Pattern Generation of Biological Ligands on a Biodegradable Poly(glycolic acid) Film

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The micropatterns of biological ligands (biotin and RGD peptides) were generated on a flat surface of biodegradable polymer, poly(glycolic acid) (PGA). The immobilization of biological ligands onto the surface of biodegradable polymers (especially aliphatic polyesters) is usually hampered by the absence of functionalizable groups on the polymer backbone. We demonstrate herein that PGA polymer films were modified by surface hydrolysis to introduce carboxylic acid groups on the film surfaces, which were subsequently used for patterning amine-terminated ligands by microcontact printing. Fluorescence microscopy was used to verify the pattern of biotin on the surface of the PGA films after complexation with fluorescein-conjugated streptavidin. In addition, the cellular micropatterns were obtained from micropatterns of RGD peptides on the surface-hydrolyzed PGA films.

Introduction

Micropatterns of biological molecules (e.g., biotin, DNAs, saccharides, peptides, and proteins) and cells on solid substrates have been one of the intensively studied topics due to potential applications in biosensors,[1−5] modulation of cell−substrate interactions,[6−8] neuroelectronics,[9] high-throughput drug screening,[10] and microarrays.[11,12] Among the methods for generating two-dimensional patterns on solid substrates, a soft lithographic technique called microcontact printing (μCP) has intensively been used to generate patterns of self-assembled monolayers (SAMs) and been applied to the pattern generation of biomolecules and cells based on the patterns of the SAMs.[13−16] Although the technique of μCP is relatively simple to perform in ordinary chemistry and biology research laboratories and does not require any special equipments or apparatus, its use has largely been restricted to the SAMs on gold, silver, or silicon oxide.[17]

Biomedical devices are mostly manufactured from polymers and metals. For these materials, the main limitation is the lack of sufficient functional groups on the surfaces for surface engineering. Treatment with high-energy sources including plasma, laser, or ion beam has been used to generate functionalizable groups on the surfaces.[18−20] Recently, several research groups reported the application of μCP to polymeric surfaces for the pattern generation of biological molecules and cells.[21−24] Chilkoti and collaborators patterned biotin onto various polymeric surfaces including poly(ethylene terephthalate) (PET), poly(ethylene) (PE), polystyrene (PS), and poly(methyl methacrylate) (PMMA). They functionalized the polymer surfaces chemically, presented pentafluorophenyl (or *N*-hydroxysuccinimidyl) ester groups (which are reactive to primary amine groups) on the surfaces, and performed μCP of amine-terminated biotin onto the activated surfaces.[25] Another approach was also developed to overcome the substrate limitation of μCP. Utilizing chemical vapor deposition (CVD) polymerization, Langer and collaborators first coated solid substrates with functionalized

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poly(*p*-xylylene)s (parylenes) the backbone of which contained chemically reactive functional groups such as pentafluorophenyl esters and carboxylic anhydrides, and patterned the polymer surfaces with endothelial cells by sequential self-assembly of streptavidin, biotin-conjugated anti-α_5-integrin, and endothelial cells.[26,27]

In this study, we patterned amine-terminated biological ligands (biotin and RGD peptides) on the surface of a biodegradable polymer, poly(glycolic acid) (PGA). Synthetic aliphatic polyesters of poly(α-hydroxy acids), such as poly(lactic acid) (PLA), PGA, and copolymers of lactic and glycolic acids (PLGAs), have widely been used for drug delivery systems and tissue engineering.[28,29] In the fabrication of biomaterials, it is an important technical challenge to develop techniques for engineering biodegradable polymer surfaces in two and three dimensions.[21] The surface engineering includes functionalization and control over chemical, physical, and biological properties of biodegradable polymers. For example, attaching cell-recognizing ligands onto polymer surfaces would promote cell adhesive property of the polymer, which is the most important factor in tissue engineering. The previous reports on the pattern generation of biomolecules and cells dealt with polymers that are not directly relevant to the biomedical applications,[22−27] and this report is the first attempt for generating patterns on the surface of a biomedically important biodegradable polymer, PGA.

