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Quantitative Photochemical Immobilization of Biomolecules on Planar and Corrugated Substrates: A Versatile Strategy for Creating Functional Biointerfaces

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Abstract

Methods for the generation of substrates presenting biomolecules in a spatially controlled manner are enabling tools for applications in biosensor systems, microarray technologies, fundamental biological studies and biointerface science. We have implemented a method to create biomolecular patterns by using light to control the direct covalent immobilization of biomolecules onto benzophenone-modified glass substrates. We have generated substrates presenting up to three different biomolecules patterned in sequence, and demonstrate biomolecular photopatterning on corrugated substrates. The chemistry of the underlying monolayer was optimized to incorporate poly(ethylene glycol) to enable adhesive cell adhesion onto patterned extracellular matrix proteins. Substrates were characterized with contact angle goniometry, AFM, and immunofluorescence microscopy. Importantly, radioimmunoassays were performed to quantify the site density of immobilized biomolecules on photopatterned substrates. Retention of function of photopatterned proteins was demonstrated both by native ligand recognition and cell adhesion to photopatterned substrates, revealing that substrates generated with this method are suitable for probing specific cell receptor-ligand interactions. This molecularly general photochemical patterning method is an enabling tool that will allow the creation of substrates presenting both biochemical and topographical variation, which is an important feature of many native biointerfaces.

Introduction

Surface chemical approaches to the generation of substrates presenting relevant biomolecules in a controlled manner hold enormous promise for advances in research areas including biointerface science,^{1,2} microarray technology,^{3,4} biosensors,^{5–9} microfluidics and point-of-care applications,¹⁰ and nanotechnology.¹¹ More specifically, biomolecular patterning tools have been rapidly developing due to a growing interest in exercising spatio-temporal control over protein patterns,¹² characterizing immobilized protein site density for quantitative biointerface studies,^{13,14} and generating substrates presenting multiple ligands to model complex physiological environments.^{15–20} Ideally, patterning approaches will be simple, molecularly general, quantifiable, and easily extendable to create substrates with higher levels of biomolecular complexity.

Our approach involves the use of light as a reagent, allowing facile control over the spatial distribution and surface density of immobilized biomolecules. Previous reports show biomolecular patterning strategies based upon either a combination of photolithographic patterning followed by bioconjugation^{21–27} or direct photochemical activation of the

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surface.^{28–32} Others have generated photopatterned biomolecular surfaces by using fluorescently labeled small molecules³¹ or photochemically labeled peptides^{33,34} and proteins,³⁵ followed by excitation with the appropriate wavelength of light to achieve conjugation to the surface.

We chose to pursue an approach that incorporates the photocrosslinking agent onto the surface rather than to the biomolecules in order to avoid the possible formation of solution-phase aggregates, due to crosslinking of photoactive biomolecules in solution, and to minimize the risk of creating a multilayer of biomolecules on the surface. We preliminarily demonstrated that glass substrates functionalized to present the photocrosslinking molecule benzophenone (BP) could be utilized to directly immobilize proteins and carbohydrates onto the surface as governed by spatially controlled incident photon flux and solution phase biomolecule concentration.³⁶ BP-modified substrates were previously applied to the photoimmobilization of polymers^{37,38} and biomolecules.^{6,39–51}

Herein we present an important extension of this method of biomolecular photopatterning onto corrugated substrates and also for the first time demonstrate the quantitative nature of this immobilization strategy by quantifying protein deposition using radioimmunoassays. This ability accurately control the spatial density of biomolecules on solid support is of critical importance for quantitative studies on cell-substrate interactions. We also demonstrate that photoimmobilized whole proteins (not peptides) are readily recognized by native ligands present both in solution and in the form of cell surface receptors. Furthermore, by optimizing the underlying surface chemistry to incorporate the non-fouling linker poly(ethylene glycol), we engender sufficient biofouling resistance to allow patterning of adhesive cells on a representative native extracellular matrix protein, fibronectin.

