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Local pH-Responsive Diazoketo-Functionalized Photoresist for Multicomponent Protein Patterning

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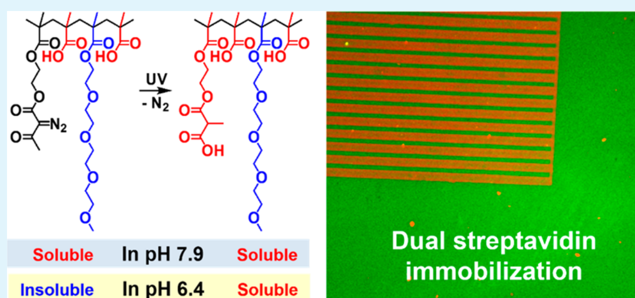
Supporting Information

ABSTRACT: Selective surface immobilization of multiple biomolecule components, under mild conditions where they do not denature, is attractive for applications in biosensors and biotechnology. Here, we report on a biocompatible and pH-responsive photoresist containing diazoketo-functionalized methacrylate, methacrylic acid, and poly(ethylene glycol) methacrylate monomers, where the photolithographic process may be carried out in a local pH range to minimize biomolecular denaturation. The polymer is insoluble or sparsely soluble in pH 6.4 or more acidic solution or deionized water, but soluble in a basic solution, pH 7.9 or more. After UV exposure, however, carboxylic acid groups are generated by Wolff rearrangement and photodissociation of the diazoketo groups in the polymer chain, leading to dissolution of UV-exposed polymer at pH 6.4. Using the property of the pH-solubility switching, we demonstrate dual streptavidin patterning using only biological buffers, pH 6.4 and 7.9 solutions, and double exposure patterning to confirm the sustainability of the diazoketo groups in unexposed regions despite carrying out several wet processes.



KEYWORDS: *diazoketo, biocompatible photoresist, pH-responsive photoresist, protein patterning*

INTRODUCTION

Biomolecular patterning has gained tremendous attention due to its applications in biosensors, bioMEMS, bioengineering, and fundamental biological studies.[1−5] Several patterning techniques including photolithography, soft-lithography, dip-pen lithography, and ink jet printing have been reported.[6−10] Each method has its own merits and demerits. Photolithography, in most cases, requires process steps including post-exposure bake and development in harsh organic solvents or strong bases that can denature the sensitive biomolecules.[11−15] Soft lithography involves an elastomeric stamp (typically poly(dimethylsiloxane)) that is used to transfer the ink to a given substrate. Despite its simplicity and cost-effectiveness, soft-lithography suffers from low speed and pattern deformation upon contact with the substrate due to the flexibility of the elastomer.[16−18] In recent years, there have been great advancements to circumvent these problems. As an example, dip-pen lithography exhibits great potential for patterning biomolecules in nanoscale dimensions but is not suitable for large-scale pattern formation.[19−21]

A plethora of literature about single component biomolecular patterning exists, where a single type of biomolecule is patterned on a given substrate.[22−24] Various developments have been achieved based on each patterning technique. In recent years, there is increasing interest in multicomponent biomolecular patterning, where more than one type of biomolecule is patterned on a substrate.[25−30] Such substrates are highly desirable for applications in complex biomolecular arrays and biosensors, along with the study of protein−protein and protein−cell interactions.[31,32] Photolithography is a promising approach due to its high-throughput and well-established industrial infrastructure. In order to make the photolithography-based approaches suitable for biomolecular patterning, mild processing conditions must be achieved.

Recently, new attempts using various photoresists for multicomponent biomolecular patterning have been reported. Chemically amplified resists have been used that require post-exposure bake below 50 °C and development in mild conditions to minimize the denaturation of the biomolecules.[33−35] Even so, there is a certain risk of denaturation since this is not a room temperature process and the photoacid generator present in the photoresist produces strong acid upon UV irradiation. In addition, dilute tetramethylammonium hydroxide was used as a developer, which might still be

