Sub-micrometer Patterning of Proteins by Electric Lithography

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We report in this paper an electric lithographic (EL) technique to generate protein patterns with sub-micrometer resolution on a poly(*N*-*t*BOC-2-aminoethyl methacrylate) surface. In the EL process, an electric potential is applied between metal patterns on a mask and the poly(*N*-*t*BOC-2-aminoethyl methacrylate) layer to electrochemically induce the dissociation of the *t*BOC from the amine functional groups. Proteins are then selectively attached to the amine functional groups in the modified polymer surface areas to form protein patterns. This technique can reliably generate high-resolution protein patterns down to ∼300 nm on the polymer surface at a high speed with a simple process/system.

Introduction

The microfabrication techniques that revolutionized semiconductor devices have recently been applied to biological systems. Microscale patterns of DNAs and proteins have been fabricated for micro-biosensing arrays and lab-on-a-chip systems.[1−3] These biological devices have been used extensively in gene profiling, disease analysis, and drug development.[4−6] Various lithographic techniques have been developed to reduce biomolecular patterns further to sub-micrometer scale. Optical lithography has been used to pattern biomolecules by modifying acid-facilitated functional groups on polymer surfaces to micrometer scale.[7−9] Microcontact printing can transfer biomolecules from a patterned stamp to a reactive substrate with a sub-micrometer resolution.[10−12] Dip-pen lithography, which uses an atomic force microscope probe to deliver molecules to a surface via a solvent meniscus, can reach a resolution down to ∼10 nm.[13] Scanning probe microscopy techniques can also be used to modify the surface atoms and molecules electrochemically to form sub-10 nm biomolecular patterns.[14−18] Although these techniques can reduce bimolecular patterns down to nanoscale, each has certain disadvantages. The patterns made by optical lithography can hardly reach sub-micrometer scale due to the limitation of optical diffraction. It is challenging to use a microcontact printing stamp to transfer multiple different biomolecules to different locations on a surface to form a biosensing array. The patterning speeds in dip-pen lithography and scanning probe microscopy techniques are very slow due to the serial process of the tip scan. This paper describes a novel electric lithographic (EL)[19] technique in which metal patterns on a mask replace the scanning probe microscope probe to locally modify the polymer surface electrochemically to generate specific chemical functional groups, and proteins are then selectively attached to the functional groups on the modified polymer surface areas to form protein patterns. The EL is a high-speed parallel process operated with a simple system. Large areas on the polymer surface can be patterned simultaneously, and it can reliably generate protein patterns with a high resolution down to ∼300 nm. This technique can potentially lead to patterning of multiple different biomolecules on a polymer surface by delivering different biomolecules to different exposed areas.

Experimental Section

Fabrication of EL Mask. The metal patterns on an EL mask are made by e-beam lithography and/or optical lithography on an insulating glass substrate. For micrometer-scale metal patterns, Au wires with a width ranging from 2 to 10 μm are made by photolithography. For sub-micrometer-scale metal patterns, multiple parallel linear Au nanowires with a width of 250 nm, a spacing of 750 nm, and a thickness of 20 nm are made on the glass substrate by e-beam lithography.[20] The nanowires are electrically connected with 20 μm wide and 20 nm thick Au wires made by photolithography.

EL Resist. The EL resist solution consists of 10% (w/w) configurable polymer, 0.5% (w/w) reducing agents, 0.5% (w/w) oxidizing agents, and 89% (w/w) solvent. Poly(*N*-*t*BOC-2-aminoethyl methacrylate) is used as the configurable polymer in which the *t*BOC functional groups can be dissociated from its amine groups during the EL process. The reducing and oxidizing agents in the resist are 1,2-diphenylhydrazine and tetrabutylammonium hexafluorophosphate, respectively.[21] Either acetonitrile or propylene glycol methyl ether acetate are used as the solvent.