To generate biomolecular patterns, substrates are immersed in a solution containing the biomolecule of interest, exposed to light through a photomask (350–365 nm), resulting in the direct, covalent attachment of biomolecules to the surface in a spatially controlled manner (Scheme 1). When illuminated with UV light, BP undergoes an $n \rightarrow \pi^*$ molecular transition, generating a triplet diradical that can eventually form a new C-C bond between the BP-modified substrate and the molecule of interest via a proton abstraction/radical recombination mechanism.⁵² If an excited BP molecule does not abstract a proton from a neighboring molecule, it will relax back to the ground state, enabling re-excitation in the presence of a different biomolecule. In this manner, we demonstrated that BP-based photoimmobilization is a versatile technique for generating two-component gradients by sequential exposures in the presence of different biomolecule solutions.³⁶

Herein, we extend this methodology to create substrates that present up to three discrete biomolecules in distinct spatial patterns, and for the first time demonstrate, via a radioimmunoassay, that this approach affords quantitative control over the density of biomolecules deposited onto the underlying substrate. Subsequent ligand-binding assays and cell adhesion experiments showed that specific ligand recognition occurs and that cells adhere selectively to immobilized capture ligands, indicating that the photopatterning scheme does not render the covalently attached molecules biologically inactive. The incorporation poly(ethylene glycol) into the backbone of the underlying chemical monolayer enabled the extension of cell patterning from suspension cells to adhesive cells, which is a significant advance given that they are much more prone to non-specific binding on account of their ability to secrete extracellular matrix proteins. Furthermore, we extend this photochemical immobilization scheme to the attachment of biomolecules onto corrugated surfaces, which are not typically compatible with contact- or flow-based patterning schemes since conformal contact and uniform fluid flow are difficult to achieve on corrugated surfaces. Together with the molecular generality afforded by the C–H bond insertion

mechanism, we feel that advantages of this direct photochemical attachment via BP, such as quantitative deposition, substrate versatility, retention of protein functionality, compatibility with multiple cell types, and ability to deposit multiple biomolecules onto the same surface, make this an attractive methodology for a wide range of biomaterials research applications that rely upon precisely defined biomolecular interfaces.

Experimental

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received, unless otherwise noted.

Chemical Functionalization: BP-Modified Substrates

Preparation of BP-modified glass substrates has been previously reported.³⁶ Briefly, glass microscope slides (Fisher Scientific, Philadelphia, PA) were cleaned with Piranha solution (4:1 (v:v) concentrated H₂SO₄ : 30% H₂O₂).⁵³ Substrates were rinsed extensively with water (ELGA LabWater Reservoir, Veolia Water Systems, Buckinghamshire, UK), absolute ethanol (Decon Laboratories, King of Prussia, PA), and dried under a stream of nitrogen. Slides were baked in an oven at 120 °C for 1 hr, cooled to room temperature, and positioned upright along the wall of a vacuum dessicator, with 100 µL of 3-(triethoxysilyl)butyl aldehyde (Gelest, Morrisville, PA) placed in the center of the chamber. Vacuum was applied to the sealed chamber and vacuum deposition of silane onto the glass slides was allowed to occur for 2.5 hrs. Slides were then cured at 120 °C for 1 hr, soaked in absolute ethanol for 30 min, rinsed with absolute ethanol, and dried under a stream of nitrogen. Successful silanization was confirmed via measurement of water contact angles (Rame~Hart Goniometer, Netcong, NJ).

Slides were then incubated in the dark for 4 hrs at room temperature in the presence of 20 mM 4-benzoyl benzylamine hydrochloride (Matrix Scientific, Columbia, SC) and 200 mM NaCNBH₃ in a solution of 4:1 DMF:MeOH, followed by immersion in aldehyde-blocking buffer (0.1 M Tris, 200 mM ethanolamine, pH 7.0) for 1 hr at room temperature. Slides were rinsed thoroughly with water, DMF, methanol, and ethanol, and then dried under a stream of nitrogen. The resulting BP-modified substrates were stored in a dessicator in the dark until further use.

Chemical Functionalization: BP-Modified Substrates Incorporating Poly(ethylene glycol)

Glass microscope slides were cleaned with Piranha solution, rinsed, and silanized with 3-(triethoxysilyl)butyl aldehyde as described above.

