Patterning Multiple Aligned Self-Assembled Monolayers Using Light

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This work describes a method for patterning a gold substrate with multiple, aligned self-assembled monolayers (SAMs) using light at different wavelengths. It describes the synthesis and characterization of an alkanethiolate SAM that is photosensitive to light at both 220 and 365 nm. A photomask acts as an area-selective filter for light at 220 and 365 nm, and a single set of exposures at these two wavelengths through one photomask, without steps of alignment between the exposures, can produce three aligned SAMs on one gold substrate. We demonstrate the versatility of this method of photopatterning by modifying individual aligned SAMs chemically to produce surfaces having different properties. We characterize the modified SAMs using immunolabeling, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy, and surface plasmon resonance spectroscopy. We also describe the patterning of two aligned SAMs that resist the adsorption of proteins and a third region that does not resist the adsorption of proteins. The ability to produce multiple, aligned patterns of SAMs in a single step, without alignment of photomasks in separate steps, increases the versatility of SAMs for studying a range of physical phenomena.

Introduction

This work describes a method for patterning a gold substrate with three aligned regions of self-assembled monolayers (SAMs) using one photomask and one set of exposures to light at different wavelengths, but without alignment of photomasks (Figure 1). This method uses a polyfunctional alkanethiol that forms a SAM on a gold substrate and that presents two types of photocleavable bonds: an *o*-nitrobenzyl amine-protecting group that cleaves on exposure to light at 365 nm[1−4] and a thiolate bond (Au−S) that cleaves on exposure to light at 220 nm.[5−11]

These two bonds can be cleaved selectively in different regions of the SAM using an area- and wavelength-selective mask (i.e., a photomask that transmits different wavelengths in different areas). Light at 365 nm cleaves the amine-protecting group; light at 220 nm removes the entire SAM (regardless of functionality) from the surface and produces a region of unprotected gold. The cleavage of thiolates is probably a result of the photo-oxidation of the thiolate to a sulfonate and/or of sulfur−carbon bond scission.[7,10,12] A different SAM can be formed in regions that were exposed to light at 220 nm upon incubation with a solution of a different alkanethiol. In regions that are protected from exposure to light at 220 and 365 nm, the SAM remains intact.

The photocleavable amine-protecting *o*-nitrobenzyl groups with either one or two reactive termini are commonly used in solid-phase synthetic chemistry.[13,14] Initially, we used 6-nitroveratryloxychloroformate (NVOC) as a photocleavable amine-protecting group but observed, using matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS), that NVOC did not cleave quantitatively on exposure to light at 365 nm in the absence of solution-phase scavenger molecules. The requirement of a scavenger solution is a drawback because the presence of a solution between the mask and the substrate limited the features produced by photopatterning SAMs to sizes on the order of 100 μm. We therefore used an alternative photocleavable amine-protecting group, 1-(3,4-(methylenedioxy)-6-nitrophenyl) ethylchloroformate (MeNPOC, $(CH_3O)_2C_6H_2NO_2CH(CH_3)OCOCl$), which cleaves quantitatively on photolysis using near-UV light (365 nm) and regenerates the amines (Figure 1A). MeNPOC does not require the use of solution-phase scavengers. It is possible to use this surface for further modification: the amines generated by deprotection can be modified using traditional solid-phase synthetic methods.

A second approach using a photocleavable linker, 3-[5-(1-amino-ethyl)-2-methoxy-4-nitro-phenoxy]-propionic acid (NPOP, $H_2NCH(CH_3)C_6H_2(OCH_3)O(CH_2)_3CO_2H$), permits

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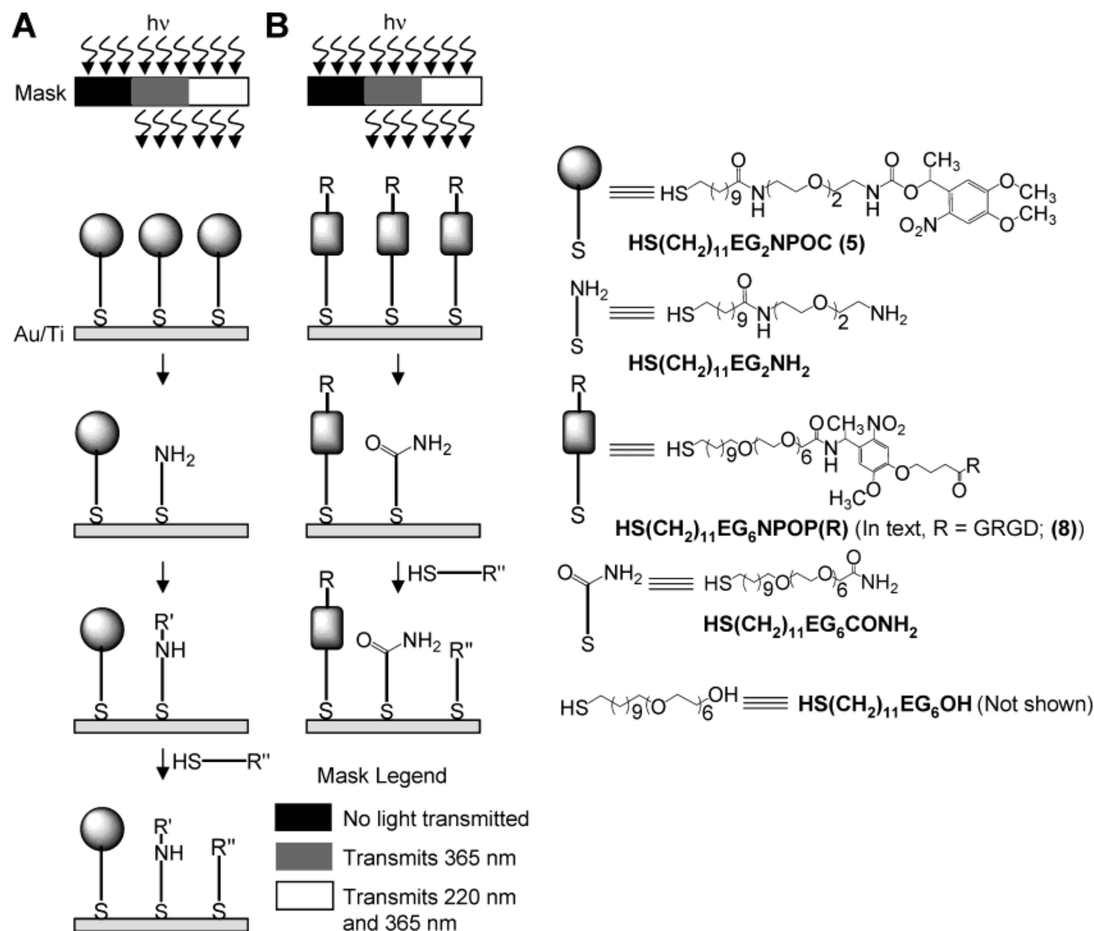

Figure 1. Patterning of a gold substrate with multiple, aligned SAMs using a photomask. Two methods are described: the photopatterning method described by process A produces a SAM that terminates in amines after exposure to light at 365 nm, while the method described by process B produces a SAM that terminates in primary amides after exposure to light at 365 nm. The method described by process B also permits the original SAM to present arbitrary functionality beyond the photocleavable linker. A new SAM can be formed in regions that are exposed to light at 220 nm in both approaches. R represents any group that can be coupled to a carboxylic acid, e.g., amine, alcohol, etc.; R′ represents any group that contains a carboxylic acid, aldehyde, etc. that can be coupled to an amine; R″ represents an arbitrary functionality that terminates with a thiol group. (Note: alkanethiol SAMs on gold substrates are tilted 30° to the normal and are shown here schematically without any tilt.)

the alkanethiol to include functional groups beyond the photosensitive group (Figure 1B); this capability based on NPOP provides an advantage relative to MeNPOC. The *o*-nitrobenzyl component can be modified to present another chemical functionality, either before (as demonstrated in this paper) or after photopatterning. A SAM that contains NPOP and that is exposed to light at 365 nm is converted to a SAM that terminates in primary amides; although primary amides can, in principle, be functionalized further (e.g., by reduction to amines with lithium aluminum hydride), the conditions used for these reactions are too harsh to be practical.

