cause undesirable nonspecific adsorption.

Mixed SAMs consisting of **1** and activated **2** have two advantages over SAMs presenting anhydride as substrates on which to print ligands. (i) The density of ligands in the SAM can be easily manipulated by varying the ratio of **1** to **2**. While it is possible to control the surface density of



ligands using the interchain anhydride method by printing a mixture of the ligand and a diluting amine, it is difficult to predict and control the incorporation of these two amines into the SAM because of potential differences in reactivity and solubility in the PDMS stamp. (ii) It is straightforward to prepare mixed SAMs incorporating low mole fractions of **2** (typically, $\chi(\mathbf{2}) < 0.10$), and it is, thus, easy to prepare SAMs presenting low densities of carboxylic acid groups. These carboxylic acid groups can be activated and caused to react with the amino groups on ligands in high yield (>75%; also see below);[18,19] these reactions generate a surface with few residual carboxylic acid groups. The method described here and the interchain anhydride approach provide two complementary methods for patterning ligands of biological interest onto well-defined surfaces provided by SAMs.

Results and Discussion

We printed two ligands—amino derivatives of biotin (**3**) and benzenesulfonamide (**4**)—to illustrate the usefulness of our method. We used biotin because (i) a monoclonal antibody against biotin conjugated to a fluorophore (mouse IgG_1, Alexa 488 conjugate; Molecular Probes) is commercially available that makes it possible to use fluorescence microscopy to detect binding and (ii) the interaction between biotin and streptavidin is widely used in analytical biochemistry.[20] Streptavidin (Sv) is a tetrameric protein; binding of Sv to immobilized biotin ligands leaves unoccupied sites on Sv that can be used to "capture" biotinylated ligands or biotin-labeled proteins in so-called sandwich experiments. The very strong interaction between Sv and biotin ($K_d \sim 10^{-14}$ M) ensures that the ligand or protein is effectively irreversibly coupled to the surface.[20] To test the generality of the method, we also printed the benzenesulfonamide ligand **4** onto these activated, mixed SAMs. Benzenesulfonamides bind to carbonic anhydrase (CA) with $K_d \cong 10^{-6}-10^{-9}$ M;[21-23] this interaction has been widely studied in solution and at the surfaces of mixed SAMs.[24] It is more representative of the ligand–receptor interactions encountered in biochemistry than is the biotin–avidin interaction.

The procedure for printing biotin-containing ligands (Scheme 1) is described in the Experimental Section. Briefly, substrates presenting mixed SAMs of **1** and **2** were activated by immersion in a solution of DMF containing 1-ethyl-3-(dimethylamino)propylcarbidiimide (EDC) (0.1 M) and pentafluorophenol (0.2 M) for 10 min. The ligand was printed by bringing a freshly oxidized PDMS stamp, inked with the ligand, in contact with the activated SAM for 5 min. Pentaflurophenyl esters in regions of the SAM not in contact with the stamp and other residual unreacted esters were hydrolyzed to carboxylic acid groups by immersion of the patterned substrates in phosphate buffer (pH 8.6, 25 mM, 20 min). The patterned substrates were rinsed with deionized water and ethanol and were dried under a stream of nitrogen.

To detect the formation of patterns containing biotin ligands, we incubated the surfaces in a solution of the antibiotin mouse IgG_1-Alexa 488 conjugate (0.13 μM) for 1 h. Figure 1A shows images of these surfaces obtained using the fluorescence detection mode on the microscope—the patterned fluorescence indicates that μCP was successful. We also detected the formation of patterned biotin in a format analogous to a sandwich experiment—by sequentially incubating the substrates (presenting biotin groups) in a solution of Sv (1.0 μM; 20 min), a solution of protein G-conjugated biotin (1.0 μM; 20 min), and a solution of fluorescently labeled goat antirabbit IgG (1.0 μM; 1 h). The patterned fluorescence (Figure 1B) confirms that μCP accomplished the pattern of biotin ligands. Protein G binds to the Fc region of antibodies[25] and is