Slides were then incubated for 4 hrs at room temperature in the presence of 10 mM H₂N-PEG-CM (1000 MW, Laysan Bio, Inc., Arab, AL) and 100 mM NaCNBH₃ in PBS (pH 7.4) followed by immersion in aldehyde-blocking buffer for 1 hr at room temperature. Slides were rinsed thoroughly with water, methanol, and ethanol, and dried under a stream of nitrogen.

Slides were then incubated in the dark for 4 hrs at room temperature in the presence of 20 mM 4-benzoyl benzylamine, 75 mM *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC), 30 mM *N*-hydroxysuccinimide (NHS) in PBS (pH 7.4) followed by immersion in NHS-quenching buffer (0.1 M Tris, 100 mM ethanolamine, pH 8.5) for 1 hr at room temperature. Slides were rinsed in water, methanol, and ethanol, dried under a stream of nitrogen. The resulting BP-modified substrates were stored under in a dessicator in the dark until further use.

Fabrication of Corrugated Substrates

Glass microscope slides were sonicated in acetone for 15 min and dried under a stream of nitrogen. Chromium metal was evaporated onto the glass substrates to a thickness of 150 nm using an electron beam evaporator (Temescal six pocket E-Beam Evaporation System, Commonwealth Scientific, Clayton South VIC, Australia). The glass substrates were then coated with approximately 2 μm of s1813 photoresist (MicroChem, Newton, MA) using a spin coater (PWM 32-PS-R790, Headway Research Inc., Garland, TX) at 2000 rpm for 60 sec and soft baked for 60 sec at 115 °C. The Cr-coated glass substrate was then exposed to UV light from a CF2000 UV LED array source (350–360 nm, Clearstone Technologies, Minneapolis, MN) through a photomask for 1 min at 35 mW/cm². The resulting photoresist pattern was developed using MF 319 developer (Rohm and Haas Electronic Materials, Marlborough, MA) and the exposed Cr was removed using a CR-9 etchant (Cyantek, Fremont, CA) for 2 min. The glass substrate with patterned Cr was then rinsed three times in purified water and dried under a stream of nitrogen. The back of the glass substrates were taped with PVC sealing tape (McMaster-Carr, Aurora, OH) to prevent etching, and the substrate was then immersed into a plastic container with glass etching solution (1 M HF: 0.5 M NH₄F: 0.75 M HNO₃) held at a constant temperature of 40 °C for 1 hr. The taped glass substrate was removed from the etching solution and immersed in purified water three times, rinsed in ethanol to strip the remaining photoresist and dried under a stream of nitrogen. The remaining Cr layer was removed using Cr etchant for 2 min as described above, rinsed in purified water and dried under a stream of nitrogen. The depths of the channels created were determined to be approximately 35 μm deep via profilometry (Sloan Detak3, Veeco, Plainview, NY). Corrugated glass substrates were cleaned, silanized and functionalized with BP as described for planar substrates.

Photoimmobilization of Proteins and Carbohydrates

The following biomolecules were used in photoimmobilization studies: biotinylated Concanavalin A (ConA-biotin), purchased from Vector Laboratories (Burlingame, CA); mannan isolated from *Saccharomyces cerevisiae*; recombinant human P-selectin (CHO cell-derived), purchased from R&D Systems (Minneapolis, MN); and fibronectin (FN), purchased from Invitrogen (Carlsbad, CA). Protein stock solutions were prepared by resuspending lyophilized protein in the recommended buffer solutions (1x PBS for proteins) to a concentration of 1 mg/mL. Aliquots were stored frozen at –20 °C until further dilutions in the respective buffers were freshly prepared to yield solutions of 5 $\mu\text{g}/\text{ml}$ for photopatterning applications. A solution of mannan was prepared by diluting lyophilized powder to a final concentration of 20 mg/ml in purified water for photopatterning applications.