Experimental Section

Poly(glycolic acid) (PGA, nuggets), pentafluorophenol (PFP), 1-ethyl-3-(dimethylamino)propylcarbodiimide (EDC), and 2-(2-aminoethoxy)ethanol (EG_2-amine) were purchased from Aldrich. Poly(ethylene glycol)amine (mPEG-amine, MW 5000) was purchased from Shearwater Co. (+)-Biotinyl-3,6,9-trioxaundecanediamine (biotin-amine) and fluorescein-conjugated streptavidin were purchased from Pierce. Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK) peptide (RGD peptide, FW = 715.7 amu) was purchased from American Peptide Company, Inc.

PGA nuggets (T_m = 225−230 °C) were placed between a silanized silicon wafer and an aluminum foil that had been heated at 230 °C for 1 min. The polymer sample was compressed (2 ton) for 1 min and allowed to cool to room temperature. The wafer and foil were carefully peeled off. The cleaned PGA films were immersed in 1 N NaOH solution at room temperature for a few seconds in order to hydrolyze ester groups only at the surface of PGA films. The surface of the hydrolyzed PGA films was activated by immersing the films into an ethanol solution of EDC (0.1 M) and PFP (0.2 M) for 30 min. The PFP-activated PGA films were then rinsed with ethanol, dried under a stream of argon, and used immediately thereafter.

PDMS stamps were prepared according to the literature method using Sylgard 184 silicone elastomer (Dow Corning).[30] Before its use, the PDMS stamp was oxidized by an oxygen plasma cleaner (Harrick PDC-002, medium setting) for 1 min. After inking, the amine-terminated biotin ligand (biotin-amine, 10 mM in ethanol) was printed by contacting the PDMS stamp onto the PFP-activated PGA films for 60 s. The sample was then immersed immediately in a solution of EG_2-amine (10 mM, 0.1 M sodium bicarbonate) for 30 min and rinsed with distilled water. After the pattern generation of biotin, the samples were immersed in a solution of fluorescein-conjugated streptavidin (0.1 mg/mL) in phosphate-buffered saline (PBS, pH 7.4) containing 0.1% (w/v) BSA and 0.02% (v/v) Tween 20 at room temperature. After 60 min, the samples were removed, washed several times with PBS and distilled water, and vacuum-dried. Fluorescence confocal microscopy was used to examine the streptavidin-bound PGA films (λ_{ex} = 488 nm, λ_{em} = 520 nm).

For the pattern generation of RGD peptides on the PGA films, the PDMS stamp was inked with mPEG-amine (1 mg/mL in distilled water). The inked stamp was brought into contact with the PFP-activated PGA film for a few seconds. The sample was immersed immediately in a solution of RGD peptides (1 mg/mL in PBS) for 60 min. The sample was cleaned with PBS and rinsed with distilled water.

Human epidermoid carcinoma A431 cells were purchased from American Tissue Type Collection and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. Cells were seeded on the RGD peptide-patterned PGA films at a density of 1×10^5 cells/mL. After 1 h, loosely adhered cells were gently rinsed with cell culture media and attached cells were cultured for 24 h. The cells were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature, and cell nuclei were stained with blue-fluorescent Hoechst 33342. Immunofluorescent images were recorded on the fluorescence microscope (λ_{ex} = 364 nm, λ_{em} = 460 nm).

X-ray photoelectron spectroscopy (XPS) study was performed with a VG-Scientific ESCALAB 250 spectrometer (UK) equipped with an Al Kα X-ray source. Tapping-mode atomic force microscopy (AFM) images of intact PGA and surface-hydrolyzed PGA films were obtained on a MultiMode SPM (Digital Instruments). Fluorescence images were acquired on an LMS 510 laser scanning confocal microscope (Carl Zeiss, Germany).

Results and Discussion

We patterned biological ligands (biotin and RGD peptides) on a surface of biodegradable PGA films via microcontact printing (μCP) (Figure 1). Synthetic aliphatic polyesters (e.g., PGA) have widely been used for drug delivery systems and tissue engineering as temporary scaffolds and as materials for surgery since the introduction of surgical suture fabricated from PGA.[28,29] The immobilization of biological ligands onto the surface of biodegradable polymers (especially, aliphatic polyesters) is hampered by the absence of functionalizable groups on the polymer backbone.[31,32] We demonstrate herein that PGA polymer films were activated by surface hydrolysis leading to the introduction of carboxylic acid groups on their surface, which were subsequently used for patterning amine-terminated ligands through an amide bond formation between amines (ligands) and carboxylic acid groups.