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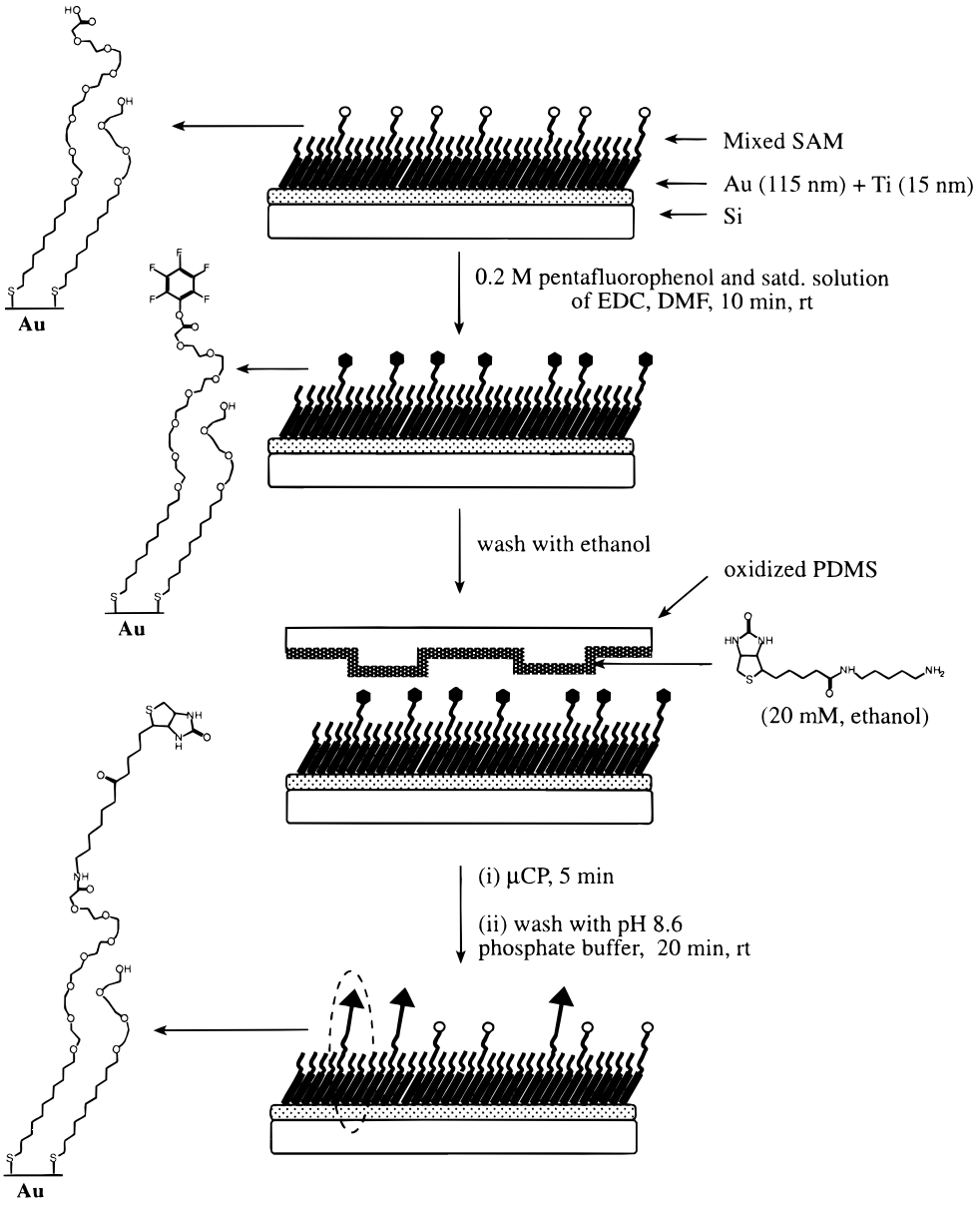
Scheme 1. Schematic Representation of the Procedure Used for Patterning Biotin Ligands onto SAMs Consisting of 1 and 2



used to immobilize antibodies on surfaces. These immobilized antibodies can be used for binding antigens to surfaces through their antigen binding (Fab) domains.