A rectangular parallel-plate flow chamber (GlycoTech; Gaithersburg, MD) was assembled with a BP-modified glass substrate or a BP-PEG modified glass substrate separated by a silicone gasket of 127 μm thickness, 6 cm length, and 1 cm width. Vacuum was applied to hold together the substrate-chamber assembly. Appropriate connectors and tubing for the solution inlet and outlet were assembled, and a syringe pump (Harvard Apparatus, Holliston, MA) was utilized to pull biomolecule solutions through the chamber. Prior to irradiation with UV light, the biomolecule solution is flowed through the chamber at a rate of 3 mL/min. During UV irradiation biomolecular solution flow was stopped. The assembled device was positioned face-down (allowing for illumination through the back of the glass substrate) beneath the UV output ($\lambda = 351.1\text{--}363.8\text{ nm}$) of an Argon ion laser (Coherent Innova 90-4, Laser Innovations, Santa Paula, CA). The laser light was homogenized using refractive beam-shaping optics (π -Shaper, Molecular Technologies, Berlin, Germany) and expanded to give a uniform illumination plane. The laser power was adjusted to give a final illumination intensity of 14 mW/cm² at the substrate. For photopatterning of biomolecules, a Cr-coated

glass photomask having 100 μm stripe features separated by 400 μm , or a relief mask presenting 800 μm circles with 200 μm spacing, was placed onto the back of the substrate and irradiated for the following times: 5 min for ConA-biotin, 1 min for P-selectin, 30 sec for FN, and 2 min for mannan.

Following UV exposure, the chamber was immersed in a rinse solution and the substrate was separated from the flow chamber device. The following rinse solutions were utilized: 0.5% (v/v) Tween 20 in PBS buffer for substrates presenting ConA-biotin and fibronectin; 0.5 mg/mL sodium dodecyl sulfate (SDS) in Dulbecco's PBS for substrates presenting mannan; and 0.5% (v/v) Tween 20 and 1% (w/v) BSA in Dulbecco's PBS with Ca^{2+} and Mg^{2+} for substrates presenting P-selectin.

For generation of three-component patterns, irradiation with the first component was performed, then a low-pH Glycine solution (pH 2.2) was flowed through the device to remove non-specifically adsorbed biomolecules. After flowing through PBS to remove residual rinse solution components, the second biomolecule solution was introduced to the substrate, irradiated under the pre-determined conditions, followed by rinses with a low-pH Glycine solution and PBS, disassembly of the flow chamber device, and subsequent rinse/sonication steps. Photopatterned substrates were stored in 1% BSA/PBS solutions.

AFM Analysis of Surface Roughness

AFM measurements were performed using an MFP-3D microscope (Asylum Research, Santa Barbara, CA). Changes in the surface roughness of the substrates after each chemical treatment were monitored by obtaining $1 \times 1 \mu\text{m}$ topographic images in AC mode using a silicon probe with $<10 \text{ nm}$ tip radius and a cantilever nominal force constant of 40 N/m (BudgetSensors, Bulgaria). Roughness values were calculated from the root mean square of the height amplitudes after the images were corrected for sample tilt using the MFP-3D imaging analysis and statistical software.

Fluorescence Imaging of Photoimmobilized Biomolecules

After photopatterning, rinsing, and BSA-blocking, substrates were placed in a solution containing fluorescently labeled binding partners and incubated overnight in the dark at 4 $^{\circ}\text{C}$. Substrates presenting one-component patterns of ConA-biotin were incubated with a solution of 0.05 $\mu\text{g/mL}$ Alexa Fluor647-conjugated streptavidin (Invitrogen) in 1% BSA/PBS.

Substrates presenting P-selectin were incubated overnight in a solution containing: native ligand PSGL-1 (8 $\mu\text{g/mL}$, F_c chimera, R&D Systems), mouse anti-human IgG (4 $\mu\text{g/mL}$, Abnova), and AlexaFluor647-conjugated goat anti-mouse IgG (2 $\mu\text{g/mL}$, Invitrogen), in 1% BSA/PBS with Ca^{2+} and Mg^{2+} . Substrates were rinsed in PBS, water, dried under a stream of nitrogen, and imaged on a fluorescence slide scanner. The specific ligand-binding ability of P-selectin was demonstrated by the fact that incubation in the same solution lacking PSGL-1 results in baseline fluorescence levels in the resulting images (data not shown).