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harmful for sensitive proteins. A nonchemically amplified type of photoresist (non-CAR) was reported by Doh and co-workers, which does not require photoacid generator and post-exposure bake.[36,37] They synthesized polymers containing photosensitive *o*-nitrobenzyl ester groups and demonstrated two-component protein patterning.[38] They also patterned T-cell receptor ligands surrounded by a field of tethered intercellular adhesion molecule-1 and studied their immunological response to T-cells.[39] One limitation of their nitrobenzyl-type photoresists is their low photosensitivity, requiring UV-irradiation at more than 2000 mJ cm^{-2} for pattern generation.[36,37] In addition, for biotinylation on a substrate, they used polyanion poly(acrylic acid) for adsorption by ionic interaction with the amine-treated surface. The resulting photogenerated aldehyde groups may react with functional groups on the substrate surface such as amines and get trapped in the underlying substrate.[40]

Novolac resists are also typical non-CARs for i- and g-line UV, which upon UV exposure undergo Wolff rearrangement to generate carboxylic acid groups. However, they are poorly photobleached in deep UV and have to be developed by dissolution in a strong basic solution, which may be harmful for sensitive biomolecules.[41]

The objective of our study is to develop a highly photosensitive and pH-responsive resist with improved utility for biomolecular patterning that is functional within a local buffer range where there is minimal stimulus to the sensitive biomolecules. In our study, we designed and synthesized a diazoketo-functionalized non-CAR that has much higher photosensitivity to be reacted to deep UV and EUV to be feasible to design nanopatterning[41,42] and predominant photobleaching effects in the deep UV region demonstrated in previous literature.[41,43−46] A major advantage of this photoresist is that it releases only molecular nitrogen as a byproduct,[41,42,47,48] thereby leaving the surface uncontaminated. Further, it provides an eco-friendly process, carried out in aqueous solutions, without toxic chemicals such as photoacid generators and developers. In our previous publications, we reported photoresist systems comprising 2-(2-diazo-3-oxo-butyryloxy)ethyl methacrylate (DOBEMA) as one of the main components and their applicability in single component biomolecular patterning.[41,42,47,48] DOBEMA has a photosensitive diazoketo group, which upon UV exposure undergoes Wolff rearrangement to generate a carboxylic acid group. In this study, we synthesized a new photo- and pH-responsive polymer consisting of DOBEMA, methoxy terminated poly(ethylene glycol) monomethacrylate (PEGMA), and methacrylic acid (MAA), whose pH solubility in the local region can be switched by UV irradiation, but which is insoluble in deionized water regardless of UV exposure. Figure 1 shows the schematic structure of the biocompatible photoresist polymer and switching structure of diazoketo groups (Figure 1a) by UV exposure to generate carboxylic acid units, which undergo Wolff rearrangement mechanism (Figure 1b). In what follows, we show the potential of our system for multicomponent biomolecular patterning via 3- and 6-μm dual protein patterns. Further, we demonstrate the efficacy of this novel polymer as a photoresist material for double UV exposure patterning and dual streptavidin patterning.

■ EXPERIMENTAL SECTION

Materials and Methods. Unless specified, all chemicals in this study were analytical grade and used as received. PEGMA (M_n = 475), triethylamine, 3-aminopropyltrimethoxysilane, 2-(methacryloyloxy)-ethyl acetoacetate, 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride, sodium phosphate monobasic monohydrate (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), and MAA were purchased from Sigma-Aldrich Chemical Co. *p*-Toluenesulfonyl azide was prepared from *p*-toluenesulfonyl chloride and sodium azide according to procedures in the literature.[49] The photosensitive monomer DOBEMA was synthesized as reported in our previous publication.[42] Deionized water (18.2 MΩ cm) was prepared in a Millipore Milli-Q Plus 185 purification system. Sulfo-NHS-LC-biotin, fluorescein isothiocyanate-conjugated streptavidin (SAv-FITC), and tetramethylrhodamine isothiocyanate-conjugated streptavidin (SAv-TRITC) were purchased from Pierce and stored in a refrigerator. Microscope glass slides (1.1 mm thick, 24 mm × 50 mm) were purchased from Polysciences, Inc. Sulfuric acid (H_2SO_4 96%) and hydrogen peroxide (30% H_2O_2) were purchased from Daejeong Chemical Company. 2,2′-Azobisisobutyronitrile (AIBN) was purchased from Junsei Chemical Company and purified by recrystallization in methanol.