The smallest features that we have resolved are squares with a 5 μm side (the smallest features in the inset in parts A and B of Figure 1); the edge resolution of features obtained from the sandwich experiment was higher than that obtained using antibiotin antibody. The ability to resolve features with this length scale is sufficient for most studies in cell biology and for biosensor applications.

We used surface plasmon resonance (SPR) spectroscopy to compare the yield of coupling of the biotin ligand to the mixed SAMs obtained by μCP relative to that obtained by immersion. Figure 2 shows SPR sensorgrams of the binding of the antibiotin mouse IgG_1-Alexa 488 to mixed SAMs presenting biotin groups; these biotin ligands were coupled to the SAM either by immersion of the activated SAM in a solution containing **3** (20 mM) or by μCP of **3** using a flat, unpatterned, oxidized PDMS stamp. For surfaces prepared both by immersion and by μCP, increasing the mole fraction of **2** in the SAM from 0.005 to 0.020 resulted in an increase in the SPR signal from a response of ~2200 RU (at time = 300 s) to ~4100 RU (at time = 300 s); increasing χ(**2**) further (to χ(**2**) = 0.10) did not result in any further increase in the SPR signal. We infer that at χ(**2**) ~ 0.02, these surfaces are completely covered by bound antibody. At each value of χ(**2**), the amount of binding of antibiotin to surfaces obtained by μCP is greater than 90% of that obtained on surfaces by immersion; we infer that for printing **3**, the coupling yield is greater than 90% of that accomplished by immersion.

Benzenesulfonamide groups were coupled to the SAM by μCP or by immersion, using procedures similar to those described for the biotin ligand. Figure 3 shows SPR sensorgrams for binding of CA to SAMs presenting benzenesulfonamide ligands. For mixed SAMs with χ(**2**) ~ 0.01, the binding was more than 90% reversible on surfaces obtained both by immersion and by μCP; we estimate $k_{on} \sim 0.005\ s^{-1}$, $k_{off} \sim 10^4\ M^{-1}\ s^{-1}$, and $K_d \sim 0.2$ μM. The amount of binding of CA on surfaces obtained by μCP was ~75% of the signal on surfaces obtained by immersion. While these data demonstrate the generality of the described μCP procedure, they also indicate that there can be differences in the efficiency of coupling using