Poly(*N*-*t*BOC-2-aminoethyl methacrylate) Synthesis. Poly(*N*-*t*BOC-2-aminoethyl methacrylate) is polymerized from *N*-(*t*-

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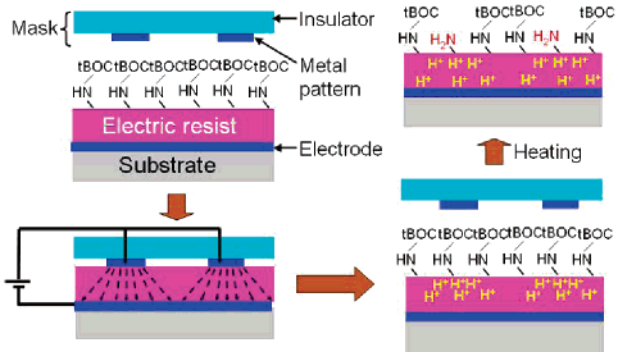

Figure 1. Schematic illustration of the process of the pattern generation of amine function groups on the surface of polymer electric resist by electric lithography. The electric field generated by the metal patterns is marked by dashed lines.

butoxycarbonyl)aminoethyl methacrylate, which is synthesized by following the published procedure.[22] *N*-BOC-2-aminoethyl methacrylate (3.0 g) is dissolved in propylene glycol monomethyl ether acetate (7.2 mL, 7.0 g) containing AIBN (60 mg) and purged with N_2 for 3 min. The reaction mixture is stirred at 70 °C for 24 h. The resulting viscous solution is diluted with acetone and poured into methanol/deionized water. The precipitate is filtered and dried under vacuum at 40 °C. The polymer identity is confirmed by 1H NMR spectroscopy (400 MHz, DMSO-d_6): δ 6.8−6.9 (bs, 0.9H), 3.7−3.9 (bs, 2.0H) 3.1−3.3 (bs, 2.1 H), 1.6−2.1 (bs, 1.8H), 1.39−1.41 (bm, 9.5 H), 0.7−1.0 (bm, 3.1H).

EL Process. The EL process is shown schematically in Figure 1. The aforementioned EL resist solution is spin-coated onto a thin Au conductive layer on a glass substrate at 2000 rpm for 20 s, followed by a soft-bake of the resist at 60 °C for 30 s. The EL resist film thickness is measured to be ∼1 μm by a stylus profiler. A mask, which consists of the micro/nanoscale Au metal patterns on the insulative glass substrate, is firmly pressed against to the EL resist layer by our homemade presser to ensure the contact between the EL resist and the metal patterns on the mask. A dc voltage with an amplitude of ∼25 V is applied between the metal patterns on the mask and the metal layer on the substrate for 1 min. The EL resist film has *t*BOC-protected amine functional group on the surface. The electric potential applied between the metal patterns on the mask and the electrode beneath the resist layer generates protons electrochemically inside the resist near the surface of the conductive patterns on the mask. After the electric potential exposure, the mask is removed. Both the mask and resist surface remain clean after the exposure, and the mask can be used repeatedly. Finally, the exposed resist is heated at 110 °C for 1 min. During the annealing, the protons inside the resist then catalyze cleavage of the *t*BOC groups from the amines, and the cleavage products are carbon dioxide and isobutylene, which evaporate away from the resist during the annealing. After the annealing process, the amine groups are revealed on the resist surface areas where the protons are generated by the conductive patterns on the mask, while the amine groups in the rest areas remain *t*BOC protected.

Observation of the Patterns of Amine Functional Groups Generated by EL on the EL Resist Surface. The EL resist is incubated with amine reactive fluorescent probes (7 mM sulfo-succinimidyl-7-amino-4-methylcoumarin-3-acetate; Pierce) in 0.01 M phosphate buffer saline (PBS, pH 7.4) for 1 h. Following rinsing with deionized water for 3 min, fluorescent patterns of the amine reactive fluorescent probes on the resist surface is observed with a Nikon E400 fluorescent microscope equipped with a Spot Camera (7.2 Color Mosaic).