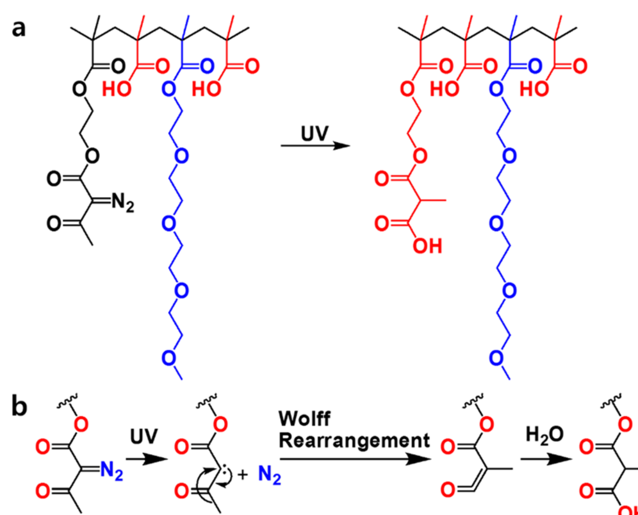

Figure 1. (a) Schematic photoresist polymer composed of DOBEMA, MAA, and PEGMA monomers. The UV-exposed diazoketo group is changed into the hydrous carboxylic acid. (b) For the photoreactive mechanism, the diazoketo group releases a nontoxic nitrogen molecule by UV absorption, transformed to carbene and then ketene as an intermediate according to Wolff rearrangement method, and finally changed into a carboxylic acid group by H_2O.

A portable pH meter (Hana Instrument) was used for pH measurements of a phosphate buffered saline (PBS) solution balancing NaH_2PO_4 and Na_2HPO_4 compounds to a desired pH value (Supplementary Table S1). The ^{1}H NMR spectra of the synthesized compounds were recorded on a Bruker Fourier Transform AVANCE 400 nuclear magnetic resonance spectrometer in $CDCl_3$ and deuterated-DMSO. Infrared spectra (IR) were recorded on a Bruker EQUINOX 55. The average molecular weight was determined in tetrahydrofuran (THF) by a Waters GPC-150C calibrated with polystyrene standards. The resist film thickness was measured with an Alpha-Step 500 Profiler (Tencor Instrument). UV exposure was done in contact mode using a deep UV exposure tool (Oriel Corporation, Model 82531) fitted with a filter transmitting light between 220 and 260 nm. For fluorescence microscopy, prepared samples were mounted on glass slides and images were obtained using an LSM 510 confocal microscope. Scanning electron microscope (SEM) images were obtained with a Hitachi model S-2280N SEM.

Polymerization. Polymerization proceeded via free radical copolymerization of DOBEMA, MAA, and PEGMA in dry THF. Monomers were mixed in various molar ratios to a total concentration of 20 mg mL^{-1} and subsequently purged with nitrogen for 30 min, polymerized by the addition of 5 mol % AIBN (based on total number

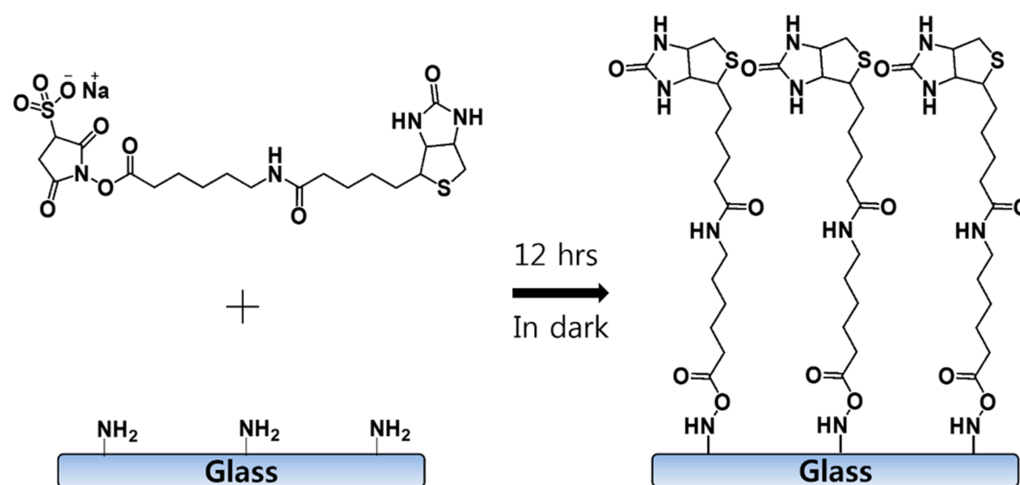

Figure 2. Scheme of the biotin surface covalently bonded by sulfo-NHS-LC-biotin on the amine-functionalized glass surface.