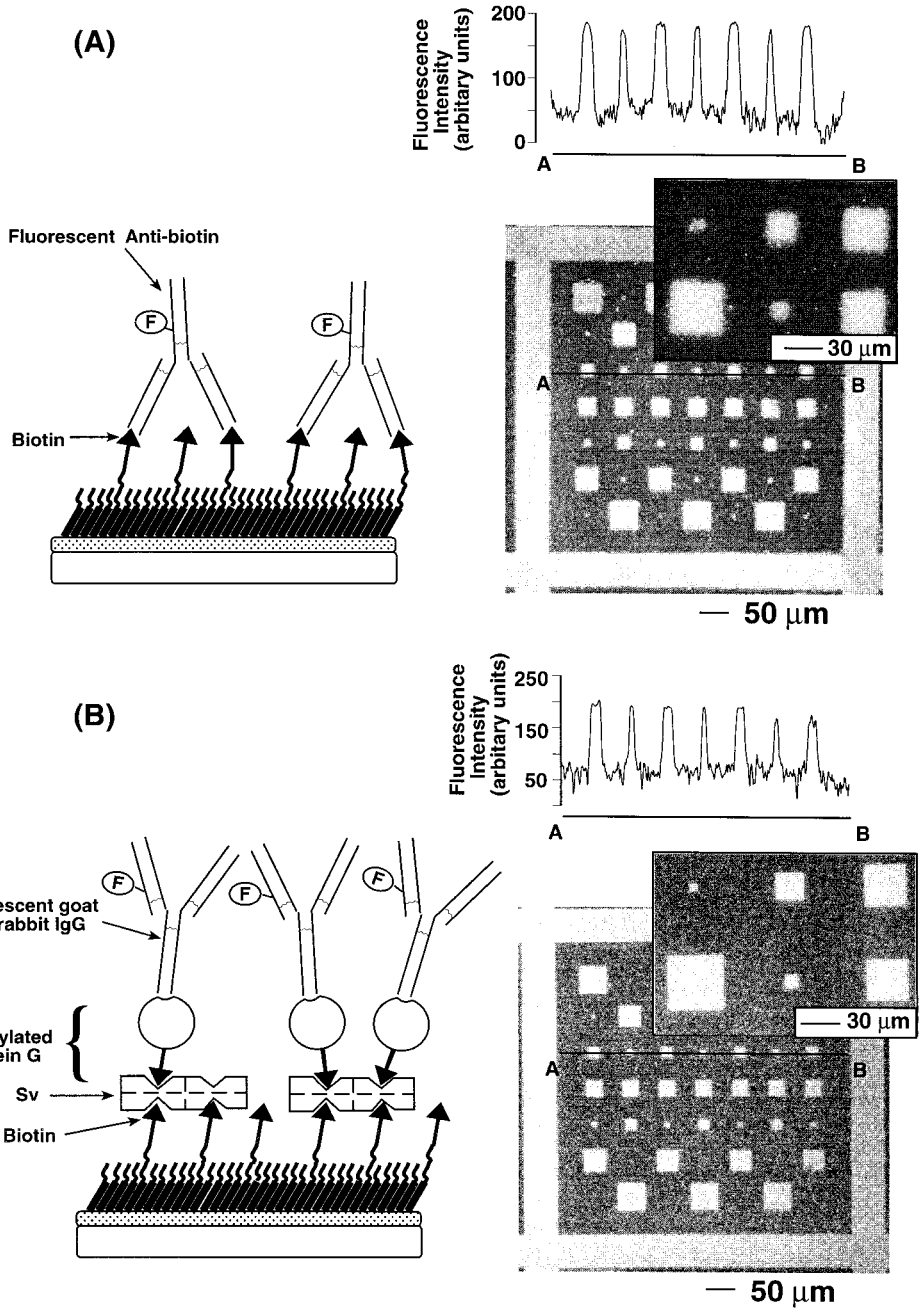


Figure 1. Fluorescence microscopy images of surfaces with patterned biotin groups (χ(**2**) ~ 0.02) and schematic representations of the surfaces during fluorescence detection (F = fluorophore). The substrates were prepared according to the procedures described in the Experimental Section. We estimated the intensity of fluorescence in regions with and without biotin groups across the line (AB) on these images using the NIH image software. (A) Images of fluorescently labeled antibiotin bound to SAMs presenting patterned biotin groups. (B) Images of fluorescently labeled goat antirabbit IgG bound to SAMs presenting biotin groups that were sequentially incubated in a solution of Sv and a solution of biotin−protein G conjugates.

μCP for different ligands (e.g., 75% for **3** and 90% for **4**, relative to the corresponding yields by immersion). We also used polarized infrared internal reflectance spectroscopy (PIERS) to compare the yields of coupling of the benzenesulfonamide ligand (χ(**2**) ~ 0.20) by immersion and μCP, and by activation by NHS/EDC followed by immersion in a solution of the ligand in aqueous buffer. Figure 4 shows the IR spectra in the carbonyl stretching region of the mixed SAM (1500−1850 cm^{-1}) to which the ligand **4** has been coupled by three methods: activation by pentafluorophenol/EDC followed by immersion in a solution of the ligand in ethanol (b); activation by pentafluorophenol/EDC, followed by μCP (c); and activation by NHS/EDC followed by immersion in a solution of the ligand in aqueous buffer (d).[18,19] We see no bands corresponding to residual carboxylic acid groups (~ 1735 cm^{-1}) in (b)—we infer that coupling is quantitative. Therefore, the yield of coupling by μCP *relative* to that obtained by immersion (~75% as obtained by SPR) is a good estimate of the *absolute* yield. Morevover, based on the relative intensities of the bands at 1550 cm^{-1} (NH bending modes) or 1663 cm^{-1} (C=O amide stretches) to the C=O stretch of residual carboxylic groups, we infer that the yields of coupling by μCP and (c) are ~75 and 85% respectively, in accordance with the SPR data and previous observations.[18,19]