PGA is a highly crystalline and linear aliphatic polyester. It has a high melting point (T_m = 225−230 °C) and a very low solubility in most common organic solvents with an exception of hexafluoro-2-propanol. The PGA films were prepared by melt-casting PGA nuggets between the silicon wafer and aluminum foil.[31] Before casting the PGA film, the silicon wafer was treated with (tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane (United Chemical Technologies, Inc.) for 1 h under vacuum at room temperature to functionalize the surface with a fluorocarbon. This functionalization aided in release of the PGA film from the silicon wafer.

For the generation of carboxylic acid groups on the surface of PGA films, we used an aqueous solution of 1 N NaOH.[31,32] The degradation of PGA polymer involves chain scissions of ester bond linkages in the polymer backbone by hydrolytic attack of water molecules. The cleavage of

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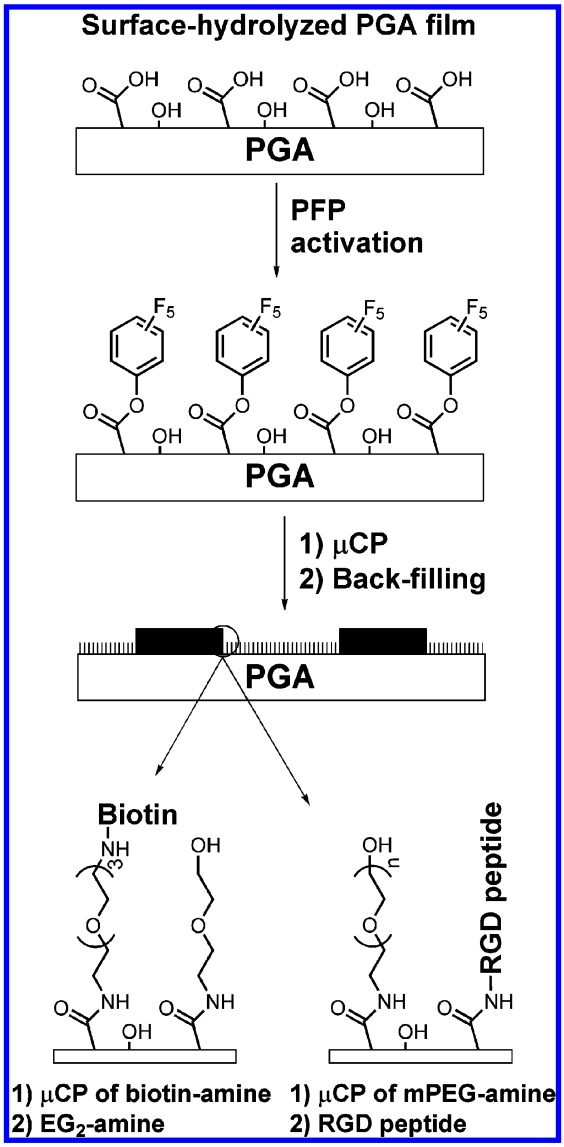

Figure 1. Schematic representation of patterning biological ligands onto the surface of biodegradable PGA films.

the ester bonds of the polymer results in the generation of carboxylic acid and hydroxyl groups at the newly exposed chain ends.[33] The hydroxide anion acts as a catalyst and cleaves the ester bonds of the polymer main chain more effectively than water, leading to surface hydrolysis.[31] The surface-exposed carboxylic acid and hydroxyl groups could be used for further functionalizations of the polymer surfaces.[32] In this study, we utilized an amide bond formation between the carboxylic acid groups (on the surface-hydrolyzed PGA films) and amine-terminated ligands (biotin and RGD peptides).

To achieve the covalent attachment of amine-terminated molecules onto the surface-hydrolyzed PGA films, we used a method of common reactive intermediates, pentafluorophenol (PFP) esters (Figure 1).[25−27] The surface-exposed carboxylic acid groups on the PGA films were converted to reactive PFP ester groups using EDC and PFP. The PFP ester is easily coupled with amines, leading to amide bonds. Molecules presenting a primary amine can, therefore, be covalently attached to the surface via the amide bond formation between the amine and the carboxylic acid groups (on the surface-hydrolyzed PGA films).

