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Immobilization of Ligands with Precise Control of Density to Electroactive Surfaces

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Abstract: We report a broadly applicable surface chemistry methodology to immobilize ligands, proteins, and cells to an electroactive substrate with precise control of ligand density. This strategy is based on the coupling of soluble aminooxy terminated ligands with an electroactive quinone terminated monolayer. The surface chemistry product oxime is also redox active but at a different potential and therefore allows for real−time monitoring of the immobilization reaction. Only the quinone form of the immobilized redox pair is reactive with soluble aminooxy groups, which allows for the determination of the yield of reaction, the ability to immobilize multiple ligands at controlled densities, and the in−situ modulation of ligand activity. We demonstrate this methodology by using cyclic voltammetry to characterize the kinetics of a model interfacial reaction with aminooxy acetic acid. We also demonstrate the synthetic flexibility and utility of this method for biospecific interactions by installing aminooxy terminated FLAG peptides and characterizing their binding to soluble anti−FLAG with surface plasmon resonance spectroscopy. We further show this methodology is compatible with microarray technology by printing rhodamine−oxyamine in various size spots and characterizing the yield within the spots by cyclic voltammetry. We also show this methodology is compatible with cell culture conditions and fluorescent microscopy technology for cell biological studies. Arraying RGD−oxyamine peptides on these substrates allows for bio-specific adhesion of Swiss 3T3 Fibroblasts.

Introduction

Strategies to immobilize biomolecules onto solid supports is important for a wide variety of applications ranging from the development of small molecule and protein microarrays to model substrates for mechanistic studies of cell behavior.[1−5] There have been several immobilization methods developed to tailor surfaces for a variety of diagnostic and high-throughput assays. The most common conjugation methods employ radical, mediated photoimmobilization,[6] acid−base chemistry,[7−9] Staudinger ligation,[10−12] Click chemistry,[13,14] Diels−Alder reaction,[15] Michael addition,[16,17] and other chemoselective ligation strategies.[18] However, to extend the utility of these surfaces to generate new types of biosensors and to study complex biological processes, it is necessary to develop substrates with more sophisticated and flexible surface properties. Surfaces for these applications would require the precise control of ligand density, the ability to immobilize multiple ligands, and the in-situ modulation of ligand activity. For cell biological applications, the ability to precisely control low density of ligands is particularly important. The key criteria to successfully preparing these types of substrates rely on the surface to resist nonspecific protein adsorption, the selective chemistry for ligand immobilization, and the nature of the underlying substrate for spectroscopic characterization of its interactions with biomolecules.

Our strategy utilizes the unique chemical properties of an immobilized *p*-benzoquinone on a conductive surface. This redox-active molecule provides many desirable features for bioconjugation of molecules to surfaces. For example, the quinone molecule reacts in high yield with a number of

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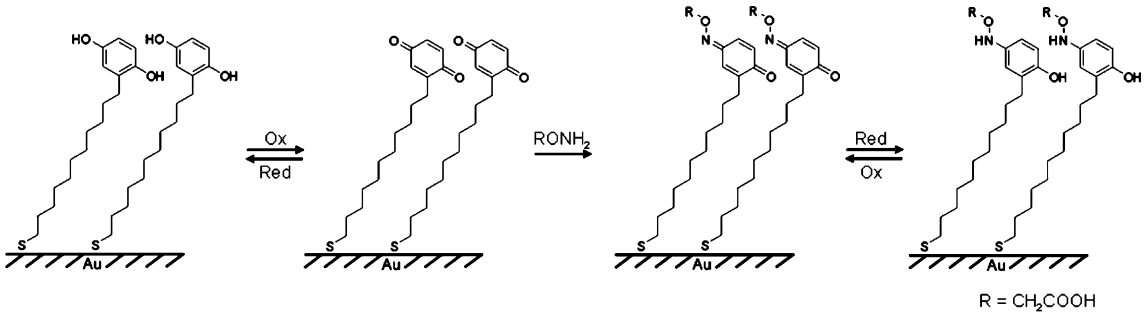