Figures 2B and 3B show the importance of using oxidized PDMS during μCP of ligands such as **3** (or **4**); the

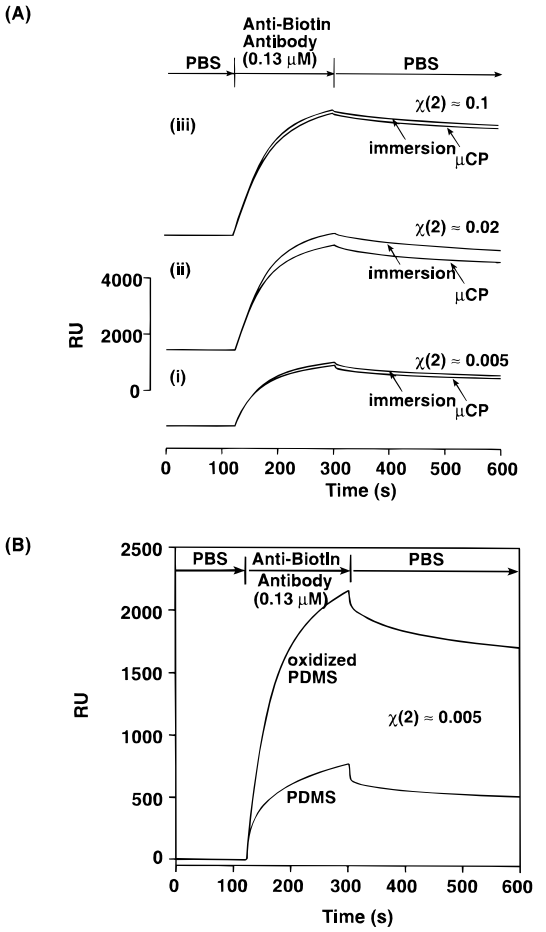


Figure 2. (A) SPR response curves for the binding of antibiotin IgG on SAMs prepared by immobilizing biotin (according to Scheme 1) by μCP or by immersion on SAMs with $\chi(\mathbf{2}) \approx 0.005$ (i), 0.020 (ii), and 0.10 (iii). For coupling by immersion, the activated SAMs were immersed in an ethanolic solution of the biotin ligand (20 mM) for 5 min; all other steps including activation and hydrolysis of unreacted pentafluorophenyl esters were carried out as in Scheme 1. We have carried out control experiments in which antiFITC IgG_1 was injected over surfaces presenting the biotin groups; no detectable binding was observed. (B) SPR sensorgrams showing the relative amounts of binding of antibiotin to surfaces presenting biotin groups that were prepared by μCP on SAMs ($\chi(\mathbf{2}) \approx 0.005$) using PDMS and oxidized PDMS.