of moles of all monomers), and then heated at 65 °C for 15 h. The resulting polymers were purified by precipitation of the high molecular weight polymers in petroleum ether to dissolve mixed low molecular weight polymers and residual monomers, followed by filtration and drying. The monomer compositions of synthesized copolymers were determined by ^{1}H NMR (Supplementary Figure S1).

Preparation of Biotin-Functionalized Substrates. Glass slides were cleaned for 20 min in Piranha solution (7:3 ratio of conc H_2SO_4 and 30% (w/v) H_2O_2), then rinsed with deionized water and ethanol, and dried in nitrogen gas. Amine-functionalization was accomplished by immersing the cleaned glass slides in a 3% (v/v) 3-aminopropyltrimethoxysilane solution in ethanol for 3 h.[50] After removal from the silane solution, substrates were rinsed with ethanol to remove noncovalently adsorbed silane molecules and then blown dry with nitrogen. The substrates were kept at 110 °C for 1 h to stabilize the amine-functionalized layer. Biotin-functionalization was achieved by incubating the slides in the sulfo-NHS-LC-biotin aqueous solution (1.0 mM) for 12 h in dark. The substrates were subsequently washed with double distilled water and ethanol and then dried with nitrogen. Figure 2 shows the reaction scheme between sulfo-NHS-LC-biotin and the aminated glass surface.[51]

Lithographic Evaluation and Solubility Study. Lithographic evaluation and solubility studies of the polymers were done on thin photoresist films prepared by spin-coating the polymers from a 10 wt % solution in THF onto a glass coverslip at 1200 rpm for 60 s to obtain a film thickness of about 0.25 μm. Coated photoresist films were prebaked at 90 °C for 60 s to remove the residual solvent. The film dissolution was observed by immersing the photoresist films in a buffer solution (different pH PBS solutions shown in Supplementary Table S1) with gentle shaking while the time required for complete dissolution of the film was measured. The PBS (pH 6.4) buffer solution was used as a developer for pattern formation.

Dual Protein Patterning. For dual protein patterning, biotin-functionalized glass substrates were coated with a 10 wt % photoresist solution in THF and soft-baked at 90 °C for 60 s. Following UV exposure and development in the pH 6.4 PBS buffer solution, the first fluorescently tagged streptavidin solution (0.1 mg mL^{-1}) in PBS (pH 6.4) containing 0.1% (w/v) bovine serum albumin and 0.02% (v/v) Tween 20 was applied to the substrates for 1 h at room temperature. After dissolving the unexposed photoresist with pH 7.9 PBS buffer and washing with double distilled water, the second fluorescently tagged streptavidin solution in PBS (pH 7.4) (similar composition mentioned above) was applied onto the substrates to obtain dual protein patterns for 1 h at room temperature. Fluorescence images were obtained with an LSM 510 confocal microscope.

RESULTS AND DISCUSSION

Synthesis of pH-Responsive Polymers. pH-sensitive polymers composed of DOBEMA, MAA, and PEGMA were prepared by free radical polymerization using 5 mol % of AIBN as a free radical initiator, and the results are summarized in Table 1. The average polymer molecular weights were in the range of 4900–5700 g mol^{-1}, while polydispersity indices were in the range of 3.98–4.61. The polymerization yields were 82–91%. PEGMA and MAA were incorporated into the polymer structure in order to exploit the hydrogen bonding between ether oxygen atoms of PEGMA and carboxylic groups of MAA, which plays a crucial role in controlling the pH-dependent solubility. A number of previous studies have been reported that characterized hydrogen bonding interactions in polymer systems possessing MAA and PEGMA units.[52−55]