Figure 1. Redox-active hydroquinone monolayer undergoes electrochemical oxidation to the benzoquinone. The resulting quinone then reacts chemoselectively with aminooxy acetic acid to give the corresponding oxime.

functional groups in solution, such as thiol,[19] cyclopentadiene,[20] hydrazide,[21] and hydroxyamine.[22] The reversible redox coupling of quinone also provides a sensitive probe to monitor the extent of interfacial reaction in-situ by cyclic voltammetry.[23,24] Furthermore, only the quinone form of the reversible redox reaction is reactive; this important feature permits modulation of the ligand activity by an electrochemical potential.[25,26] Previous work has shown that a quinone monolayer reacts with a cyclopentadiene (cp) via a Diels−Alder reaction to immobilize a variety of biologically active ligands.[27,28] Although this strategy provides a rapid and selective immobilization of biomolecules, the syntheses of cyclopentadiene tethered molecules are cumbersome. In particular, the use of cyclopentadiene for common solid-phase peptide synthesis is not feasible because the cp readily decomposes under cleavage conditions. Alternatively, the use of sulfhydryl groups to conjugate biomolecules onto quinone surfaces via Michael addition provides a general and straightforward route. Unfortunately, the resulting quinone−thiol adduct has a redox-active signal similar to that of the quinone starting material.[29,30] Because there is no diagnostic peak that distinguishes between the quinone and the product, it is not possible to determine the surface density of immobilized ligands electrochemically with this coupling strategy for a variety of ligands. To extend the utility of immobilizing molecules to electroactive substrates, it would be desirable to develop other coupling chemistry for the quinone molecules that allows for the determination of the yield of coupling and therefore the ligand density on the surface. In this article, we report a novel chemical approach to immobilize ligands with precise control of density onto an electroactive self-assembled monolayer (SAM) of alkanethiolates on gold. This approach is based on the coupling between the ketone-bearing quinone monolayer and soluble aminooxy terminated ligands. We further

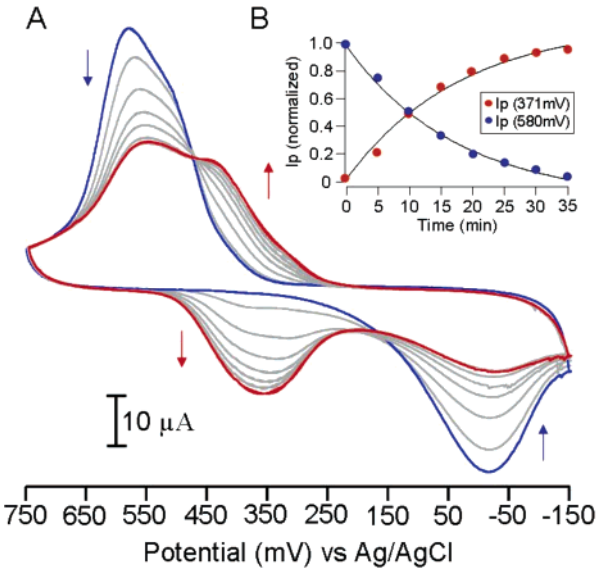

Figure 2. (A) Cyclic voltammograms recorded in 5-min intervals at a scan rate of 50 mV·s^{-1} showed the extent of the interfacial reaction between soluble aminooxy acetic acid (150 mM) and quinone monolayer. The peaks at 580 and −30 mV correspond to the redox coupling of the quinone, whereas the peaks at 450 and 371 mV correspond to the redox peaks of the product oxime. (B) A plot of the peak currents versus time compares the relative rates between the decrease of quinone monolayer and the formation of oxime on the surface.

demonstrate the utility of this methodology to immobilize ligands for selective protein binding and cell attachment on inert surfaces.

