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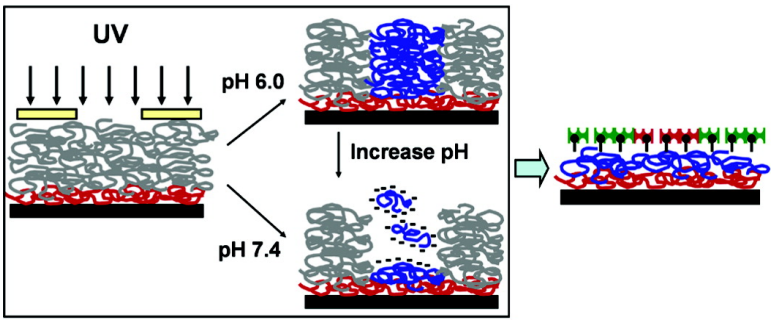

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Photogenerated Polyelectrolyte Bilayers from an Aqueous-Processible Photoresist for Multicomponent Protein Patterning

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Surface immobilization of proteins in micrometer-scale patterns has importance for bioengineering, biosensors, and fundamental studies of cell biology,[1−4] but it is made challenging by the fragile structure of proteins and their propensity for nonspecific binding to surfaces. Several techniques such as photolithography,[5−7] soft lithography,[3,8,9] photochemical methods,[10] and dip-pen nanolithography[11] have been developed, which have primarily focused on the immobilization of one protein in defined regions surrounded by a "background" that lacks protein (and may be additionally resistant to the adsorption of other proteins from solution). However, to mimic complex cell−cell and cell−extracellular matrix interactions, patterned surfaces comprising multiple functional protein regions on cellular and subcellular length scales would be useful. Few methods have been reported that allow patterning of multiple proteins on surfaces, and these may have limitations in spatial resolution,[12,13] in patterning fragile proteins that cannot withstand dehydration,[14,15] or exposure to organic solvents.[16] To address these issues, we developed a photolithographic approach that utilizes a novel aqueous-processible, pH-sensitive photoresist (PR) to pattern two different proteins onto defined regions of a surface without exposing either protein to irradiation, organic solvents, or dehydration.

The application of photolithography to patterning of biomacromolecules is limited by the harsh processing conditions required: typically, PRs are developed with organic solvents or strong bases, which can denature proteins and destroy their activity.[1,2,6,16] To obtain a PR that could be processed using biological buffers, a random terpolymer was synthesized (Figure 1a) by free radical polymerization of *o*-nitrobenzyl methacrylate (*o*-NBMA) with methyl methacrylate (MMA) and poly(ethylene glycol) methacrylate (PEGMA, $M_n \approx 360$; synthesis details are reported in the Supporting Information). These repeat units were used to tailor the properties of the resulting polymer: upon UV exposure, the *o*-nitrobenzyl protective group is cleaved to a pH-sensitive carboxylic acid,[17,18] while the PEGMA units tune the hydrophilicity of the resist and serve as a barrier to nonspecific protein binding to PR films. In addition, biotin was conjugated to hydroxyl endgroups of the PEGMA units to allow streptavidin−biotin-mediated coupling of proteins to the ends of flexible PEG tethers at a PR film surface. At an optimal composition (*o*-NBMA ≈ 43 wt %, MMA ≈ 38 wt %, PEGMA ≈ 19 wt %), thin films of the terpolymer could be dissolved by phosphate-buffered saline (pH 7.4 PBS, 10 mM sodium phosphate, 140 mM sodium chloride) after brief exposure to UV irradiation.