Table 1. Radical Polymerization of DOBEMA, MAA, and PEGMA

	DOBEMA:MAA:PEGMA				
polymer[a]	molar feed ratio	copolymer composition[b]	yield (%)	M_n[c]	PDI
DMP-1	60:30:10	52:34:14	91	5400	3.98
DMP-2	30:60:10	27:51:23	88	4900	4.61
DMP-3	25:60:15	27:48:25	82	5700	4.54

[a]DMP is indicative of the random copolymer with DOBEMA, MAA, and PEGMA. [b]Determined by integration of the corresponding peaks in the ^{1}H NMR spectra (Supplementary Figure S1). [c]Measured by GPC using THF as an eluent and polystyrene as a standard.

pH Sensitivity of the Polymers. Several studies exist that examine the hydrogen bonding effect between ethylene oxide and methacrylic acid under various pH conditions.[52−54] In one such system, complexation due to hydrogen bonding between ethylene glycol and methacrylic acid units was observed in the acidic conditions that rendered the overall system hydrophobic.[55] In basic conditions, hydrogen bonds ruptured to render the system hydrophilic. In our study, the solubility of the polymers in pH 4.0–7.9 PBS buffer solutions and deionized water was investigated for the polymer films before and after UV exposure; the results are summarized in Table 2.

All polymer films were completely insoluble in deionized water before and after UV exposure, which could be attributed to the fact that due to the low ionic strength the deionized

Table 2. Solubilities[a] of the Prepared Polymers in PBS Solutions at Different pH Levels before and after UV Irradiation

	before UV irradiation						after UV irradiation							
polymer	water	6.0	6.4	6.6	6.8	7.9	water	4.0	4.5	5.0	5.5	6.0	6.4	7.9
DMP-1	−	−	−	+	++	++++	−	−	−	+	++	+++	++++	++++
DMP-2	−	+	+	+++	+++	++++	−	−	+	+	++	++++	++++	++++
DMP-3	−	+	++	+++	+++	++++	−	+	+	++	+++	++++	++++	++++

[a] −, sparsely soluble; +, soluble after 1−3 h; ++, soluble after 20−60 min; +++, soluble after 2−20 min; ++++, soluble in less than 2 min.

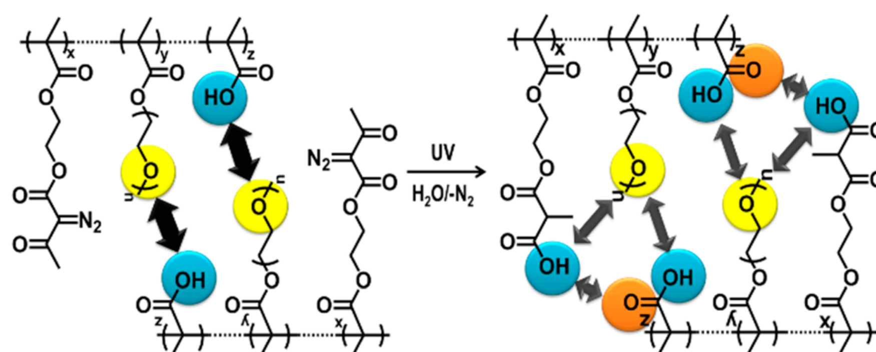

Figure 3. Schematic illustration of hydrogen bonding effect before and after UV irradiation. Before UV irradiation, the hydrogen bonding interaction mainly exists between hydrogen atoms of carboxylic acid groups and oxygen atoms of ethylene glycol segments. After UV irradiation, the hydrogen bonding sites have been increased and the interaction is distributed due to extra carboxylic acid groups generated from the diazoketo groups.