Several techniques allow the alignment of SAMs on surfaces. Microcontact printing (μCP) of alkanethiols on a gold or silver surface using a poly(dimethylsiloxane) (PDMS) stamp allows one type of alkanethiol SAM to be patterned in the background of a second type of alkanethiol.[15,16] Multiple printing steps can produce multiple patterns of molecules on a surface, but most patterns require the alignment of a stamp.[17] Micromolding in capillaries (MIMIC) is a general method for depositing polymers, SAMs, and proteins in continuous patterns on a substrate,[18−20] but MIMIC is unable to align multiple, discontinuous patterns. Three-dimensional networks of channels in PDMS can generate multiple, discontinuous patterns of proteins and cells,[21] but the microfluidic networks require alignment in fabrication. Chen et al. have demonstrated an elegant technique using a multilevel PDMS stamp to print multiple, aligned regions of proteins.[22] This technique involves several complicated steps (in fabrication of the stamp, in inking, and in printing or patterning).

A number of procedures for multicolor patterning have used photolithography. Cremer et al. coupled different fluorescent dyes in solution to a surface coated with bovine serum albumin (BSA) using irradiation with UV light through a photomask.[23] Where light passed through the photomask, the fluorescent dye molecules were excited

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and produced radicals; fluorescent dye radical molecules coupled to BSA in regions defined mostly by the pattern of illumination. Although this method permitted multiple molecules to be patterned by using different wavelengths to excite different fluorescent dyes, alignment of individual patterns was not demonstrated, and the resolution of the features was limited by the diffusion of the fluorescent dyes. Another report described the use of diarylethene derivatives that undergo photoinduced structural rearrangements depending on the wavelength of light used.[24] Two diarylethene derivatives were cast as a film and exposed sequentially through individual masks to UV or visible light.

The fabrication of multiple patterns on a surface requires alignment of two (or more) features when fabricating either a photomask or a microfluidic device (e.g., photolithography, MIMIC) or aligning a stamp when printing patterns of molecules (e.g., μCP). The work described in this article describes a method that requires alignment of two patterns in the fabrication of the photomask, but *not* in the exposure. The fabrication of the photomask need be carried out only once and uses materials and techniques that are routine in microfabrication. The method has two characteristics that may be useful: (i) the method can produce two discontinuous patterns of alkanethiolates on a surface without alignment of the photomasks; (ii) the method does not require the surface to be brought into contact with another surface to produce a pattern of molecules and therefore can pattern SAMs that resist the adsorption of proteins. We make a final comparison between the photolithographic technique introduced here and photography. The photographic process generates multiple patterns of different colors on film and is distinct from the method described in this work; in photography, each pattern is generated independently of another. Unlike photography, the approach described in this article is unable to generate single patterns at each wavelength; upon exposure to light at 220 nm, photocleavage of the alkanethiolate bond removes the *o*-nitrobenzyl component from the surface.

Results and Discussion

Synthesis of Alkanethiols Used for Patterning Multiple, Aligned SAMs. Figure 2 describes the synthesis of the photocleavable alkanethiols. Briefly, a trityl-protected form of mercaptoundecanoic acid was coupled to one amino group in $H_2N(CH_2CH_2O)_2CH_2CH_2NH_2$. The terminal amine of this compound was allowed to react with MeNPOC; removal of the trityl protecting group generated $HS(CH_2)_{11}(EG)_2$NPOC (**5**). Figure 1 describes the use of this alkanethiol to pattern multiple, aligned SAMs.

The second photocleavable alkanethiol, $HS(CH_2)_{11}EG_6$-NPOP(GRGD), contained a photocleavable linker that allowed functional groups to be added beyond the photocleavable group, NPOP. We synthesized the peptide sequence Gly-Arg-Gly-Asp (GRGD) on a Wang resin,[25] coupled the photocleavable linker to this peptide, and then coupled a trityl-protected alkanethiol to the photocleavable linker on the solid support. The removal of all protecting groups (except the photocleavable linker) produced HS-$(CH_2)_{11}EG_6$NPOP(GRGD) (**8**). Figure 1 also shows the use of this alkanethiol to pattern multiple, aligned SAMs.

Fabrication and Use of the Photomask Used for Patterning Multiple, Aligned SAMs. A microfabricated mask was used as an area- and wavelength-selective filter. In fabricating the mask, we started with a commercially available quartz substrate coated with indium tin oxide (ITO). ITO blocks light at 220 nm; quartz transmits light at 220 nm (Figure 3A). Using electron-beam metal evaporation, we deposited films of either chromium or gold on the ITO to block light at 365 nm, in addition to light at 220 nm.

To demonstrate photopatterning of SAMs using two different wavelengths of light from a Hg(Xe) arc lamp, we fabricated a mask with an array of squares in quartz and an array of triangles in ITO; we rendered the remainder of the mask opaque using chromium (Figure 3B). The photomask, once fabricated, could be used repeatedly.

The photomask was typically placed 10−50 μm above the gold surface to avoid damaging the SAM, although only minimal damage (e.g., submicron scratches) was observed in experiments where the mask was placed in contact with the SAM. We exposed the photomask to light at 220 nm (15 mW/cm^2, 15 min) and 365 nm (34 mW/cm^2, 3 min) in two separate steps because our Hg(Xe) lamp setup required separate mirrors to reflect these two wavelengths. In principle, a single exposure would be required if a mirror that reflected light at 220 *and* 365 nm were used. The need for two exposures in patterning multiple, aligned SAMs represents a limitation of the mirrors used in this work and is not a fundamental limitation of the method described here.

Patterning Multiple, Aligned SAMs Using Photolithography. We used a mixed SAM[26] containing HS-$(CH_2)_{11}EG_2$NPOC (**5**) and $HS(CH_2)_{11}EG_6OH$ on a gold substrate (Figure 4A). Upon exposure to light at both 220 and 365 nm through the photomask (but without repositioning the mask or substrate between the two exposures), the substrate was patterned into regions presenting the original SAM, a SAM that terminated in primary amines, and bare gold. The region that presented a SAM that terminated in amines was modified by reaction with (+)-biotin (as the (+)-biotin *N*-hydroxysuccinimide ester), and the exposed gold surface was modified with a mixed SAM by incubation with a solution containing $HS(CH_2)_{11}$-EG_6OH (1.9 mM) and $HS(CH_2)_{11}EG_2$DNP (**7**) (0.1 mM; DNP, dinitrophenyl). The substrate was incubated with anti-biotin mouse IgG and anti-DNP rabbit IgG and then with a mixture of fluorescently labeled anti-mouse IgG (green in Figure 4B) and fluorescently labeled anti-rabbit IgG (blue in Figure 4B). Using these antibodies, a fluorescence signal with an intensity that was indistinguishable from the background was recorded from the original SAM (black in Figure 4B). The resulting immunofluorescence image showed three aligned SAMs in the pattern of the photomask used and, thus, demonstrated the ability to pattern multiple, aligned SAMs using light and a photomask without alignment. A control experiment where anti-DNP rabbit IgG and anti-biotin mouse IgG were replaced by anti-BSA rabbit IgG and anti-BSA mouse IgG did not show any pattern. Thus, we conclude that the image shown in Figure 4B is a result of specific interactions between antigens and antibodies.