Results and Discussion

Our interest in using oxyamine for the bioconjugation is motivated by several considerations: (1) Oxyamine readily reacts with quinone in high yield at physiological pH and room temperature. (2) The two partners react covalently to form a chemically stable oxime linkage.[31,32] (3) The aminooxy groups can be easily introduced into most biomolecules through straightforward solution or solid-phase synthesis.[33]

We first demonstrate that oxyamine can undergo selective immobilization onto quinone monolayer in high yield. In this model study, we used the coupling between aminooxy acetic acid and a monolayer presenting quinone groups (Figure 1). Characterization of the redox reaction by cyclic voltammetry

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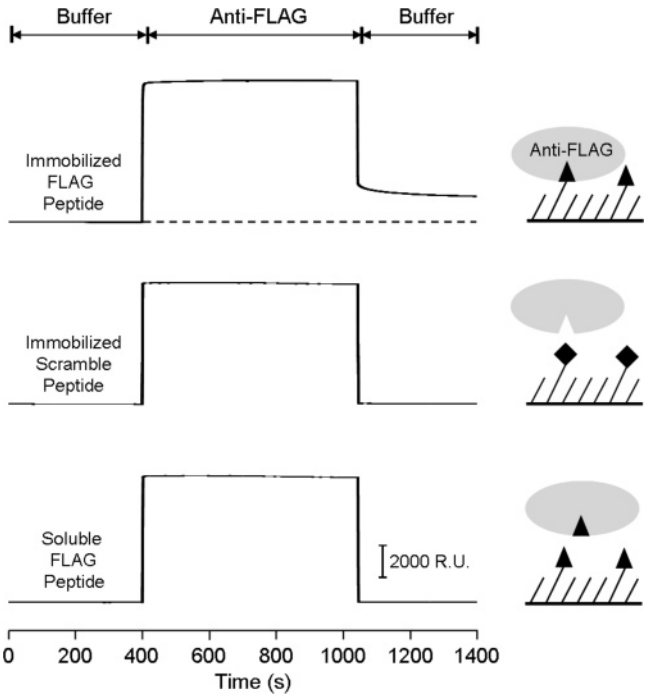


Figure 3. Surface plasmon resonance characterization for the selective immobilization of anti-FLAG onto mixed monolayer presenting tetra(ethylene glycol) groups and chemoselectively immobilized FLAG peptides. The results showed that the antibody associated only to the monolayer presenting the FLAG peptide ligands (top), whereas the use of a scrambled peptide ligand (middle) and the addition of soluble FLAG peptide (bottom) completely inhibited binding.

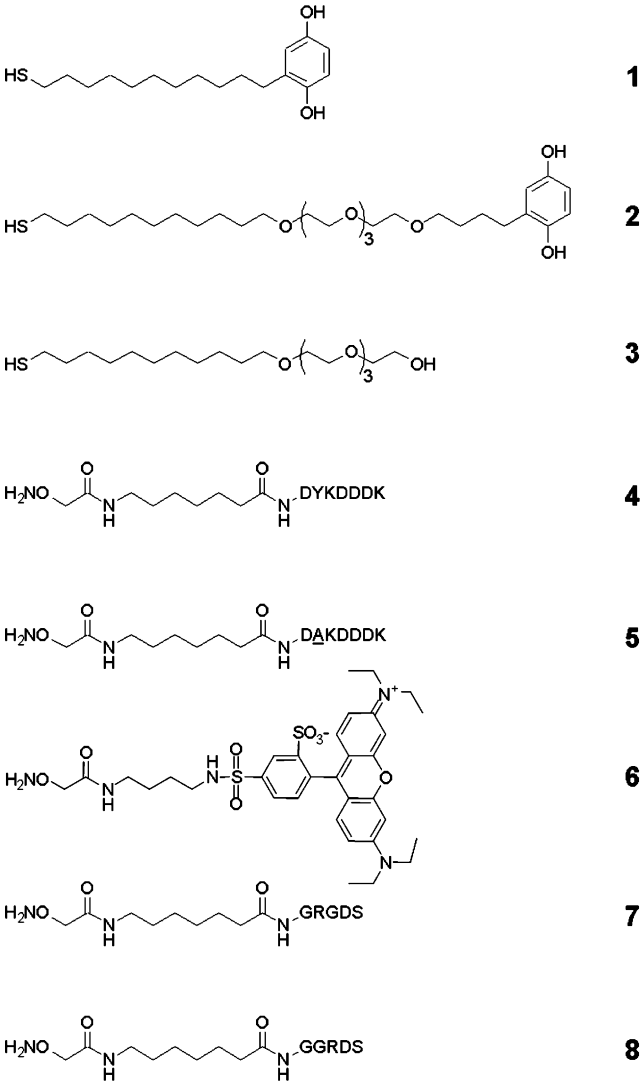
showed the monolayer presenting hydroquinone groups (**1**) underwent a reversible oxidation at 580 mV and reduction at −30 mV in 1 M perchloric acid. Addition of soluble aminooxy acetic acid resulted in the loss of peak currents for the quinone monolayer, and an increase in peak currents corresponding to the oxidation (450 mV) and reduction (371 mV) of the oxime product (Figure 2A). To confirm that the product redox-active peaks are due to the reaction of aminooxy acetic acid with the quinone monolayer, we independently synthesized the aminooxy acetic acid tethered quinone alkanethiol. Cyclic voltammograms of the monolayer for the control molecule showed identical peak currents as the immobilization product. Furthermore, we used X-ray photoelectron spectroscopy to show the characteristic nitrogen peak at 401 eV after surface immobilization of the aminooxy acetic acid to the quinone monolayer.