water was incapable of breaking the hydrogen bonding between carboxylic acids and ethylene oxide segments in the polymer chains. However, in PBS buffer solutions the solubility and dissolution rate changes between weakly acidic to weakly basic conditions. In general, more acidic buffers beget less soluble polymer films and vice versa. DMP-1 was sparingly soluble in the weakly acidic PBS solution in the pH range between 6.0 and 6.4 before UV irradiation. However, its solubility increased with increasing pH above 6.4. After UV irradiation, DMP-1 was readily soluble in the pH range between 6.0 and 7.9, whereas its dissolution rate was slower when the pH was less than 6.0. This increasing solubility in weakly acidic conditions is because the carboxylic groups that give rise to strong hydrogen bonds, inhibiting dissolution of DMP-1, are protonated. The hydrogen bonds are disrupted with increasing pH of the PBS solution, resulting in complete dissolution. Both DMP-2 and DMP-3, which contain more MAA than DMP-1, were soluble in the PBS solution in the pH range between 6.0 and 6.4. This can be attributed to the increased hydrophilicity due to the abundant carboxylic acids in the polymers. Furthermore, the higher ionic strength of the PBS buffer solution relative to that of deionized water (due to the presence of salts) may also give solubility in the mild acidic range by disrupting the hydrogen bonding. However, it is noteworthy that it still requires nearly 1 h or more for these polymers to be dissolved in this pH range. In pH 6.8 PBS solution, DMP-2 and DMP-3 were readily dissolved in less than 10 min, which can be explained by the easier disruption of hydrogen bonding compared to when pH 6.4 PBS buffer solution is used. After UV exposure, additional carboxylic acid groups were generated in the polymer due to the photorearrangement of the diazoketo groups, which led to the increased hydrophilicity (Figure 3). The excess carboxylic acid groups, which did not hydrogen bond with ethylene oxide segments, exist as free acids or weakly hydrogen-bonded dimers. Therefore, they could be hydrated in the weakly acidic PBS solution, leading to complete dissolution.[52−55]

Only DMP-1 showed controlled dissolution switching behavior as a function of pH between 6 and 7.9. Therefore, this polymer was used as a photoresist material for the patterning experiments. From the solubility measurement study (Table 2), pH 6.4 PBS buffer solution was found to offer excellent solubility switching control, and therefore this solution was used for photosensitivity and patterning studies.

Photosensitivity and Lithographic Evaluation of DMP-1. The photosensitivity of the DMP-1 film was studied by measuring the remaining film thickness after UV exposure and development steps. For development, pH 6.4 and pH 7.9 PBS buffer solutions were used as the weak acid and weak base developers, and the development time was fixed as 2 min. As shown in Figure 4, a sharp contrast in the solubility behavior of the DMP-1 film was observed when pH 6.4 PBS buffer solution was used as the developer, and the required exposure dose was found to be >40 mJ cm^{-2}.

Lithographic evaluation was performed using a thin film of DMP-1, which was spin-coated on a silicon wafer and soft-

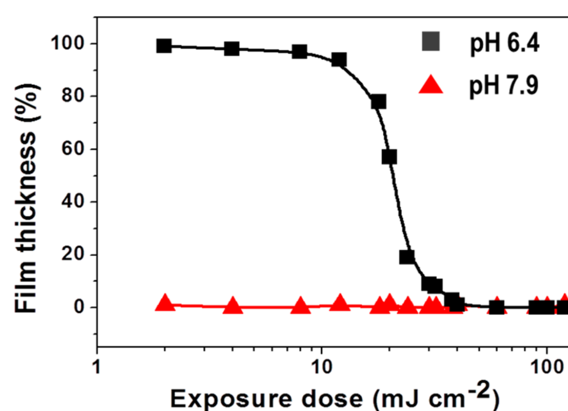

Figure 4. Normalized plot of exposure dose versus film thickness for DMP-1 (about 0.25 μm thickness).

baked. This film was exposed at a dose of 50 mJ cm^{-2} in the deep UV wavelength region through a photomask, followed by development in the PBS (pH 6.4) solution for less than 5 min to remove the film in the exposed region. When an UV-exposed circle-patterning DMP-1 sample was immersed in deionized water, the pattern image was not exhibited (Supplementary Figure S2a). However, after moving and immersing it in pH 6.4 PBS buffer solution, the 50 μm circle patterns appeared (Supplementary Figure S2b), and then the patterned image was made to disappear by dipping in pH 7.9 PBS buffer solution (Supplementary Figure S2c). The UV-exposed region is well developed in subacid condition and the UV-unexposed region in basic condition. The well-defined circle and stripe patterns obtained with DMP-1 were shown in Figure S2b and S2d as microscope images and Figure 5 as SEM images. The second

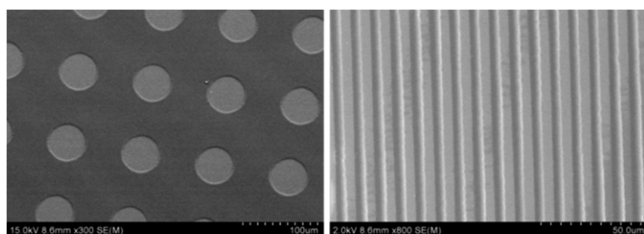

Figure 5. SEM images of 50 μm circle and 5 μm line features obtained with DMP-1 through one photolithographic process.