The minimum feature size we have been able to produce is on the order of 10 μm (Figure 4B). This limitation reflects the method used to fabricate the regions of the mask that transmitted light at 220 nm. These regions were fabricated by wet-etching ITO to expose quartz; this etch was difficult

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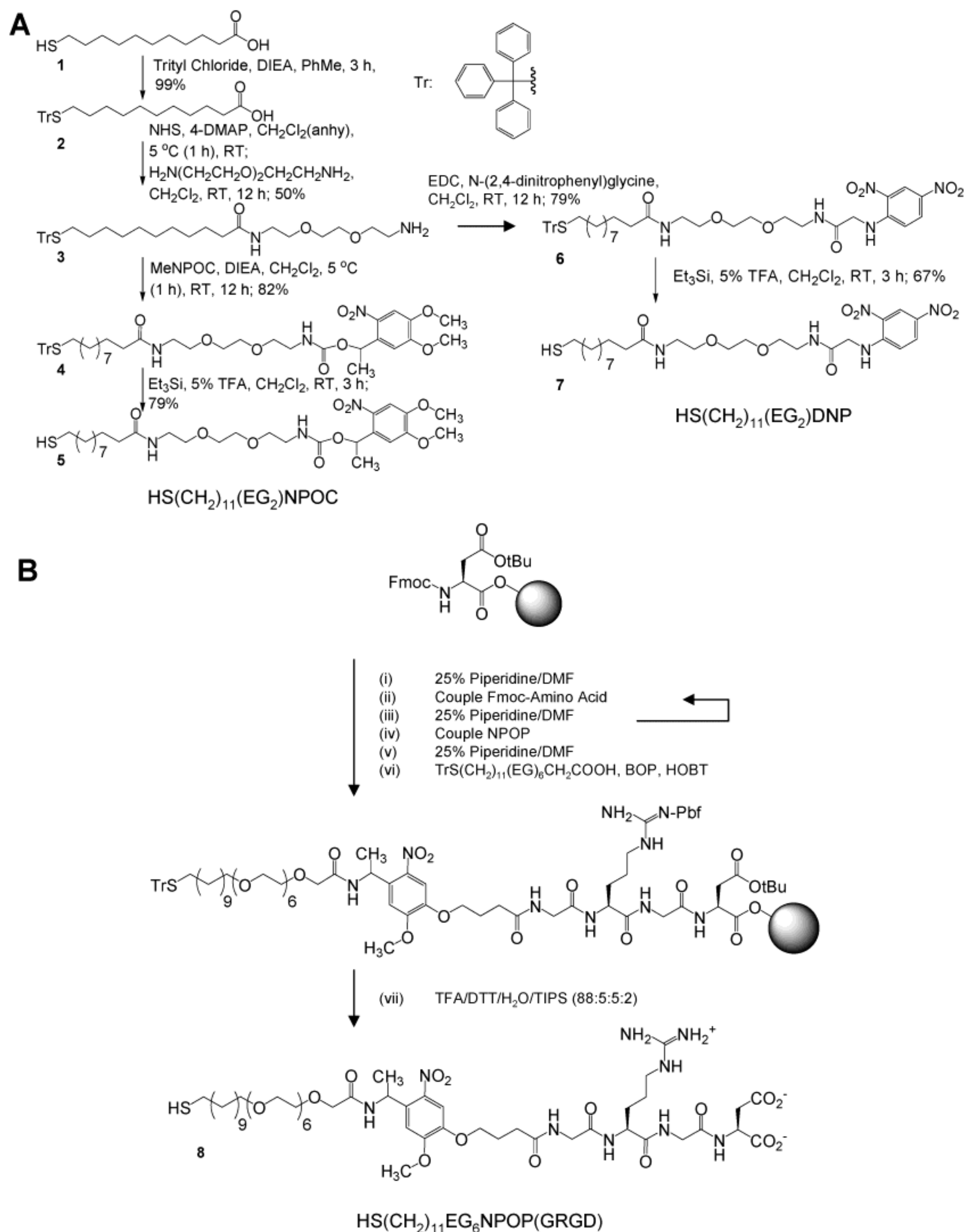

Figure 2. Synthetic strategies for (A) $HS(CH_2)_{11}(EG_2)NPOC$ and $HS(CH_2)_{11}(EG_2)DNP$ and for (B) $HS(CH_2)_{11}EG_6NPOP(GRGD)$ (on a solid-phase support).

to control.[27] Alternative fabrication processes including dry-etching (e.g., deep reactive ion etching) would allow patterning of such features at a resolution comparable to that of currently available photolithographic methods (<1 μm).

Patterning Multiple Aligned SAMs That Resist the Adsorption of Proteins. A drawback of the procedure described in Figure 1A is that the original SAM terminating in MeNPOC could not be functionalized further. We developed a second approach that also used a photocleavable linker (Figure 1B) but allowed the alkanethiol to include functional groups beyond the photocleavable linker. We demonstrated this capability by adding a peptide sequence, Gly-Arg-Gly-Asp (GRGD), to an alkanethiol that contained a photocleavable linker. The RGD sequence is found within many extracellular matrix (ECM) proteins (fibronectin, laminin, vitronectin, collagens, and proteoglycans), and SAMs that incorporate this peptide sequence are relevant for studies of adhesion of cells to surfaces.[28-30]

Here, we formed a SAM from an ethanolic solution of $HS(CH_2)_{11}EG_6NPOP(GRGD)$, **8** (0.05 mM), and $HS(CH_2)_{11}$-

(27) We etched ITO for 10 min, which was the optimal time to expose a uniform quartz region; however, the etchant partially removed metal near the quartz region, and a semitransparent region was produced around metal regions that failed to absorb light at 220 and 365 nm as efficiently as metal that was not etched (see Figure S1, Supporting Information). These partially exposed regions of ITO are responsible for the blue border around the black pattern in Figure 4B.

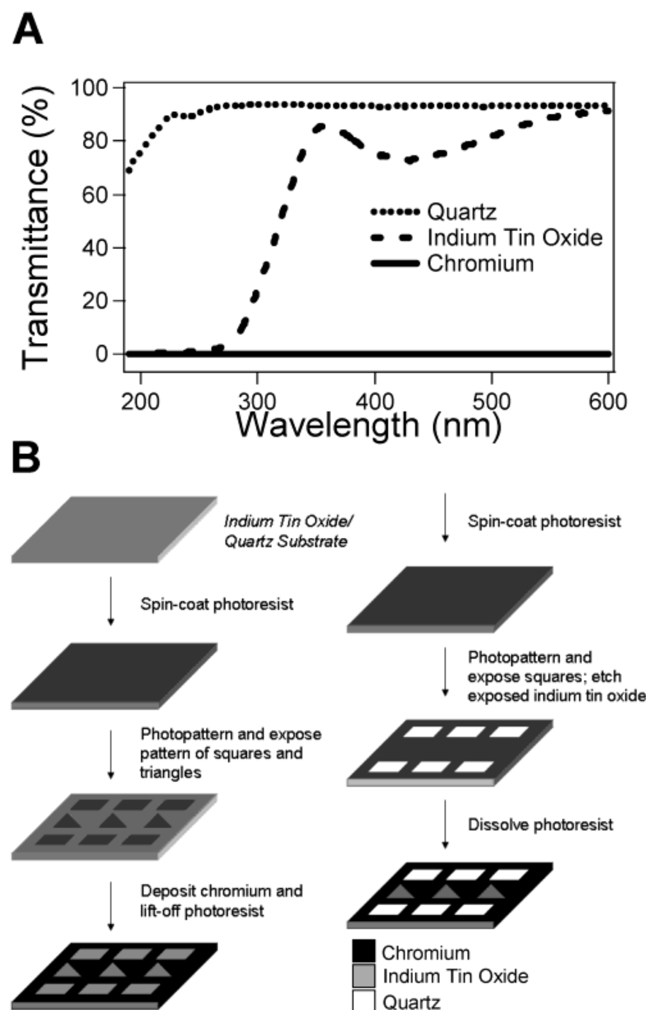

Figure 3. Fabrication and characterization of a photomask. (A) The transmission spectra of each component used in the mask. Chromium is sufficiently opaque to prevent significant transmission of light at 220 or 365 nm. Indium tin oxide is sufficiently transparent to light at 365 nm and opaque to light at 220 nm to filter deep UV wavelengths during exposure. Quartz is transparent to light at all wavelengths used in this study. (B) The procedure used for fabricating a photomask that allows area-selective transmission of 220 nm light, 365 nm light, and neither of these wavelengths.