Figure 2B shows a plot of the peak currents versus time for the cyclic voltammograms shown in 2A. We expected the rate for the loss in peak currents of the quinone monolayer to follow pseudo first-order kinetics because the aminooxy acetic acid was present in large excess relative to the immobilized quinone. The data were fit to an exponential decay (eq 1) to give a pseudo first-order rate constant (k') of 0.056 min^{-1}, where I_t is the peak current at time t, I_0 is the initial peak current and I_f is the residual

$$I_t = I_f + (I_0 - I_f)\exp^{-k't} \quad (1)$$

$$I_t = I_0 \cdot (1 - \exp^{-k't}) \quad (2)$$

nonfaradaic current. To compare the difference in the rates between the loss of quinone and the formation of oxime, we fitted the data for the product oxime peak currents to an integrated first-order rate law (eq 2). The fit was excellent and gave a pseudo first-order rate constant of 0.055 min^{-1}. The rate constants for the decay of the hydroquinone−quinone signal and the formation of the product oxime are well within experimental error. This result shows that the quinone monolayer reacts chemoselectively with aminooxy acetic acid to give the corresponding oxime on the surface.



To demonstrate the utility of this methodology for biological ligand immobilization, we used the association of anti-FLAG antibody to a monolayer presenting immobilized FLAG peptide. We prepared a mixed monolayer containing 1% hydroquinone groups (**2**) and 99% tetra(ethylene glycol) groups (**3**). The hydroquinone monolayer was electrochemically oxidized at 750 mV for 10 s in phosphate buffer saline (PBS) to give the corresponding quinone. To demonstrate the reaction was chemoselective in the presence of complex protein mixtures the substrates were treated with a 0.1 M solution of an aminooxy functionalized FLAG peptide (**4**) in HeLa cell lysates for 4 h to ensure completion of the ligand immobilization. We characterized the antibody immobilization by surface plasmon resonance (SPR) spectroscopy. Figure 3 shows SPR sensorgrams for the selective binding of anti-FLAG (0.1 mg/mL) onto the monolayer presenting immobilized FLAG peptides. When a