UV exposure was also carried out with the patterned film, with the photomask placed in an orthogonal fashion to the stripe patterns formed in the first UV exposure process (Figure 7a). The second UV exposure proceeded by development in the PBS (pH 6.4) solution showed the potential of this photoresist for multicomponent biomolecular patterning. Figure 6 shows

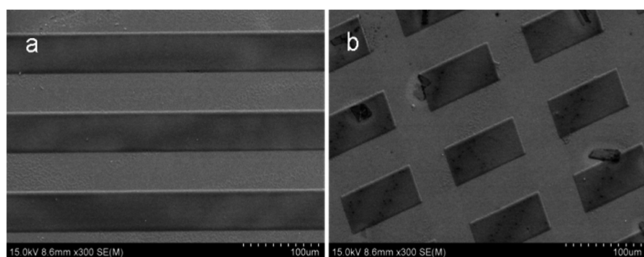

Figure 6. SEM images obtained from the double UV exposure process using DMP-1: (a) after first photolithographic process, 50 μm/50 μm line and space features and (b) after second photolithographic process, 50 μm × 100 μm rectangular patterns by 50 μm/100 μm line and space photomask that were placed perpendicular to the first pattern direction.

the SEM images of the patterns formed by the double UV exposure process. This photoresist has high photosensitivity with UV irradiation, 50 mJ cm^{-2}, and rapid switching solubility in a mild pH range at room temperature before and after UV irradiation. Also since the diazoketo-functionalized groups remain in the unexposed regions, it is possible to obtain complex patterns by a multiexposure process.

Dual Protein Patterning. To demonstrate the applicability of this novel photoresist for multicomponent biomolecular patterning, a dual streptavidin patterning was performed after UV exposure in order to prevent denaturation of biomolecules by deep UV (Figure 7b). A thin DMP-1 film on a biotin-functionalized glass slide was first patterned by UV exposure through a photomask according to the above-mentioned conditions. Patterned slides were then immersed in a PBS solution containing SAv-TRITC at pH 6.4 for 1 h at room temperature, with unexposed regions acting as a protective layer. The SAv-TRITC was found to be specifically immobilized on the UV-exposed regions due to the 'biotin–avidin' specific binding, which additionally proved that the photoresist in the exposed regions was completely dissolved, leaving the underlying biotin layer available for binding with streptavidin. The slides were then rinsed with a pH 7.9 PBS solution to remove the remaining photoresist. The SAv-TRITC immobilized slides were subsequently immersed in a PBS solution containing SAv-FITC at pH 7.4 for 1 h at room temperature. The SAv-FITC was found to be immobilized to the masked regions in the first exposure step. Figure 8 and Supplementary Figures S3 and S4 show the confocal fluorescence images of the different width dual protein pattern images obtained by this procedure. In the images, the noticeable fluorescence contrasts of the SAv-TRITCs were observed, while the SAv-FITCs' contrasts were less due to the nonspecific adsorption of the SAv-FITCs onto the first immobilized region. However, this procedure has proven the potential of the photoresist system for multicomponent protein patterning.

CONCLUSIONS

A photosensitive and pH-responsive non-CAR was designed and synthesized in this study. As our photoresist system does not release any potentially detrimental byproducts and shows good photosensitivity and pH-switching ability, this system is highly suitable for biological applications. Due to the diazoketo functional group's high photosensitivity, the photoresist generated carboxylic acid groups by only 50 mJ cm^{-2} deep UV irradiation, compared with others (more than 2000 mJ cm^{-2} UV irradiation for the nitrobenzyl-type photoresist and less photobleaching effect of the novolac resist in deep UV). Also, unlike the bioapplicable photoresists soluble in an aqueous solution after light exposure,[36] the solubility of the photoresist was dependent on UV exposure in a local region of pH 6 to 7.9. Hydrogen bonds between carboxylic acids and ethylene glycol segments present in the photoresist enabled us to tune the solubility in mild pH conditions. DMP-1, which was not well dissolved in the PBS solution with pH between 6.0 and 6.4, became readily soluble in the same pH range after UV exposure. It was found that hydrogen bonding prevented dissolution and removal in water regardless of UV exposure. Using this photoresist, dual protein patterning was successfully demonstrated. A double exposure lithographic evaluation of the photoresist additionally showed the potential of this photoresist for multicomponent biomolecular patterning.[32−37] Due to the conversion of diazoketo functional groups into carboxylic acid groups in a short time UV exposure (50 mJ cm^{-2}), it may be possible to apply multibiopatterning by multiple UV exposure if biomolecules that are less UV-sensitive over short exposure time are used.