EG_6OH (0.95 mM) on a gold substrate (Figure 5). Assuming that the concentration of alkanethiols in solution approximates the concentration of alkanethiols in a SAM, this mixture of alkanethiols produces a SAM containing ~5 mol % $HS(CH_2)_{11}EG_6$NPOP(GRGD) and ~95 mol % $HS(CH_2)_{11}EG_6OH$ (mol % refers to the ratio of the number of moles of an individual alkanethiol relative to the number of moles of both alkanethiols, expressed as a percentage); we did not directly measure these mol % values in the mixed SAM. In favorable cases, a mixed SAM that comprises at least 50 mol % $HS(CH_2)_{11}EG_6OH$ resists the adsorption of proteins.[26]

We illustrate the use of a photocleavable linker to pattern *two* aligned SAMs that are resistant to the adsorption of proteins and a *third* region that does not resist the adsorption of proteins. The two aligned SAMs that are resistant to the adsorption of proteins contained, before exposure to light at 365 nm, $HS(CH_2)_{11}EG_6$NPOP(GRGD) and $HS(CH_2)_{11}EG_6OH$ and, after exposure to light at 365 nm, $HS(CH_2)_{11}EG_6CONH_2$ and $HS(CH_2)_{11}EG_6OH$.

Characterization of Multiple, Aligned SAMs That Resist the Adsorption of Proteins. Using surface plasmon resonance (SPR) spectroscopy, we characterized the ability of SAMs exposed to different wavelengths of light to adsorb proteins. A SAM composed of hexadecanethiol on gold was used as an internal standard; this SAM adsorbs a monolayer of protein, and the change in reflectance due to this monolayer of protein is assigned a value of 100% ML (ML, monolayer; i.e., one monolayer of adsorbed protein).[31,32] SPR established that a mixed SAM we assumed to consist of 5 mol % $HS(CH_2)_{11}$-EG_6NPOP(GRGD) and 95 mol % $HS(CH_2)_{11}EG_6OH$ on a gold substrate was resistant to the adsorption of proteins prior to irradiation (Figure 5). We characterized this mixed SAM after exposure to light at 220 and 365 nm. As expected, after exposure to light at 365 nm, the surface remained resistant to the adsorption of proteins, and after exposure to light at 220 nm, the surface no longer resisted the adsorption of proteins. The loss in ability to resist the adsorption of proteins after exposure to light at 220 nm was evidence that the SAM containing $HS(CH_2)_{11}EG_6$-NPOP(GRGD) and $HS(CH_2)_{11}EG_6OH$ was photocleaved. We note that the amount of protein adsorbed on bare gold results in an increased %ML value compared with proteins adsorbed on hexadecanethiol SAMs. We speculated that proteins are denatured to a lesser extent on bare gold surfaces than on hexadecanethiol SAMs; a denatured protein probably presents a larger footprint on the surface and, in turn, limits the amount of protein adsorbed. From SPR, we concluded that a mixed SAM composed of HS-$(CH_2)_{11}EG_6$NPOP(GRGD) and $HS(CH_2)_{11}EG_6OH$ (before exposure to light at 365 nm) as well as a mixed SAM composed of $HS(CH_2)_{11}EG_6CONH_2$ and $HS(CH_2)_{11}EG_6OH$ (after exposure to light at 365 nm) resisted the adsorption of proteins.

We characterized these surfaces using MALDI-TOF MS.[33,34] MALDI-TOF MS provided the molecular weights of the components (as the sodium adducts of disulfides, primarily) containing the original SAM (containing HS-$(CH_2)_{11}EG_6$NPOP(GRGD) and $HS(CH_2)_{11}EG_6OH$), the SAM after exposure to light at 365 nm, and the SAM after exposure to light at 220 nm (Figure 6). In the case of the SAM before exposure to light at 365 and 220 nm (containing $HS(CH_2)_{11}EG_6$NPOP(GRGD) and $HS(CH_2)_{11}$-EG_6OH), a peak at *m*/*z* 1659 corresponded to the asymmetric disulfide $HOEG_6(CH_2)_{11}SS(CH_2)_{11}EG_6$NPOP-(GRGD). After exposure to light at 365 nm, a peak appeared at *m*/*z* 1015, which corresponded to the asymmetric disulfide $HOEG_6(CH_2)_{11}SS(CH_2)_{11}EG_6CONH_2$. The presence of this peak and the absence of a peak at *m*/*z* 1659 indicated the photocleavage of the peptide sequence and showed that the remaining SAM terminated in primary amides and hexaethylene glycol groups. The peak at *m*/*z* 958, found in each of the previous spectra, corresponded to the symmetric disulfide $HOEG_6(CH_2)_{11}$-$SS(CH_2)_{11}EG_6OH$ expected to be present in both samples. Since no organic species with *m*/*z* greater than 350 (lower

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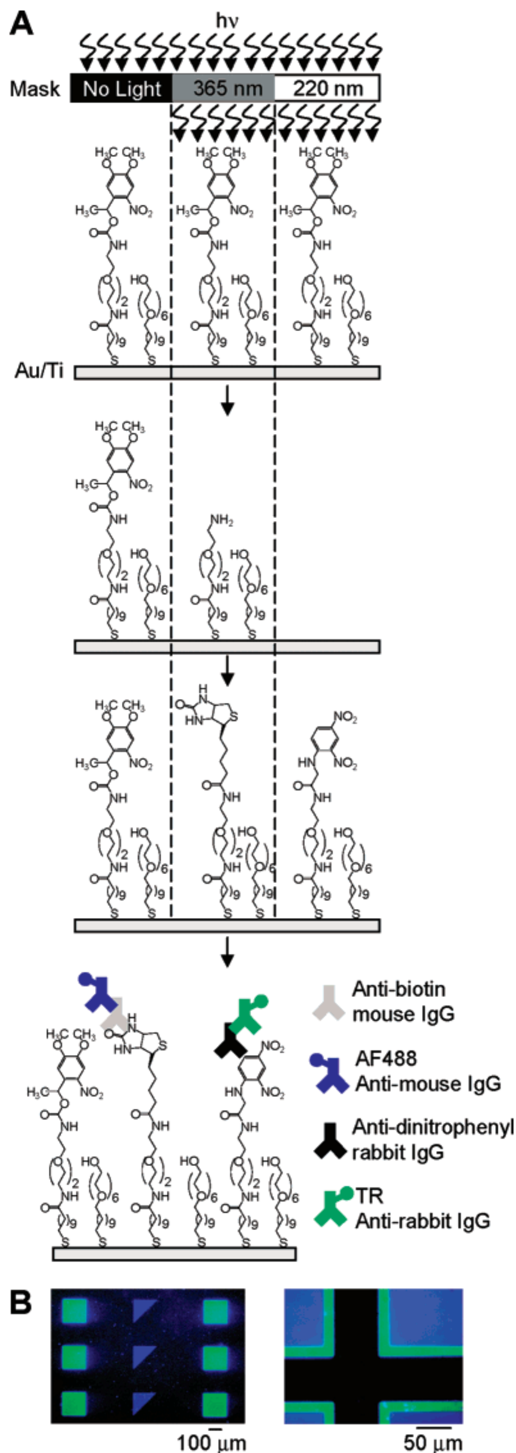

Figure 4. Preparation and immunolabeling of multiple, aligned SAMs. (A) Schematic illustration of patterning multiple, aligned SAMs using a photomask. Using the strategy outlined in Figure 1A, we illuminated a mixed SAM containing $HS(CH_2)_{11}EG_2$-NPOC and $HS(CH_2)_{11}EG_6OH$ through an area-selective mask that transmitted light either at 220 or 365 nm only or that blocked light at all wavelengths, to produce a region containing the original SAM, a SAM that terminated in primary amines, and a region of bare gold (or oxidized gold). We allowed (+)-biotin *N*-hydroxysuccinimide ester to react with the primary amines and also formed a new SAM composed of $HS(CH_2)_{11}$-EG_2DNP and $HS(CH_2)_{11}EG_6OH$ on the exposed gold. We labeled the SAMs using anti-biotin mouse IgG (followed by fluorescently labeled anti-mouse IgG) and anti-DNP rabbit IgG (followed by fluorescently labeled anti-rabbit IgG). (B) Fluorescence images of patterns of multiple, aligned SAMs.