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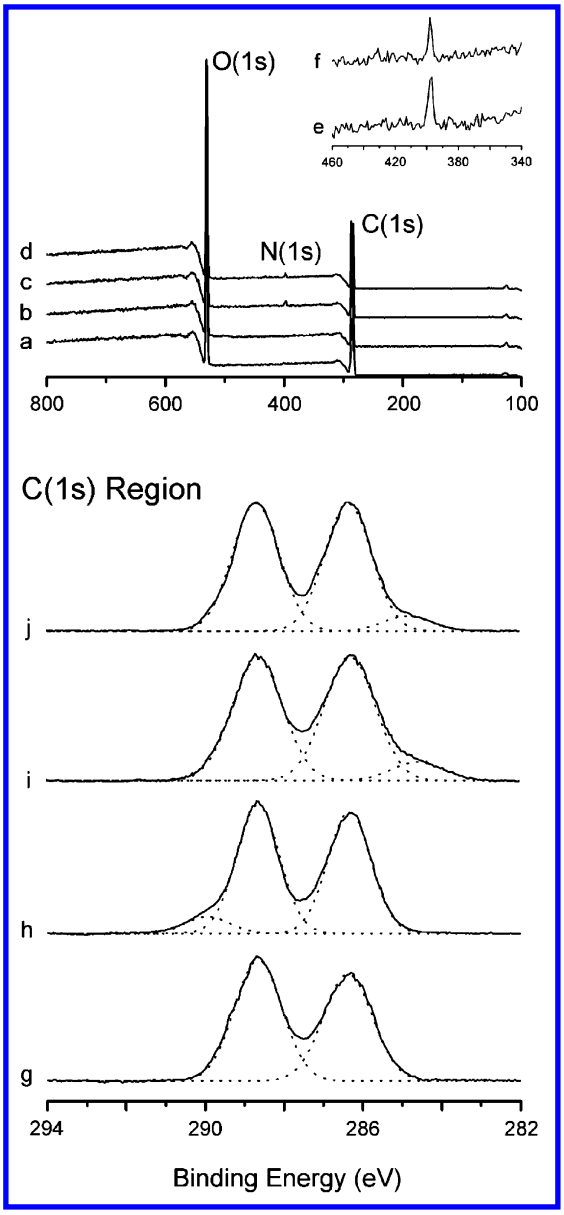

Figure 2. Wide-scan XPS spectra of (a) intact PGA, (b) surface-hydrolyzed PGA, (c) biotin-attached PGA, and (d) RGD peptide-attached PGA films. (e and f) Magnified XPS spectra of N_{1s} region of c and d. C_{1s} region of XPS spectra of (g) intact PGA, (h) surface-hydrolyzed PGA, (i) biotin-attached PGA, and (j) RGD peptide-attached PGA films.

To confirm the feasibility of the amide bond formation on a *degradable* polymer, biotin and RGD peptides were coupled with PFP-activated PGA films in solution. X-ray photoelectron spectroscopy (XPS) was used to monitor the amide bond formation between the carboxylic acid groups (on the surface-hydrolyzed PGA films) and amine-terminated ligands (biotin and RGD peptides) (Figure 2). For intact PGA films, the surface was composed of 54% carbon and 46% oxygen (Figure 2a). After the surface hydrolysis for 30 s, the surface elemental composition changed to 51% carbon and 49% oxygen, which is a similar result to the previous work (Figure 2b).[31] The change of surface elemental composition between the intact PGA and surface-hydrolyzed PGA films is consistent with the oxygen insertion from water (and hydroxide ion) by nucleophilic attack of water molecules to form hydroxyl and carboxylic acid groups. A relative increase in the composition of oxygen is expected as a result of hydrolytic degradation. The presence of biotin and RGD peptides on