Additional works are underway to fabricate prototype biosensors using this method for the detection of multiple target biomolecules using DNA hybridization and antigen/antibody interaction.

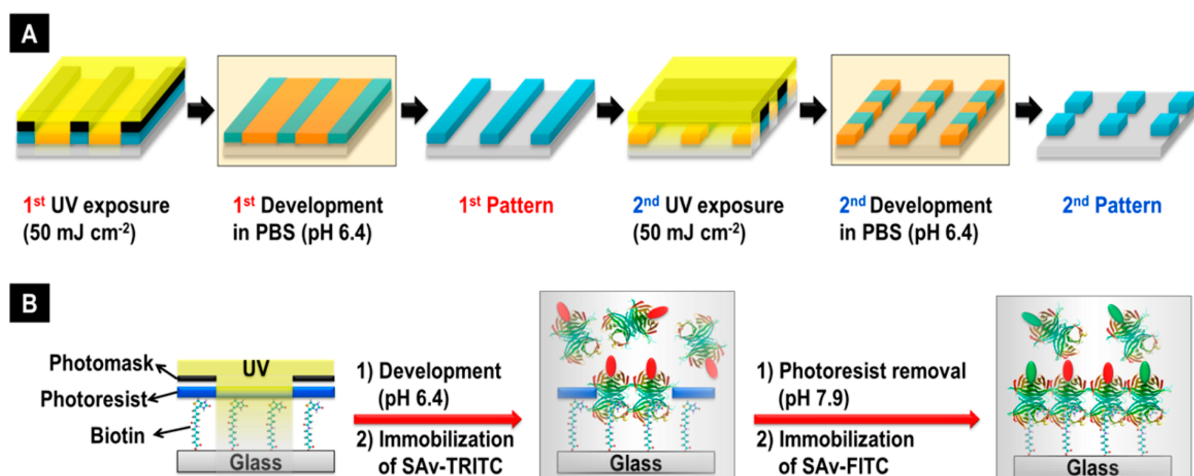

Figure 7. Schematic representation of (a) the double UV exposure process and (b) dual protein patterning used in this study.

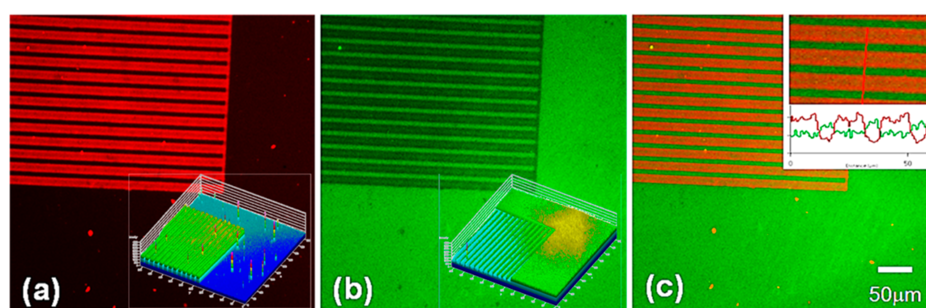

Figure 8. Fluorescence micrographs of the dual streptavidin patterned surface: (a) first immobilization of SAv-TRITC, (b) sequential immobilization of SAv-FITC, and (c) overlay. Insets show the 3D intensity images for TRITC (a) and FITC (b) and the intensity profile of the dual pattern (c).

■ ASSOCIATED CONTENT

Supporting Information

Proton NMR spectra for each synthesized polymer, microscopic images for each double patterning process, confocal images for dual protein patterning, and PBS preparation table. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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