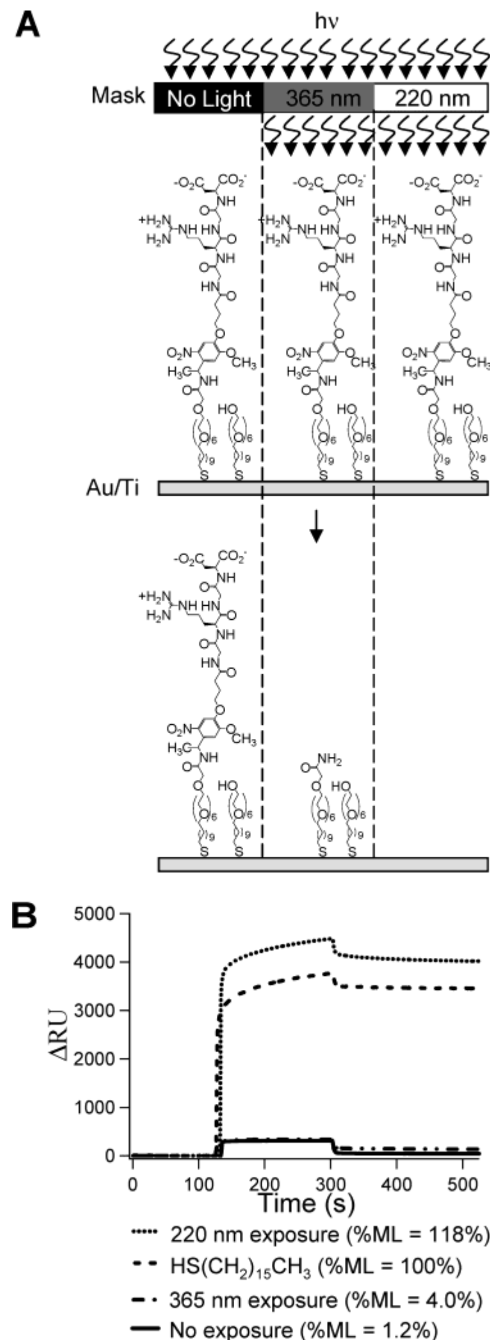

Figure 5. Fabrication and characterization of surfaces containing two SAMs that resist the adsorption of proteins and a surface that does not resist the adsorption of proteins. (A) Schematic illustration of the approach used to pattern multiple, aligned SAMs that resist the adsorption of proteins. Using the strategy outlined in Figure 1B, we illuminate a mixed SAM containing $HS(CH_2)_{11}EG_6$NPOP(GRGD) and $HS(CH_2)_{11}EG_6OH$ through an area-selective mask that transmits selectively light at 220 or 365 nm or blocks light at these wavelengths, to produce a bare gold (or oxidized gold) region, a SAM that terminates in primary amides, and a region containing the original SAM. (B) An SPR sensorgram of the mixed SAMs for substrates that have not been exposed to light, that have been exposed to light at 365 nm, and that have been exposed to light at 220 nm. The original SAM, protected from exposure to light by the opaque, chromium area of the mask, remains resistant to the adsorption of fibrinogen (1 mg/mL, PBS buffer). After exposure to light at 365 nm, the SAM region that terminates in primary amides resists the adsorption of proteins, and after exposure to light at 220 nm, the gold (or oxidized gold) surface (the monolayer is cleaved entirely) is unable to resist the adsorption of proteins.

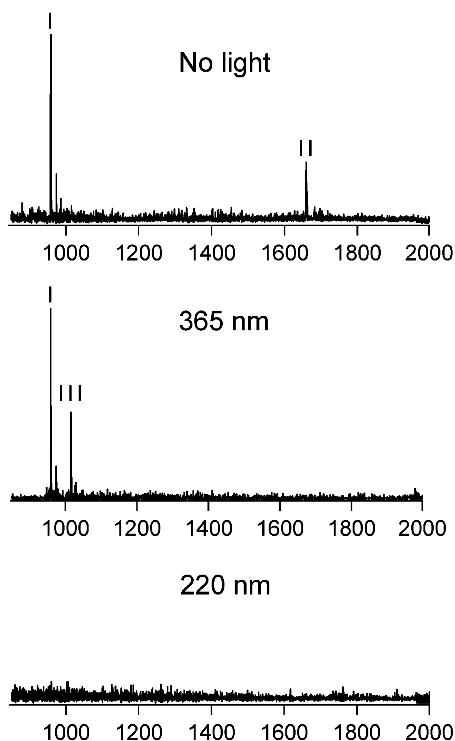

Figure 6. MALDI-TOF mass spectra of samples containing mixed SAMs that have not been exposed to light, that have been exposed to light at 365 nm, and that have been exposed to light at 220 nm. The peak at *m*/*z* 958 (**I**) corresponded to the symmetric disulfide $HOEG_6(CH_2)_{11}SS(CH_2)_{11}EG_6OH$ that is expected to be abundant in the spectra of the mixed SAMs. The initial monolayer displayed a peak at *m*/*z* 1659 (**II**) corresponding to the asymmetric disulfide $HOEG_6(CH_2)_{11}SS(CH_2)_{11}EG_6$-NPOP(GRGD) (expected *m*/*z* is 1659). After exposure to light at 365 nm, a peak at *m*/*z* 1015 (**III**) corresponding to the asymmetric disulfide $HOEG_6(CH_2)_{11}SS(CH_2)_{11}EG_6CONH_2$ (expected *m*/*z* is 992 plus Na^+, *m*/*z* 23, gives *m*/*z* 1015) was observed. After exposure to light at 220 nm, no peaks were observed, indicating photocleavage of the mixed SAM. No other molecular fragments are expected to be in significant abundance in this region (800 < *m*/*z* < 2000) for all three spectra, in agreement with the observed data. The symmetric disulfides (GRGD)-$NPOPEG_6(CH_2)_{11}SS(CH_2)_{11}EG_6$NPOP(GRGD) and H_2NOCEG_6-$(CH_2)_{11}SS(CH_2)_{11}EG_6CONH_2$ are unlikely to appear in the mass spectra because of the dilute concentration of the alkanethiol $HS(CH_2)_{11}EG_6$NPOP(GRGD) present in the original mixed SAM. Although individual alkanethiols corresponding to HS-$(CH_2)_{11}EG_6$NPOP(GRGD) and $HS(CH_2)_{11}EG_6CONH_2$ were observed occasionally, most molecular fragments appeared as disulfides.

limit of detection of our mass spectrometer) was recorded from MS analysis of the SAM after exposure to light at 220 nm, we concluded that in regions exposed to light at 220 nm, the entire SAM is photocleaved.