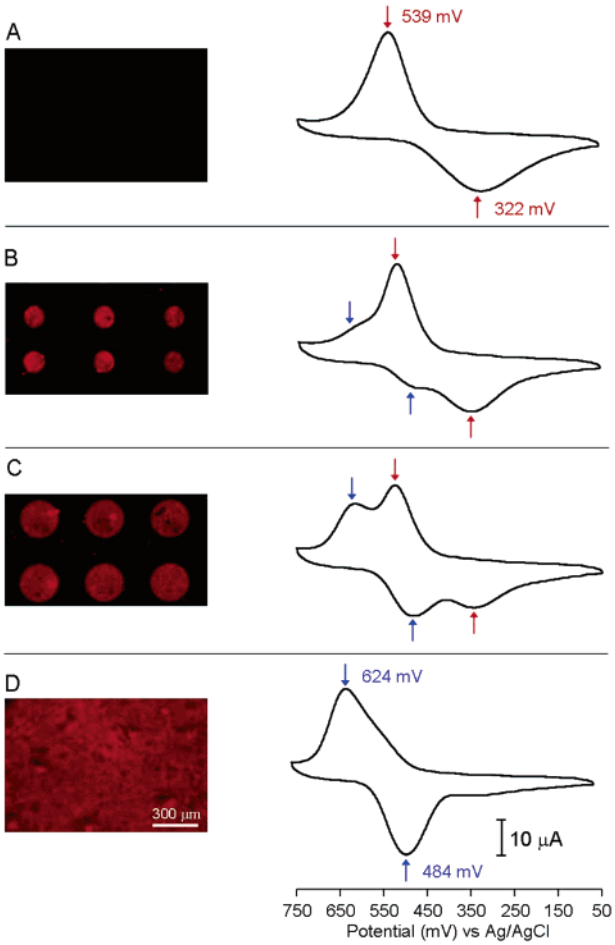


Figure 4. Fluorescent microscopy images of a quinone surface reacted with rhodamine-oxyamine and characterized by cyclic voltammetry. (A) A substrate presenting a mixed monolayer of hydroquinone and tetra(ethylene glycol) groups (1:1). Rhodamine oxyamine was spotted using a microarray on a quinone monolayer with an average spot diameter size of 120 μm (B) and 250 μm (C). (D) Complete conversion of the surface to the oxime product after rhodamine−oxyamine was reacted to the entire quinone surface.

peptide oxyamine with a scrambled non-binding sequence (**5**) was immobilized onto the quinone monolayer, no protein association to the surface was observed. Furthermore, presaturation of the antibody with soluble FLAG peptide (1 mM) completely prevented binding of the antibody to the monolayer. These results confirmed the association of the anti-FLAG to the immobilized FLAG peptide ligands was biospecific.

We next show the immobilization and electrochemical characterization strategy is compatible with microarray technology. We prepared a mixed monolayer containing 50% hydroquinone groups (**2**) and 50% tetra(ethylene glycol) groups (**3**). Cyclic voltammetry showed an oxidation peak at 539 mV and reduction peak at 322 mV corresponding to the hydroquinone−quinone redox couple (Figure 4A). A SpotBot microarrayer was then used to print rhodamine−oxyamine (**6**) (50 mM in methanol, 30 min) onto the substrate in 120 μm diameter spots (Figure 4B). Subsequent cyclic voltammetry of the entire surface showed characteristic redox active peaks for both the unreacted surface bearing the hydroquinone−quinone groups (539 and 322 mV) and the printed regions containing electroactive oxime product (624 and 484 mV). Integration and comparison of the peaks of the cyclic voltammogram allows for the determination of yield of immobilization of the rhodamine−oxyamine within the printed spots. To show the flexibility of this methodology a second substrate was printed with rhodamine−oxyamine to generate larger-sized (250 μm in diameter) spots (Figure 4C). Both large and small arrayed spots were printed on the same size substrate with the same number of spots. The only difference between the substrates is the size of the printed spots. The larger spots (250 μm) are approximately 4 times greater in surface area than the smaller (120 μm) printed spots. Comparison of the cyclic voltammograms for the smaller (Figure 4B) and larger (Figure 4C) printed spots shows the difference in yield to be approximately 4 times larger upon integration of the peaks and is consistent with the difference in surface area reacted. Figure 4D shows that upon addition of rhodamine-oxyamine to the entire surface after printing the surface is completely converted to the product oxime and characterized by cyclic voltammetry. These results indicate the use of cyclic voltammetry to precisely determine the yield of immobilization and therefore density of ligand on an arrayed substrate. This strategy can be used with microelectrode arrays to immobilize multiple ligands with the exact density of each ligand determined by electrochemistry.