Attachment and Observation of Biotin/Streptavidin Patterns Formed on the Configured EL Resist Surface. After the EL process, the surface of the EL resist is incubated with 1 mg/mL 6-(6((biotinoyl)amino)hexanoyl)amino)hexanoic acid, sulfosuccinimidyl ester, sodium salt (biotin-XX, SSE; Invitrogen) in 0.01 M phosphate buffer saline (PBS, pH 7.4) for 40 min, followed by deionized water rinsing for 3 min. The surface of EL resist is then incubated with 10 μg/mL fluorescent streptavidin [Alexa Fluor 488 conjugate (Invitrogen)] in 0.01 M phosphate buffer saline (PBS, pH 7.4) for 1 h. The PBS also contains 0.02% (v/v) Tween 20 detergent and 0.1% (w/v) bovine serum albumin as blocking agents.[23] Following final rinsing with deionized water for 3 min, fluorescent streptavidin patterns on the resist surface are observed with a Nikon E400 fluorescent microscope equipped with a Spot Camera (7.2 Color Mosaic).

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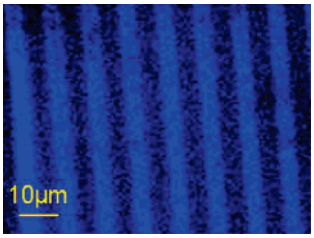

Figure 2. Fluorescent microscope image of parallel lines (bright blue) of amine reactive probes attached to amine groups generated by the electric lithography.

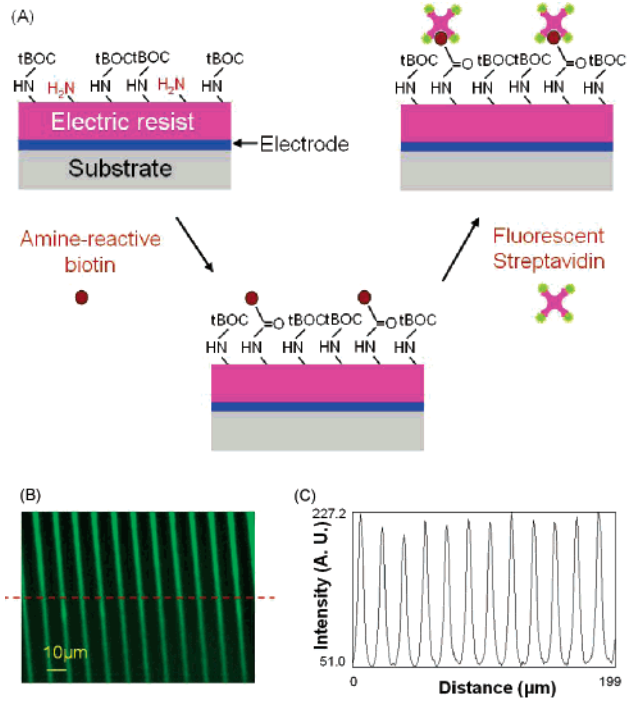

Figure 3. (A) Schematic illustration of the attachment sequence of biotin and streptavidin onto patterns of amine function groups on the surface of polymer electric resist. (B) A fluorescent microscope image of parallel linear patterns of fluorescent streptavidin (bright green) after the biotin and streptavidin attachment on EL-modified surface. (C) The fluorescent intensity profile of (B).

Results and Discussion

Amine-reactive fluorescent probes, Sulfo-NHS-AMCA (sulfo-succinimidyl-7-amino-4-methylcoumarin-3-acetate; Pierce), are incubated with the amine groups on the resist surface. Figure 2 shows a fluorescent microscope image of the attached amine reactive probes to the amine patterns generated by the EL process using a mask with 5 μm wide parallel metal lines. The image shows that the metal patterns on the mask have been faithfully transferred to the patterns of amine reactive probes on the resist surface.