We scanned a substrate containing multiple, aligned SAMs (1 cm × 1 cm square region) and plotted the observed *m*/*z* peaks as a function of position on the substrate (Figure 7). The resolution of the resulting plot is limited to about 100 μm, since the spatial resolution of the detector used to acquire individual mass spectra from the patterned substrate was limited to square pixels with a dimension of 100 μm. The spatially resolved plot showed three distinct regions: the original SAM (*m*/*z* arising from $HOEG_6$-$(CH_2)_{11}SS(CH_2)_{11}EG_6$NPOP(GRGD)), the SAM after exposure to light at 365 nm (*m*/*z* arising from $HOEG_6(CH_2)_{11}$-$SS(CH_2)_{11}EG_6CONH_2$), and the SAM after exposure to light at 220 nm. The expected pattern of multiple, aligned SAMs generated using our photomask is shown in Figure 7A, and the observed plot of the multiple, aligned SAMs

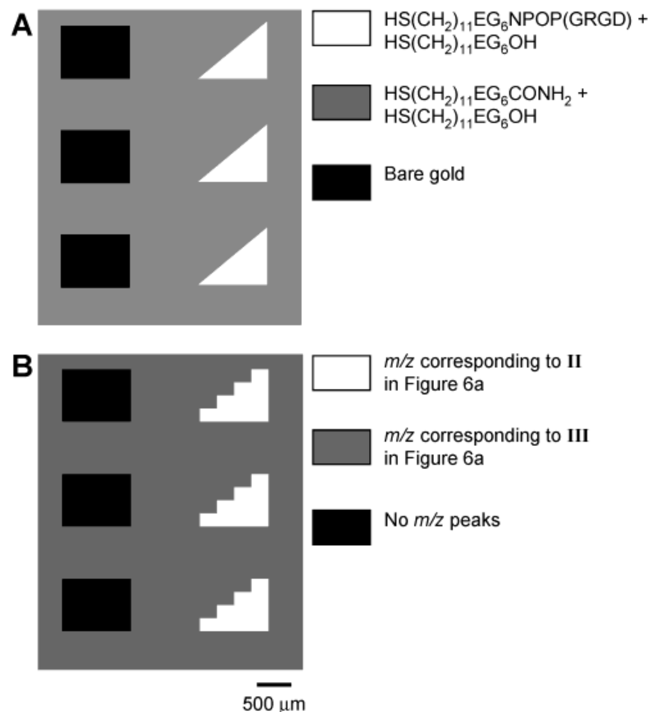

Figure 7. Patterning two aligned SAMs that resist the adsorption of proteins and a third region that does not resist the adsorption of proteins. (A) A representation of the expected pattern of multiple, aligned SAMs generated from a mixed SAM containing $HS(CH_2)_{11}EG_6$NPOP(GRGD) and $HS(CH_2)_{11}EG_6OH$. (B) A spatially resolved image of multiple, aligned SAMs constructed from the location of *m*/*z* peaks obtained using MALDI-TOF and corresponding to $HOEG_6(CH_2)_{11}SS(CH_2)_{11}$-$EG_6$NPOP(GRGD) (white), $HOEG_6(CH_2)_{11}SS(CH_2)_{11}EG_6CONH_2$ (gray), or no alkanethiol (black). The spatial resolution of our detector is limited to 100 μm (see text for discussion).

as generated from an analysis of *m*/*z* peaks in MS data is shown in Figure 7B. We conclude from both the MS and SPR data that we patterned two aligned SAMs that resist the adsorption of proteins and a third region that does not resist the adsorption of proteins. In summary, we have shown the patterning of *multiple*, aligned regions of alkanethiol SAMs that resist the adsorption of proteins.

Although we originally intended to use multiple, aligned patterns of SAMs to study cell−cell signaling (where one cell type is separated from the other), we have thus far not been successful in patterning multiple cell types (data not shown).

Conclusions

Multiple, aligned patterns of SAMs can be produced using an area- and wavelength-selective photomask and an alkanethiol that contains a photoprotecting group. The use of light at two wavelengths to effect the cleavage of two photoprotecting groups represents a conceptually new approach to patterning multiple (>2) SAMs. The technique is also capable of generating multiple, discontinuous patterns of SAMs that resist the adsorption of proteins, which is a new capability for patterning SAMs. A major advantage of this technique is that alignment is required only once: in the fabrication of the photomask. The technique, however, requires the synthesis of alkanethiols containing photoprotecting groups and the fabrication of a photomask. The ability to produce complex patterns of molecules on surfaces may be relevant for understanding a number of different interfacial phenomena, including dewetting and adhesion.

Experimental Section

Materials. All chemicals were purchased from Aldrich (St. Louis, MO) unless stated otherwise. MeNPOC was provided as a gift from Cambridge Major Laboratories (Germantown, WI). Anti-dinitrophenol delipidized rabbit anti-serum (anti-DNP rIgG), monoclonal anti-biotin mouse immunoglobulin G (anti-biotin mIgG), monoclonal anti-bovine serum albumin mouse IgG (anti-BSA mIgG), anti-bovine serum albumin rabbit IgG (anti-BSA rIgG), phosphate-buffered saline (PBS), and fibrinogen were purchased from Sigma (St. Louis, MO). Texas Red labeled anti-rabbit donkey IgG (TR-anti-rabbit IgG) was obtained from Amersham Biosciences (Newark, NJ). Alexa Fluor 488 labeled anti-mouse IgG (AF488-anti-mouse IgG) was obtained from Molecular Probes (Eugene, OR). The quantities of antibodies used are quoted as a ratio relative to the stock concentration acquired commercially, for example, 1:10 implies we used a 1:10 dilution in blocking buffer (0.05% Tween (w/v) in PBS) of the concentration provided by the supplier. H-1000 mounting medium for fluorescence was obtained from Vector Laboratories (Burlingame, CA).

Analytical HPLC was run on a Varian instrument (Walnut Creek, CA) with a Microsorb C18 column (5 μm, 4.6 $\times$ 250 mm) using a linear gradient of water with 0.1% trifluoroacetic acid (TFA) (A) followed by acetonitrile containing 0.08% TFA (B), at a flow rate of 1.2 mL/min (UV detection at 214 nm). Preparative reverse-phase HPLC was performed using a Varian apparatus on a C18 column (5 μm, 10 $\times$ 250 mm) at a flow rate of 6 mL/min (UV detection at 214 nm). Amino acids and derivatives were obtained from Novabiochem (San Diego, CA). 4-{4-[1-(Fmoc-amino)ethyl]-2-methoxy-5-nitrophenoxy}butyric acid was purchased from Advanced Chemtech (Louisville, KY). Mass spectra of organic molecules (not SAMs) were obtained by MALDI-TOF mass spectrometry on a Voyager-DE PRO (PerSeptive Biosystems, Foster City, CA). MALDI-TOF mass spectra of SAMs were analyzed on a Voyager-DE Biospectroscopy mass spectrometer using 2,4,6-trihydroxyacetophenone (5 μL of a 10 mg/mL solution in acetone) as a matrix. SPR experiments were performed using a Biacore 1000 SPR instrument (Piscataway, NJ). Solutions of fibrinogen (1 mg/mL, PBS buffer) were used in all SPR experiments, and solutions were filtered through 0.2-μm poly(vinylidene fluoride) filters immediately before use. Fluorescence images were recorded using an ORCA-ER Hamamatsu charge coupled device camera mounted on a DMIRB Leica inverted fluorescence microscope (DSC Optical Services; Newton, MA). An AB-M Mask Aligner (AB-M Inc.; San Jose, CA) was used as a light source for 220 and 365 nm wavelengths. Mirrors that select for each of these wavelengths had to be used separately during the experiments.

The alkanethiols (2-{2-[2-(11-mercapto-undecanoylamino)-ethoxy]-ethoxy}-ethyl)-carbamic acid 1-(4,5-dimethoxy-2-nitro-phenyl)-ethyl ester ($HS(CH_2)_{11}(EG)_2NPOC$) and 11-mercapto-undecanoic acid [2-(2-{2-[2-(2,4-dinitro-pheylamino)-acetyl-amino]-ethoxy}-ethoxy)-ethyl]-amide ($HS(CH_2)_{11}(EG)_2DNP$) were prepared according to Figure 2A. All reactions involving MeNPOC and NPOP were carried out in aluminum-foil-coated flasks to exclude light during reactions.

11-Tritylsulfanyl-undecanoic Acid (2). To a solution of trityl chloride (4.6 g, 17 mmol) and diisopropylethylamine (DIEA, 4.2 g, 33 mmol) in toluene (50 mL) was added 11-mercaptoundecanoic acid, **1** (6.0 g, 14 mmol), and the solution was stirred at room temperature for 3 h. The solution was evaporated, and the product separated between dichloromethane and water. The organic phase was washed with water (2 $\times$ 100 mL), dried ($MgSO_4$), filtered, and concentrated to yield crude **2** (6.3 g, 13.6 mmol, 97%). ^1H NMR ($CDCl_3$, 500 MHz): δ 1.14−1.42 (br m, 14H), 1.59−1.68 (br t, 2H), 2.06−2.09 (br m, 2H), 2.36−2.40 (t, 2H), 7.19−7.23 (m, 6H), 7.26−7.29 (m, 9H). (No mass spectrum of this compound was obtained.)