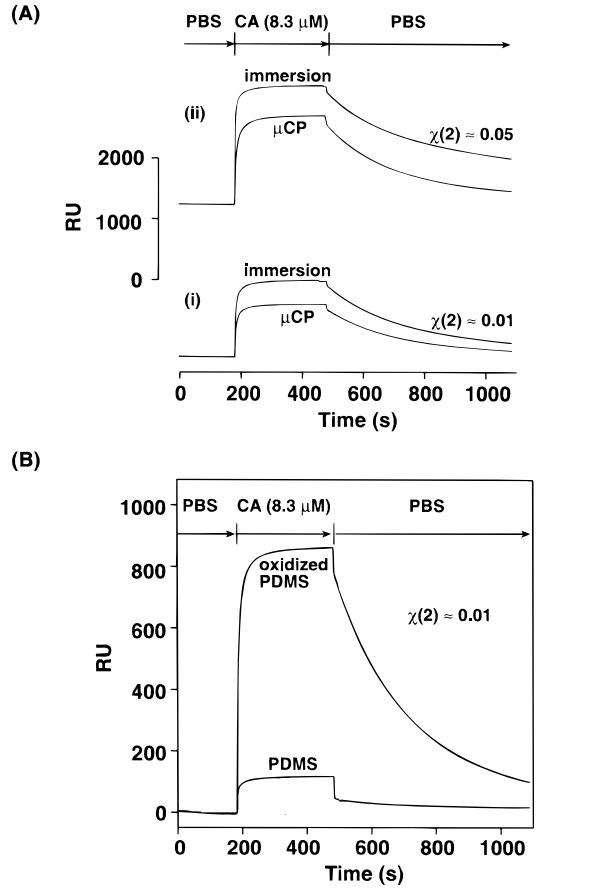


Figure 3. (A) SPR response curves for the binding of CA to SAMs presenting benzenesulfonamide groups; these SAMs were prepared by coupling of **5** to mixed SAMs ($\chi(\mathbf{2}) \approx 0.01$ (i) and 0.05 (ii)) either by μCP or by immersion. The procedures for coupling were similar to those used for biotin. (B) SPR sensorgrams showing relative amounts of binding of CA to surfaces presenting benzenesulfonamide groups that were prepared by μCP on SAMs ($\chi(\mathbf{2}) \approx 0.01$) using PDMS and oxidized PDMS.

amount of binding of antibiotin to surfaces obtained by using untreated PDMS is just ~30% of that obtained using oxidized PDMS. Similarly, the amount of binding of CA to SAMs generated using PDMS is less than 10% of that obtained using oxidized PDMS. We believe that the more hydrophilic, oxidized PDMS is wetted better by **3** and **4** than untreated, hydrophobic PDMS; better wetting leads to more efficient transfer of molecules of ligand to the stamp during the inking process.

Conclusions

We have described a method for μCP of amine-terminated ligands on mixed SAMs containing low mole fractions ($\chi(\mathbf{2}) \leq 0.10$) of activated carboxylic acid groups. The method is straightforward experimentally and the coupling yields are high (75% and 90% of the maximum obtained for that protein, based on the two ligand-protein pairs examined). The method requires the synthesis of **1** and **2**,[2,18,26] but the mixed SAM that is obtained using them can be converted to a common reactive intermediate for coupling to a variety of ligands.[18] The SAMs contain patterned regions that present ligands at defined densities in environments that resist the nonspecific adsorption of proteins; these surfaces are potentially useful for biosensors. We believe that these systems will also be useful in studying the influence of small molecules, peptides (e.g. RGD variants) and proteins on the adhesion of cells to surfaces[26] and in studying the influence of adhesion on the metabolism of cells.[27]

Experimental Section

Materials. The synthesis of compounds **1**, **2**, and **4** have been published.[2,18,26] Compound **3** and streptavidin were purchased from Pierce. Antibiotin mouse IgG_1-Alexa 488 and BODIPY FL goat antirabbit IgG (H+L) conjugate were purchased from Molecular Probes. Bovine carbonic anhydrase II (E.C. 4.2.1.1) was purchased from Worthington. Sodium dodecyl sulfate (SDS) was purchased from BioRad. Pentafluorophenol and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were purchased from Aldrich. PDMS (Sylgard 184) was purchased from Dow Chemical. Stamps were made as described previously.[14]

Preparation of Gold Substrates Presenting Mixed SAMs for Microscopy. Gold substrates were prepared by evaporating thin films of titanium (1.5 nm, to promote adhesion of gold to

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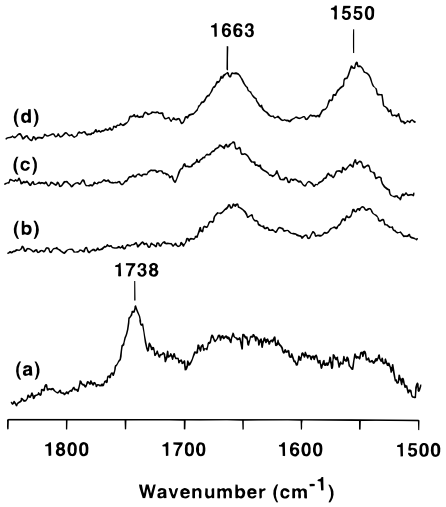