Finally, we extend this immobilization strategy by combining microarray technology, cell culture and high-throughput microscopy for cell biological studies and applications. We prepared a surface composed of a mixed monolayer of 2% hydroquinone (**2**) and 98% tetra(ethylene glycol) alkanethiol (**3**). To this surface we printed an RGD-oxyamine peptide (**7**) (30 mM in PBS, 1 h) and a control scramble peptide GRD-oxyamine (**8**) in alternating spots. The RGD peptide is found in the central binding domain of the extracellular matrix protein fibronectin and is known to facilitate adhesion through cell surface integrin receptors.[34] Addition of Swiss 3T3 fibroblasts to the entire surface resulted in cell attachment only to the RGD spots and not to the control scramble peptide GRD spots (Figure 5). To show the interaction between cell and substrate is biospecific, i.e., the only interaction between cell and substrate is a ligand−receptor interaction, soluble RGD peptide was added to the media (1 mM in PBS) as a competitive inhibitor of the immobilized peptide to detach the fibroblasts (data not shown). As a further characterization, fast scan electrochemistry was used to determine the density of immobilized RGD peptide (data not shown) and may provide in combination with microarray technology a new method to interrogate how cells behave on surfaces where the density of immobilized ligands can be precisely controlled.

Conclusion

We have demonstrated a straightforward approach to immobilize ligands onto an electroactive quinone monolayer with precise control of ligand density using high throughput microarray technology. We have further applied this methodology to immobilize peptide ligands for selective protein binding and cell attachment to inert surfaces. The use of quinone and oxyamine provides a general route for the preparation of substrates presenting a variety of biological ligands for biospecific interactions. The redox activity between the quinone and oxime groups permits the determination of the yield of reaction and therefore the density of immobilized ligands in real-time

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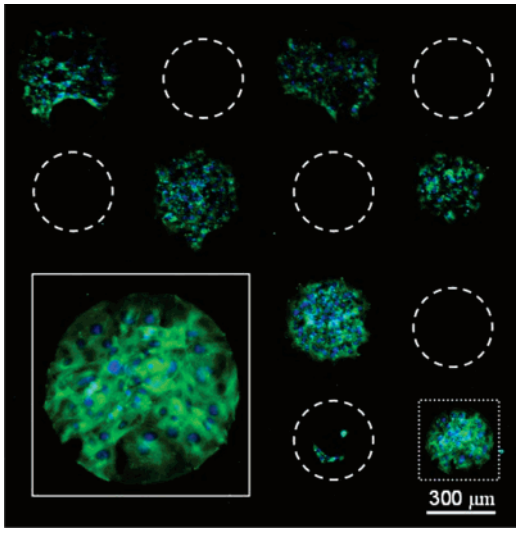

Figure 5. A fluorescent image of attached cells on a surface arrayed with immobilized RGD and GRD peptides. Cells were stained for microtubules and nuclei. Both peptides (30 mM in PBS) were printed by a microarray in alternating spots. The dotted circles represent the position where the control GRD peptide was immobilized. Cells attached only to the spots presenting the RGD peptide. A fluorescent image of attached cells on a RGD spot indicated by a dotted square (inset) is shown at higher magnification.

by cyclic voltammetry. The selective coupling between the aminooxy groups to the oxidized quinone monolayer makes possible the modulation of ligand activity by an electrochemical potential. The immobilization strategy shown here, in combination with inert surfaces, may be used with microelectrode arrays and microfluidic technology to generate complex biosensors with massively parallel analysis. We believe this methodology will provide a broad range of tailored substrates for new studies in attached cell culture and applications in biotechnology.

Experimental Section

All the solvents for the synthesis were HPLC grade. THF was distilled from sodium benzophenone under nitrogen before use. Absolute ethanol was purchased from Aaper Alcohol Chemical Company. Flash chromatography was carried out using silica gel (230−400 mesh). All amino acids and resin were purchased from Anaspec, Inc. (La Jolla, CA). All reagents were purchased from Aldrich and used as received. All reagents used in cell culture were obtained from Gibco BRL.

Synthesis of Alkanethiolates. Alkanethiolates terminated in the hydroquinone groups (**1**, **2**) and the tetra(ethylene glycol) group (**3**) were prepared as previously described.[20,35]

Synthesis of Rhodamine Oxyamine. Rhodamine oxyamine was synthesized in four steps. Rhodamine sulfonyl chloride was coupled to *N*-Boc diaminobutane. Removal of the Boc group by trifluoroacetic acid (TFA) gives the corresponding rhodamine butylamine. The resulting amine was then reacted with *N*-Boc aminooxy acetic acid in the presence of dicyclohexylcarbodiimide/*N*-hydroxysuccinimide. Boc group deprotection by TFA gives the corresponding rhodamine oxyamine conjugate.