In typical photolithographic processes, a PR is used only as a selectively removable physical barrier for transferring 2D patterns present in a mask to a substrate. Here we took advantage of the polyelectrolyte nature of the UV-exposed PR to combine development of water-soluble PR with in situ electrostatic self-assembly, by using a cationic substrate to capture polyanions generated at the film−substrate interface by the photochemical reaction (illustrated in Figure 1a). Poly(allylamine) hydrochloride (PAH, $M_w \approx 70\,000$) was adsorbed on glass coverslips or silicon substrates (dry thickness 3 nm), and a 130-nm-thick film of PR polymer was subsequently spin-coated over the polycation monolayer. PR films were then exposed under a UV lamp (254 nm, 2.25 mW/cm^2) for various times and rinsed with PBS for 1 min. The thickness of dried films (measured by ellipsometry) after UV exposures longer than 10 min was 6−10 nm, indicating dissolution of the majority of the polymer but retention of a layer significantly thicker than the initial PAH film (Figure 1b). We hypothesized that this remaining film was a polyelectrolyte bilayer formed in situ at the PR/PAH interface by electrostatic cross-linking of newly formed carboxylic acid groups to amines on the PAH during UV exposure. To test this hypothesis, PR-coated substrates were UV-exposed for 15 min through a TEM grid as a crude photomask, rinsed with PBS, then dipped in a solution of cationic methylene blue dye. Only UV-exposed regions were stained (Figure 1c), suggesting that a polyelectrolyte bilayer with a net negative surface charge had formed between photogenerated polyanions and the underlying polycation.

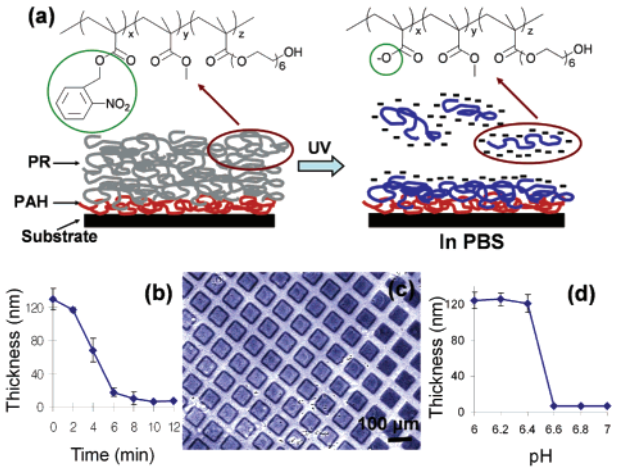

Figure 1. (a) Chemical structure of PR and its mechanism for in situ polyelectrolyte bilayer formation. (b) UV exposure time vs remaining PR film thickness. (c) UV-exposed, developed, and methylene blue-stained PR surface. (d) pH-dependent solubility of the UV-exposed PR.

The degree of ionization of weak polyelectrolytes is sensitive to pH, and thus the stability of a polyelectrolyte film in aqueous buffers can likewise exhibit pH dependence.[19,20] Protonation of the carboxylic acid groups on our UV-exposed PR at reduced pH made the polymer insoluble in acidic aqueous buffers. As shown in Figure 1d, PR-coated substrates exposed to UV for 15 min

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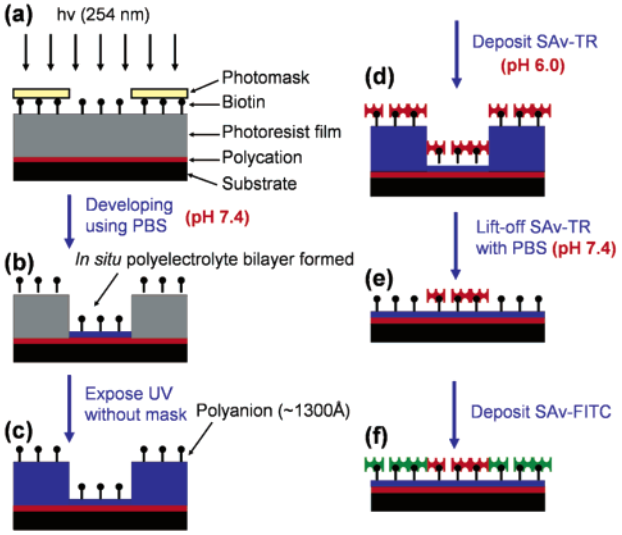

Figure 2. Schematic procedure of dual streptavidin patterning.

followed by rinsing with phosphate buffer (10 mM sodium phosphate) for 1 min exhibited dramatically different final thicknesses depending on the pH of the buffer solution. At low pH's, the UV-exposed films were stable in phosphate buffer. However, at a pH above 6.6, UV-exposed films dissolved to a constant thickness (~6−10 nm) characteristic of the polyelectrolyte bilayer structure.