11-Tritylsulfanyl-undecanoic Acid {2-[2-(2-Aminoethoxy)ethoxy]-ethyl}-amide (3). To a solution of *N*-hydroxysuccinimide (0.26 g, 2.3 mmol) and a catalytic amount of 4-(dimethylamino)pyridine in anhydrous dichloromethane (50 mL) was added crude **2** (1.03 g, 2.2 mmol). Dicyclohexylcarbodiimide (0.46 g, 2.2 mmol) was added to the solution. The reaction was cooled for the first hour at 5 °C and left to react at room temperature overnight. The solution was diluted with dichloromethane, filtered to remove dicyclohexylurea, and evaporated to dryness to yield the active ester of **2** (1.1 g, 2 mmol). To a stirred solution of this active ester (1.1 g, 2 mmol) in dichloromethane (50 mL) was added 2,2′-(ethylenedioxy)bisdiethylamine (5.1 g, 34 mmol) over a period of 30 min. The reaction was left at room temperature for 12 h. The solution was filtered, washed with water (3 $\times$ 200 mL), dried ($MgSO_4$), and concentrated to yield **3** (1.25 g, 2.1 mmol, 95%). ^1H NMR ($CDCl_3$, 500 MHz): δ 1.24−1.42 (br m, 14H), 1.59−1.68 (br t, 2H), 2.06−2.09 (m, 2H), 2.78−2.85 (br t, 2H), 3.38−3.42 (m, 4H), 3.46−3.51 (m, 4H), 3.58−3.63 (s, 4H), 7.19−7.23 (m, 6H), 7.26−7.29 (m, 9H). $C_{36}H_{50}N_2O_3S$ (590.35): *m/z* 591.1 $[M + H^+]+$.

(2-{2-[2-(11-Tritylsulfanyl-undecanoylamino)-ethoxy]-ethoxy}-ethyl)-carbamic Acid 1-(4,5-Dimethoxy-2-nitro-phenyl)-ethyl Ester (4). To a stirred solution of **3** (1.03 g, 1.74 mmol) and DIEA (0.26 g, 2.0 mmol) in dichloromethane (50 mL) was added MeNPOC (0.59 g, 2.0 mmol) over a period of 30 min. The reaction was cooled for the first hour at 5 °C and then left to react at room temperature overnight. The solution was washed with 0.01 M HCl (1 $\times$ 100 mL), 0.2 M NaOH (1 $\times$ 100 mL), and saturated aqueous NaCl solution (1 $\times$ 100 mL). The solution was dried ($MgSO_4$) and evaporated to dryness. The crude compound was chromatographed (SiO_2/EtOAc → MeOH) to yield 1.2 g (1.4 mmol, 82%) of **4** as a yellow oil. ^1H NMR ($CDCl_3$, 500 MHz): δ 1.24−1.42 (br m, 10H), 1.59−1.71 (br m, 4H), 1.82−1.93 (br t, 2H), 2.02−2.11 (br m, 2H), 2.78−2.85 (br t, 2H), 3.38−3.42 (m, 4H), 3.46−3.51 (m, 4H), 3.58−3.63 (s, 4H), 3.63−3.66 (s, 3H), 3.66−3.69 (s, 3H), 6.14 (s, 3H), 6.22−6.31 (br q, 1H), 6.95 (s, 1H), 7.14−7.24 (m, 6H), 7.31−7.39 (m, 9H), 7.45 (s, 1H). (No mass spectrum of this compound was obtained.)

(2-{2-[2-(11-Mercapto-undecanoylamino)-ethoxy]-ethoxy}-ethyl)-carbamic Acid 1-(4,5-Dimethoxy-2-nitro-phenyl)-ethyl Ester, $HS(CH_2)_{11}(EG)_2NPOC$ (5). A solution of trifluoroacetic acid in dichloromethane (5% v/v), **4** (1.2 g, 1.4 mmol), and triethylsilane (0.83 g, 7.1 mmol) was stirred for 3 h at room temperature. The solution was washed with 0.2 M NaOH (2 $\times$ 100 mL) and brine (2 $\times$ 100 mL), dried ($MgSO_4$), and evaporated to dryness. The crude compound was chromatographed (SiO_2/EtOAc → MeOH) to yield 0.46 g (0.77 mmol, 54%) of $HS(CH_2)_{11}(EG)_2NPOC$ as a thick yellow oil.

$HS(CH_2)_{11}(EG)_2NPOC$, **5**: ^1H NMR ($CDCl_3$, 500 MHz): δ 1.24−1.42 (br s, 14H), 1.57−1.68 (br t, 2H), 2.17−2.21 (br m, 2H), 2.51−2.57 (q, 2H), 3.32−3.41 (m, 4H), 3.46−3.51 (m, 4H), 3.58−3.63 (m, 4H), 3.63−3.66 (s, 3H), 3.66−3.69 (s, 3H), 6.14 (s, 3H), 6.22−6.31 (br q, 1H), 6.95 (s, 1H), 7.45 (s, 1H). $C_{28}H_{47}N_3O_9S$ (585.75): *m/z* 608.9 $[M + Na^+]+$.

11-Tritylsulfanyl-undecanoic Acid [2-(2-{2-[2-(2,4-Dinitro-phenylamino)-acetylamino]-ethoxy}-ethoxy)-ethyl]-amide (6). To a stirred solution of **3** (0.25 g, 0.42 mmol) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (0.1 g, 0.5 mmol) in dichloromethane (50 mL) was added *N*-(2,4-dinitrophenyl)glycine (0.11 g, 0.51 mmol), and the solution was left to react at room temperature overnight. The solution was washed with 0.01 M HCl (1 $\times$ 100 mL), 0.2 M NaOH (1 $\times$ 100 mL), and brine solution (1 $\times$ 100 mL). The solution was dried ($MgSO_4$) and evaporated to dryness. The crude compound was chromatographed (SiO_2/EtOAc → MeOH) to yield 0.26 g (0.33 mmol, 79%) of **6** as a yellow oil. ^1H NMR ($CDCl_3$, 500 MHz): δ 1.12−1.42 (br m, 14H), 1.57−1.62 (br m, 2H), 1.63−1.72 (br t, 2H), 2.12−2.21 (br m, 2H), 3.41−3.61 (br m, 12H), 4.17−4.21 (d, 2H), 5.87−5.92 (br s, 1H), 6.89−6.94 (d, 1H), 7.14−7.24 (m, 6H), 7.31−7.39 (m, 9H), 8.26−8.32 (d, 1H), 9.14−9.21 (br d, 1H). $C_{44}H_{55}N_5O_8S$ (813.38): *m/z* 812.7 $[M - H^+]-$.

11-Mercapto-undecanoic Acid [2-(2-{2-[2-(2,4-Dinitro-phenylamino)-acetylamino]-ethoxy}-ethoxy)-ethyl]-amide, $HS(CH_2)_{11}(EG)_2DNP$ (7). A solution of trifluoroacetic acid in dichloromethane (5% v/v), **6** (0.26 g, 0.33 mmol), and triethylsilane (0.19 g, 1.7 mmol) was stirred for 3 h at room temperature. The solution was washed with 0.2 M NaOH (2 $\times$ 100 mL) and brine (2 $\times$ 100 mL), dried ($MgSO_4$), and concentrated. The crude compound was chromatographed (SiO_2/EtOAc → MeOH) to yield 0.12 g (0.22 mmol, 67%) of $HS(CH_2)_{11}(EG)_3DNP$ as an orange solid.