Solid-Phase Peptide Synthesis. All aminooxy terminated peptides were synthesized using a peptide synthesizer (CS Bio) at 2 mM. To cleave the peptide, the resin was placed in a solution of TFA containing 20% water and 10% phenol for 2 h, and filtered. The filtrate was concentrated in vacuo to give a yellow oil, and then precipitate in cold ether to give a white solid.

Microscopy. Adherent cells were fixed in 3.7% paraformaldehyde in phosphate buffer saline (PBS) for 10 min and then permeabilized with 0.1% Triton X in PBS (PBST) for 10 min. Cells were then stained with anti-tubulin (1:1000) in PBS containing 10% goat serum for 1 h, followed by Alexa 488-conjugated goat anti-mouse IgG (1:100 in PBST) and Dapi (1:300 in PBST) for 1 h. Substrates were rinsed with deionized water before mounted onto glass cover slips for microscopy. All optical and fluorescent micrographs were imaged using a Nikon inverted microscope (model TE2000−E). All images were captured and processed by MetaMorph.

Preparation of Monolayers. All gold substrates were prepared by electron-beam deposition of titanium (5 nm) and then gold (15 nm for microarray, 50 nm for electrochemical and spr measurements) on glass cover slips (75 cm × 25 cm). All gold coated glass substrates were cut into 1 cm^2 pieces and washed with absolute ethanol. The substrates were immersed in an ethanolic solution containing the alkanethiolates (1 mM) for 12 h and then cleaned with ethanol prior to each experiment.

Electrochemical Measurements. All electrochemical experiments were performed using a Bioanalytical Systems CV−100W potentiostat. Electrochemistry on SAMs was performed in 1 M $HClO_4$, using a platinum wire as the counter electrode, Ag/AgCl as reference, and the gold/SAM substrate as the working electrode. All cyclic voltammograms were scanned at 50 mV/s.

Kinetic Measurements. The kinetic experiment was performed in a glass cylindrical cell with stirring in the electrolyte and fitted with a gold substrate presenting hydroquinone terminated alkanethiol. Hydroquinone was electrochemically oxidized to the corresponding quinone. Aminooxy acetic acid hydrochloride (150 mM) was then added to the electrolyte to initiate the kinetic experiment. Cyclic voltammograms were recorded in 5 min intervals to monitor the extent of the reaction in-situ.

Surface Plasmon Resonance Spectroscopy. All SPR measurements were conducted using a Biacore 2000 instrument. Monolayer substrates were incorporated into the Biacore cassettes by removing of the manufacturer's substrate and then fastened onto the cassettes with double side tape (3 M). Measurements were performed with Dulbecco's phosphate-buffered saline (pH 7.4) as the running buffer and reported as changes in resonance unit (R.U.).

X-Ray Photoelectron Spectroscopy. XPS characterization was carried out on an AXIS HIS 165 and ULTRA Spectrometer, using Al K_α radiation (energy 1486.6 eV) in a vacuum of 5×10^{-9} Torr.

Cell Culture. Swiss Albino 3T3 cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% calf bovine serum and penicillin/streptomycin. Cells were removed with a solution of 0.05% trypsin/0.53 mM EDTA, resuspended in serum-free culture medium (10,000 cells/mL), and plated onto the SAM substrates. After 2 h, the substrates were placed in serum containing media and maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Microarray. All molecules were printed onto monolayer substrates using a SpotBot 2 microarrayer.

Acknowledgment. This work was supported by the Carolina Center for Cancer Nanotechnology Excellence and grants from the NIH to MNY and the Burroughs Wellcome Foundation (Interface Career Award).

Supporting Information Available: Cyclic voltammogram of the monolayer presenting the control molecule; XPS characterization for the surface immobilization. This material is available free of charge via the Internet at http://pubs.acs.org.

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