This unique combination of characteristics—chemical patterning on UV exposure and pH-sensitive aqueous development—can be exploited to create multicomponent patterns of surface-immobilized proteins without exposing the proteins to UV irradiation or any solvents other than gentle near-neutral biological buffers. Using a biotinylated PR, we achieved assembly of two different fluophore-coupled proteins (Texas Red-conjugated streptavidin (SAv-TR) and fluorescein isothiocyanate-conjugated streptavidin (SAv-FITC)) following the scheme shown in Figure 2. A PR film spin-coated atop a PAH monolayer was exposed to UV through a photomask (Figure 2a) and developed with PBS rinsing (Figure 2b). Next, the substrate was reexposed to UV without a photomask (Figure 2c), and SAv-TR in pH 6.0 PBS was adsorbed (Figure 2d). Since the UV-exposed PR is not soluble in pH 6.0 PBS, the thick film of UV-exposed PR remained intact during this step, and SAv-TR was bound to the entire surface (data not shown). By subsequently washing the surface with pH 7.4 PBS, the thick PR film masking the "background" was dissolved, removing SAv-TR on that region and exposing underlying biotin groups in the retained polyelectrolyte bilayer for "backfilling" with a second type of SAv (Figure 2e). Finally, SAv-FITC was adsorbed on the newly exposed region (Figure 2f).

Fluorescence micrographs of a typical surface prepared by this process are shown in Figure 3. Figure 3, parts a and b, was taken from the same surface with excitation/emission filters matching Texas Red and FITC, respectively. Figure 3c shows an overlay of the two images. The red- and green-channel images show the clear segregation of the two proteins to their respective regions; the target region/nontarget region fluorescence ratios for the first (red) and second (green) proteins were 5.74 ± 0.74 and 4.82 ± 1.10, respectively, values which compare well to prior reports of patterning single proteins on surfaces.[5,13] In support of the role of

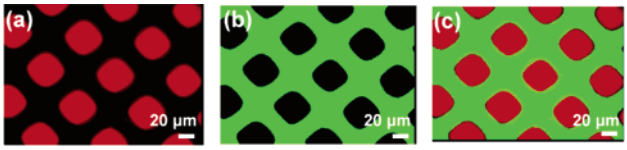

Figure 3. Fluorescent microscopy of dual streptavidin patterned surface. (a) SAv-TR fluorescence. (b) SAv-FITC fluorescence. (c) Overlay.

the resist's PEG groups in blocking nonspecific protein binding to the PR films, nonspecific binding of streptavidin was measured and found to be ~6% of the total protein bound to a biotinylated PR surface (see Supporting Information).

In summary, by utilizing the unique combination of photo- and pH-sensitivity of the described photoresist, two-component protein patterning was achieved under conditions that avoid exposing the proteins to conditions outside the narrow range of physiological pH, ionic strength, and temperature where their stability is greatest. Singly biotinylated proteins (some available commercially) are readily prepared by recombinant DNA technology, and using the process outlined in Figure 2, such proteins can be readily immobilized into two different regions of a substrate with high fidelity by simply incubating the surface with each protein following streptavidin in steps 4 (Figure 2d) and 6 (Figure 2f). This patterning strategy should have resolution limits similar to standard photolithography; we have been able to fabricate surfaces with 10-μm features using simple benchtop contact printing methods. Further, patterning of multiple protein arrays in 2D patterns should be readily achieved using contact aligners common in the silicon processing industry by step-and-repeat patterning.[13]

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Supporting Information Available: Synthesis and biotinylation of PR and characterization of protein binding to patterned surfaces. This material is available free of charge via the Internet at http://pubs.acs.org.

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