$HS(CH_2)_{11}(EG)_2DNP$, **7**: ^1H NMR ($CDCl_3$, 500 MHz): δ 1.22−1.39 (br s, 14H), 1.58−1.65 (br t, 2H), 2.18−2.21 (br m, 2H), 2.52−2.56 (q, 2H), 3.41−3.49 (m, 4H), 3.52−3.56 (m, 4H), 3.58−

3.61 (m, 4H), 4.17−4.21 (d, 2H), 6.89−6.94 (d, 1H), 8.26−8.32 (d, 1H), 9.14−9.21 (br d, 1H). $C_{25}H_{40}N_4O_8S$ (571.69): *m*/*z* 594.8 [M + Na$^+$]+.

Solid-Phase Synthesis of $HS(CH_2)_{11}EG_6$NPOP(GRGD) (8). The alkanethiol peptide $HS(CH_2)_{11}EG_6$NPOP(GRGD), **8**, was synthesized using Fmoc-tBu[25] chemistry and stepwise solid-phase methodology (Figure 2B). Synthesis of protected peptide chains was carried out on a 100-μmol scale starting from Fmoc-Asp-(OtBu)-Wang resin. The Fmoc group was removed using 20% piperidine in dimethylformamide (DMF, 1 × 5 min, 1 × 15 min) under nitrogen. The resin was filtered and washed with DMF (6 × 3 min). For each coupling step, a solution of the Fmoc-amino acid (5 equiv), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 5 equiv), and 1-hydroxybenzotriazole (HOBT, 5 equiv) in DMF and DIEA were added successively to the resin, and the suspension was stirred for 10 min at room temperature. The coupling reaction was monitored using 2,4,6-trinitrobenzene sulfonic acid (TNBS). After the removal of the last Fmoc protecting group, the resin was washed with DMF and $TrS(CH_2)_{11}(EG)_6CH_2CO_2H$ (Prochimia, Poland; 2.5 equiv) was coupled using BOP (2.5 equiv), HOBt (2.5 equiv), and DIEA (2.5 equiv) in DMF for 5 h at room temperature. The resin was washed with dichloromethane and diethyl ether and dried under nitrogen. Deprotection of the side chain and cleavage of the peptide from the resin were performed by treatment with TFA, dithiothreitol (DTT), water, and TIPS (triisopropyl-silane) in the ratio 88:5:5:2 (TFA/DTT/H_2O/TIPS). After precipitation in cold ether and centrifugation, the deprotected peptide was solubilized and lyophilized. The crude peptide derivative was purified by HPLC (linear gradient, 0−80% B, 40 min) and lyophilized to yield $HS(CH_2)_{11}EG_6$NPOP(GRGD) (**8**) as a white powder. HPLC t_R 18.84 min (linear gradient, 0−100% B, 20 min); MS (MALDI-TOF) *m*/*z* 1192.96 [M + 1]+.

Fabrication of the Mask for Patterning Multiple Aligned SAMs. The mask used for patterning multiple, aligned SAMs was fabricated as shown in Figure 2. We patterned an ITO-coated quartz substrate (Delta Technologies, 1 mm thick substrate coated with ∼200 nm of ITO, 15 ± 5 Ω) with an array of squares and triangles (200 μm or 1 mm sides) of photoresist (Shipley 1827, 2.7 μm thick), followed by deposition of 100 nm of chromium. We dissolved the photoresist using acetone (3 min) and rinsed the substrate with isopropyl alcohol. We etched selectively the ITO in the squares, using photoresist (Shipley 1827, 2.7 μm thick) as an etch-mask. The etchant solution for ITO consisted of deionized water, HCl, HNO_3 (15:4:1, v/v), and a few drops of Triton X-100 (55 °C, 10 min).[35] After etching, we rinsed the substrate with deionized water, removed the photoresist using acetone (3 min), and rinsed the substrate with isopropyl alcohol.

A similar fabrication strategy was used for obtaining patterns of multiple, aligned SAMs that resist the adsorption of proteins. The background region (that is not patterned with squares or triangles) consists of ITO, and the triangles consist of a 200 nm thick layer of gold on a 1 nm thick layer of titanium.

Immunofluorescent Labeling of Photopatterned Mixed SAMs. We formed a SAM using an ethanolic solution of $HS(CH_2)_{11}EG_2$NPOC (0.1 mM) and $HS(CH_2)_{11}EG_6OH$ (1.9 mM) on electron-beam-deposited gold on a glass slide (Ti, 1 nm; Au, 30 nm). A mask was placed on top of a 50-μm Kapton spacer resting on the gold slide and exposed to light at 365 nm (33.7 mW/cm^2, 3 min) followed by an exposure to light at 220 nm (15 mW/cm^2, 15 min). The exposed slide was rinsed with ethanol. The gold substrate was incubated (60 s) in an ethanolic solution of $HS(CH_2)_{11}(EG)_3$DNP (0.1 mM) and $HS(CH_2)_{11}(EG)_6OH$ (1.9 mM), resulting in the formation of a mixed SAM in the areas that had been exposed to 220 nm light. The gold substrate was rinsed with ethanol. (+)-Biotin *N*-hydroxysuccinimide ester (Biotin-NHS, 5 mg, 15 μmol) was dissolved in dimethyl sulfoxide (DMSO, 0.5 mL) and sonicated until a clear solution was obtained (60 s). The biotin-NHS solution was diluted immediately before use with 50 mM sodium carbonate buffer (pH 9.55) to obtain a 1.5 mM aqueous solution of biotin-NHS, and the gold substrate was incubated (300 s) in this aqueous solution. The substrate was rinsed with ethanol and dried with compressed nitrogen. Biotin-NHS is expected to couple to the amines in the region of the SAM that was exposed to light at 365 nm only. Surface blocking and antibody binding were carried out by successive incubation steps (37 °C, 1 h), each followed by rinsing with PBS, as follows. Antibody solutions were diluted to their working concentration using blocking buffer. The substrate was incubated in blocking buffer and then incubated in a mixture of anti-DNP rabbit IgG (1:3.5) and anti-biotin mouse IgG (1:35). Control experiments were carried out by incubation in a mixture of anti-BSA rabbit IgG (1:35) and anti-BSA mouse IgG (1:35). A final incubation step was carried out in a mixture of AF488-anti-mouse IgG (1:10) and TR-anti-rabbit IgG (1:10). The gold substrate was mounted on a coverslip using H-1000 mounting medium for fluorescence and imaged by fluorescence microscopy.

Photopatterning and Characterization of Multiple, Aligned SAMs That Resist the Adsorption of SAMs. We formed a SAM by incubating an electron-beam-deposited gold surface on a glass slide (Ti, 1 nm; Au, 30 nm) in an ethanolic solution of $HS(CH_2)_{11}EG_6$NPOP(GRGD) (0.05 mM) and $HS(CH_2)_{11}EG_6OH$ (0.95 mM) over a period of 12 h. Individual gold substrates were exposed to light at 365 nm (33.7 mW/cm^2, 3 min) or light at 220 nm (15 mW/cm^2, 15 min), rinsed with ethanol, and tested for resistance to the adsorption of proteins using SPR. Spatially resolved MALDI-TOF MS data were obtained by programming a scanner to obtain five mass spectra from 250 μm square regions across the substrate. Each mass spectrum corresponded to either a region containing the original SAM, the SAM after exposure to light at 365 nm, or the bare gold. For the purposes of presentation, we assigned an arbitrary color to each pixel: white for *m*/*z* signals representing the original SAM ($HOEG_6(CH_2)_{11}SS(CH_2)_{11}EG_6$NPOP(GRGD)), gray for *m*/*z* signals representing the SAM after exposure to light at 365 nm ($HOEG_6(CH_2)_{11}SS(CH_2)_{11}EG_6CONH_2$), and black for when no *m*/*z* signal was detected. We plotted a spatial representation of these data using the arbitrary values and their corresponding *x*−*y* coordinates.

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Supporting Information Available: Scanning electron microscope images of the photomask used for patterning multiple, aligned SAMs. This material is available free of charge via the Internet at http://pubs.acs.org.

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