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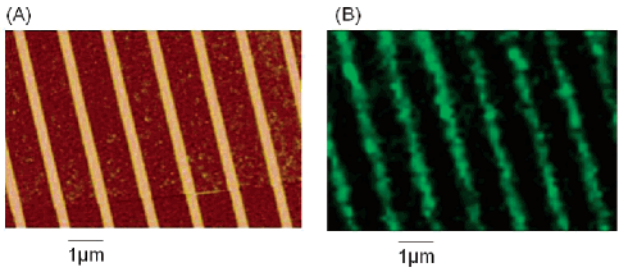

Figure 4. (A) Atomic force microscope image of sub-micrometer Au linear patterns (light yellow) on a glass substrate (brown) on a mask. (B) Fluorescent microscope image of sub-micrometer streptavidin linear patterns (bright green) generated on the polymer surface by EL with the mask shown in (A).

To further demonstrate the surface patterning of various biomolecules, after the EL process, amine-reactive biotin and fluorescent streptavidin are consecutively incubated with amine groups on the EL resist surface (Figure 3A). The fluorescent microscope image of the fluorescent streptavidin patterns shown in Figure 3B are generated by EL with 3 μm wide parallel metal lines on the mask. The image shows that the metal patterns on the mask have been faithfully transferred to the streptavidin patterns on the electric resist surface. The ratio of the fluorescent intensities in the EL modified area to that in the unmodified area is ~4.3:1 (Figure 3C). To ensure the integrity of the observed fluorescent image, control experiments are performed to investigate autofluorescence of electric resist and nonspecific binding of fluorescent streptavidin. Results show that an EL process missing either the amine reactive biotin incubation or fluorescent streptavidin step will produce negligible fluorescence.

Patterning of fluorescent streptavidin by the EL process at sub-micrometer scale is tested, and the results are shown in Figure 4. The parallel metal linear patterns with a width of 250 nm and spacing of 750 nm on the mask can be transferred to linear patterns of streptavidins on an electric resist surface. In Figure 4B, the fuzzy edges of the image of the streptavidin linear patterns are caused partially by the limitation of the optical imaging resolution of fluorescence microscope and partially by the limitation of the resolution of EL pattern transfer process.

Using predefined electrodes to replace the scanning probes for electrochemical patterning provides a rapid patterning method with a parallel batch process. However, the extensive diffusion of the active species in the liquid electrolytes during the electrochemical reaction prevents the patterning resolution from reaching sub-micrometer scale.[24] In this experiment, a semisolid polymer electrolyte is used as the EL resist facilitating the electrochemical reaction during the EL process. The acids are generated electrochemically in the EL resist underneath the surface of the conductive patterns on the mask. Compared with the metal patterns on the mask, the areas with the acids can be broadened inside the electric resist layer due to the fringe effect of the electric field (Figure 1) and thermal diffusion, but the broadening effect on the surface of the EL resist due to the fringe effect is minimal compared with that inside the resist. In order to activate the acid-facilitated dissociation of *t*BOC groups, the resist needs to be annealed at 110 °C for 1 min. During the annealing, the thermal diffusion of the acids is estimated to be ~20 nm.[25] Therefore, the ultimate limit of the spatial resolution for the EL process is ~40 nm. The further improvement of the EL resolution can be achieved by optimizing the process and the resist.

Conclusions

We have demonstrated an EL technique to generate protein patterns on a polymer surface. During the EL process, an electric potential is applied between metal patterns on a mask and the EL polymer resist layer to generate acids electrochemically inside the resist electrically contacted with the metal patterns. After annealing, the acids facilitate the detachment of *t*BOC groups from amine groups on the resist surface areas configured by the metal patterns on the mask. The further attachment and patterning of biotin/fluorescent streptavidin to the amines on the modified surface areas are observed by fluorescence microscope. The EL patterning can reach a high resolution down to 300 nm and is a high-speed parallel batch process operated with a simple system—a presser and a voltage source. By using individually addressable electrode arrays on a mask, this technique can be used to assemble biomolecules on the selected locations on the polymer surface. By selecting different electrodes in a series of EL processes, different biomolecules can be assembled to desired locations to form an array of biomolecules for bioanalytic applications. The further optimization of the EL process may lead to sub-100 nm resolution for biomolecular patterning.

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