Figure 4. PIERS spectra obtained for (a) a mixed SAM comprising **1** and **2** (χ(**2**) ~ 0.20), (b) the mixed SAM after activation with pentafluorophenol/EDC followed by immersion in an ethanolic solution of **4**, (c) the mixed SAM after activation with pentafluorophenol/EDC followed by μCP of **4** using a flat oxidized PDMS stamp as described, and (d) the mixed SAM after activation with NHS/EDC followed by immersion in an ethanolic solution of **4**.[18,19]

glass) and gold (12 nm) onto glass cover slips (Corning No. 2, used as obtained). The metallized substrates were cut into rectangles ~1 cm by 1.5 cm. Stock solutions of thiols **1** and **2** (2 mM in ethanol) were combined in different ratios in glass scintillation vials. The gold substrates were immersed in the solutions of thiols for 2 h, rinsed with ethanol, dried under a stream of nitrogen and used immediately.

Preparation of Gold Substrates Presenting Mixed SAMs for SPR. Gold substrates were prepared by evaporating films of titanium (1.5 nm) and gold (40 nm) onto glass cover slips (0.2 mm thick). SAMs were made as described before.

Microcontact Printing. The substrates presenting mixed SAMs were immersed in a solution of DMF containing 1-ethyl-3-(dimethylamino)propylcarbidiimide (EDC) (0.1 M) and pentafluorophenol (0.2 M) for 10 min. They were then rinsed with ethanol and dried under a stream of nitrogen. Printing of the ligand was accomplished by bringing a freshly oxidized (by a 1 min exposure to a plasma formed in air) PDMS stamp, inked with an ethanolic solution of the ligand, in conformal contact with the activated SAM for 5 min. The substrates were immersed in phosphate buffer (pH 8.6, 25 mM) for 20 min, rinsed with deionized water, ethanol, and dried under a stream of nitrogen.

For SPR experiments, a flat PDMS stamp was used.

Immobilization of Ligands by Immersion. Mixed SAMs, activated as described above, were immersed in ethanolic solutions of **3** (20 mM) or **4** (3.5 mM, pH adjusted to 8.5 by adding Et_3N) for 5 min. These SAMs were then washed with pH 8.6 buffer, water, and ethanol, and dried under a stream of nitrogen.

Detection of Printed Biotin Ligands by Fluorescence Microscopy. Substrates presenting biotin groups were incubated at room temperature for 1 h in a solution of the antibody (0.13 μM) in PBS buffer (with 1%, w/v, BSA and 0.01%, v/v, Tween 20), washed extensively with buffer, mounted onto glass slides with Fluoromount G (Southern Biotechnology), and then imaged. The detection (based on a sandwich experiment) of patterned biotin ligands was also accomplished by sequentially incubating the substrates in solutions of Sv (1 μM; 20 min), solutions of biotin−protein G conjugates (ImmunoPure biotinylated protein G, Pierce, 1 μM, 20 min), and finally a solution of fluorescently labeled goat antirabbit IgG (BODIPY FL goat antirabbit IgG (H+L) conjugate, 1 μM, 1 h). The surfaces were washed extensively with buffer after each immersion, mounted onto glass slides with Fluoromount G, and then imaged.

Microscopy. Images were obtained on a Zeiss Axiophot microscope, equipped with fluorescence detection, and connected to a 35 mm camera.

SPR. The BIAcore 1000 instrument was used for SPR studies. Substrates with SAMs presenting ligands were glued onto cartridges (we removed the gold substrates from commercial BIAcore cartridges) for use in the BIACore instrument.

The adsorption of antibiotin to mixed SAMs presenting biotin groups was accomplished by injecting a solution of buffer (PBS with 1% BSA and 0.01% Tween 20) over the SAM for 2 min, substituting a solution of antibody (0.13 μM) in the same buffer for 3 min, and then replacing it with the original buffer for 5 min (Figure 2). The adsorption of CA to mixed SAMs presenting benzenesulfonamide groups was carried out by injecting a solution of buffer (PBS) over the SAM for 3 min, substituting a solution of CA (0.13 μM) in the same buffer for 5 min, and then replacing it with the original buffer for 10 min (Figure 3).

PIERS. Piers spectra were recorded on a DigiLab FTS 175 spectrometer equipped with a liquid nitrogen cooled MCT detector at an 80° angle of incidence. The spectra are the result of 1024 scans.

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