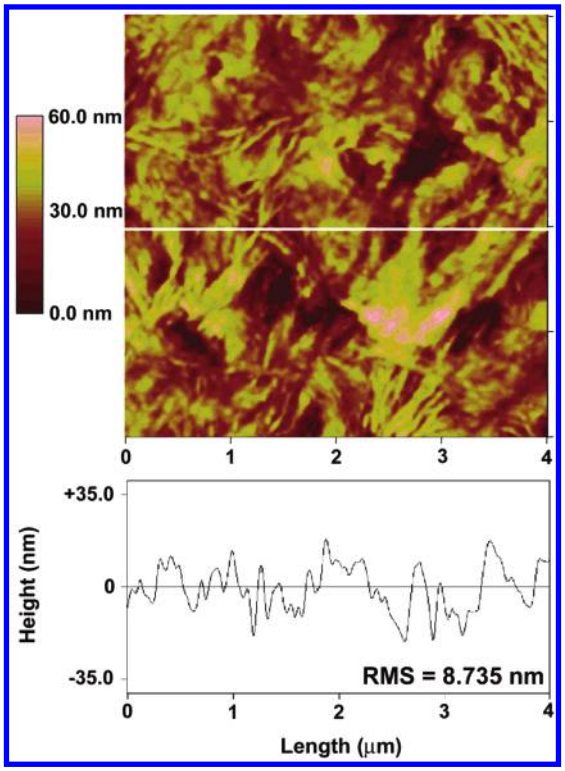

Figure 3. AFM micrograph of 5-s hydrolyzed PGA film.

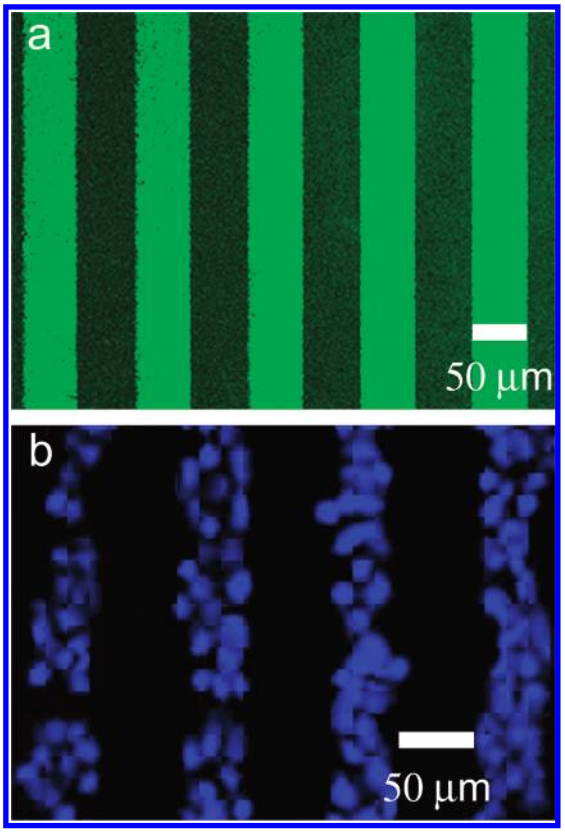

Figure 4. (a) Fluorescence micrograph of fluorescein-conjugated streptavidin bound to micropatterns of biotin on the surface of PGA films. (b) Spatially controlled adhesion and spreading of A431 cells on lines containing RGD peptides on the surface of PGA films recorded by fluorescence microscopy (cell nuclei were stained with blue-fluorescent Hoechst 33342).

the surface of PGA films was confirmed by the detection of elemental nitrogen at 399.5 eV (N_{1s}), which was introduced from biotin and RGD peptides (spectra c and d of Figure 2, respectively; spectra e and f of Figure 2 show magnified spectra of the N_{1s} region). Spectra g−j of Figure 2 show the high-resolution XPS spectra of the C_{1s} region of intact PGA, surface-hydrolyzed PGA, biotin-attached PGA, and RGD peptide-attached PGA films, respectively. The spectrum of the intact PGA film mainly consisted of a peak from C−O (at 286.4) and a peak from C═O (at 288.6), and a peak from −COOH appeared at 289.8 after the hydrolysis. We observed a peak at 284.5 eV (from C−C and C−H) after the coupling with either biotin-amine or RGD peptide but did not observe a peak from C−N. Our previous result[32] and the XPS data indicated that μCP onto the PFP-activated PGA film would be feasible.

To investigate the surface uniformity of the PGA films used, the surface morphologies were imaged by tapping-mode atomic force microscopy (AFM) in air. Figure 3 shows the morphology of PGA films after a 5-s hydrolysis in 1 N NaOH. The root-mean-square (rms) of the intact PGA film (that is, the PGA film before the hydrolysis) was measured to be 1.6 nm by AFM, and after the 5-s hydrolysis the rms of the hydrolyzed PGA film was increased to 8.7 nm. The roughness increased as the hydrolysis time was increased: for example, the 30-s hydrolysis yielded an rms value of 40 nm. We attempted to perform μCP onto PGA films with different roughnesses and selected the 5-s hydrolysis for generating reproducible patterns of ligands. Using a longer hydrolysis time, we were not able to generate micropatterns reproducibly. We also performed a control experiment to get an insight into the effect of the surface roughness. Hydrolyzed PGA films with different roughnesses were activated with pentafluorophenol, and the resulting films were reacted with biotin-amine in solution. Fluorescence microscopy showed a bright fluorescence on the films after a complexation with fluorescein-conjugated streptavidin. The result suggests that the successful pattern generation was determined at the μCP step.

Pattern generation of biotin on the PGA film was achieved by μCP of biotin-amine ((+)-biotinyl-3,6,9-trioxaundecanediamine) with a PDMS stamp that had relief features with lateral dimensions of 50 μm (width of line). After patterning the biotin-amine on the PFP-activated PGA films and passivating the films with $(EG)_2$-amine, the samples were incubated in a PBS solution of fluorescein-conjugated streptavidin containing bovine serum albumin (BSA) and Tween 20. BSA and Tween 20 were used to reduce nonspecific adsorption of streptavidin. Successful patterning was verified by fluorescence microscopy (Figure 4a). Streptavidin has two pairs of binding sites on opposite sides. One pair was used to the complexation with the pattern of biotin on the PGA film, leaving the other pair on the opposite side, which would be a useful binding site for further complexation of desired biomolecules.[27]

Tripeptide RGD (Arg-Gly-Asp) is known as an active sequence of adhesive proteins of the extracellular matrix (ECM) that binds to integrin receptors.[34] To promote cellular adhesion, RGD-containing peptides were covalently immobilized onto biomaterials.[35] We used RGD peptides, Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK), to study a spatially directed cell attachment on the surface of a biodegradable PGA. Micropatterns of RGD peptides on the surface of PGA films were prepared by (1) μCP of amine-terminated poly(ethylene glycol) (mPEG-amine) as

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a cell repellent[36,37] and (2) immobilization of RGD peptides containing lysine, which was used for covalent attachment of the peptides on the PGA films through the amide bond formation. It was not successful to generate patterns of RGD peptides by direct μCP of the RGD peptides onto the PFP-activated PGA films under various conditions.[38] Figure 4b shows patterns of A431 cells after the deposition, where cells did not attach to areas of the mPEG-amine-stamped regions (lateral dimensions of 50 μm (width of line) separated by 50 μm). The cells are confined only in the areas presenting RGD peptides. Little loss of the feature resolution and intensity was detected at the edges. In contrast, when cells were seeded onto mPEG-amine-coated PGA films all the cells were detached from the substrate.

Conclusions

In summary, we demonstrated a pattern generation of biological ligands—biotin and RGD peptides—and cells on the surface of biodegradable poly(glycolic acid). This approach involves (1) activation of the biodegradable polymer surface by surface hydrolysis to introduce reactive groups (such as carboxylic acid groups) and (2) reactive microcontact printing of biological ligands through the amide bond formation between the carboxylic acid groups and amine-terminated ligands. Considering the wide use of synthetic biodegradable aliphatic polyesters in the biomedical areas and the need of tailoring properties of the polymers, the method reported herein has a great potential because it is simple in the direct patterning of biological ligands onto the surface of biodegradable polymers and could potentially be applicable to a wide variety of biodegradable polymers that are amenable to surface hydrolysis. In addition, micropatterns of cells on the surface of biodegradable polymers will give an opportunity to study short- and long-term metabolism of cells attached onto biomedically relevant polymer surfaces.

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(38) We performed all the possible permutations of μCP and back-filling. We observed the same contrast in fluorescence under confocal microscopy when we generated a biotin pattern by μCP of mPEG-amine and back-filling with biotin-amine. However, we did not obtain a reproducible pattern of A431 cells when we used $(EG)_2$-amine instead of mPEG-amine.