For three-component patterned substrates, three solutions of fluorescently labeled binding partners were made separately and then combined immediately before incubating with substrates. These solutions were: 0.1 $\mu\text{g/mL}$ ConA-Alexa Fluor 488 (Invitrogen) in 1% BSA/PBS for mannan recognition, a solution of 1 $\mu\text{g/mL}$ anti-P-selectin (R&D Systems) and 0.5 $\mu\text{g/mL}$ Alexa Fluor 647-anti-IgG (Invitrogen) in 1% BSA/PBS with Ca^{2+} and Mg^{2+} for P-selectin recognition, and a solution of 1 $\mu\text{g/mL}$ biotinylated anti-FN (Abcam, Cambridge, MA) and 0.5 $\mu\text{g/mL}$ Alexa Fluor 568-streptavidin (Invitrogen) for FN recognition. After incubation, substrates were rinsed twice with the appropriate buffer, once with water, and dried under a stream of nitrogen. Substrates were visualized with a laser

scanning confocal microscope (LSM 710, Carl Zeiss Microimaging, GmbH, Germany) and the resulting images analyzed with Imaris 7.0 software (Bitplane AG, Zurich, Switzerland).

Quantitative Determination of Immobilized Protein Density

To determine protein loading on BP-modified substrates under various photoimmobilization conditions, a radioimmunoassay using an [^{125}I]-labeled binding partner was performed to analyze photopatterned protein substrates. In an effort to establish a relationship between the site density from the radioimmunoassay and the fluorescence intensity (F.I.), identical substrates were created and analyzed in parallel using both fluorescence and radioimmunological methods, and the resultant data were correlated. A rectangular parallel-plate flow chamber was assembled with a BP-modified glass substrate (as described above). The assembled device was positioned face-down, allowing for illumination through the back of the substrate beneath the UV LED array source, which was configured to an output of power of 44 mW/cm². A total of 8 substrates were prepared for each of the following six irradiation time points in the presence of ConA-biotin (5 $\mu\text{g/mL}$): 5, 15, 30, 60, and 120 sec. BP-modified substrates that never contacted ConA-biotin were used as controls. Four of the eight replicates at each exposure time were analyzed for F.I., as described above. The remaining four replicates were subjected to a radioimmunoassay for site density quantitation.

[^{125}I]-labeled streptavidin was generated by a standard iodination technique. Streptavidin (10 mg/mL, Pierce, Rockford, IL) was placed into the bottom of tube pre-coated with Pierce Iodination Reagent (Pierce, Rockford, IL) along with ^{125}I (5 $\mu\text{Ci}/\mu\text{g}$, in 10^{-8} M NaOH, Perkin-Elmer, Waltham, MA), followed by incubation at room temperature for 10 min with shaking. The solution was then purified using a Bio-Spin 6 purification column (Bio-Rad, Hercules, CA). The percentage of excess ^{125}I was determined via thin layer chromatography on Whatman filter paper.⁵⁴ The concentration of material obtained after purification was determined using a Bradford assay. Slides presenting ConA-biotin were incubated with saturating concentrations of [^{125}I]-labeled SA (0.05 $\mu\text{g/mL}$) in 1% BSA/PBS for 1 hr, rinsed in PBS, purified water, and dried under a stream of nitrogen. Slides were cut into four pieces and each piece placed into a scintillation tube with 1 mL of scintillation fluid (ScintiSafe Econo 1, Fisher Scientific, Pittsburgh, PA). The radioactivity for all the sample tubes were determined using a scintillation counter (Beckman LS 6500 Liquid Scintillation Counter, Beckman Coulter, Brea, CA), in units of counts per min (CPM). The number of radioactive molecules bound per area (molecules/ μm^2) was then determined by comparison of the CPM obtained from the slides to a standard curve of [^{125}I]-labeled streptavidin standards of known concentration. Plots of F.I. vs. exposure time, and CPM vs. exposure time were correlated to establish a correlation between F.I. and the density of immobilized biomolecules.

Cell Culture and Adhesion Experiments

The HL-60 cell line (Human promyelocytic leukemia, American Type Culture Collection, Manassas, VA) was cultured in RPMI 1640 supplemented with penicillin (100 U/mL), streptomycin (100 $\mu\text{g/mL}$) and 10% fetal bovine serum (Cell Media Facility, UIUC). P-selectin was photopatterned on BP-modified substrates as described above. Substrates were rinsed and soaked overnight in PBS containing 0.2% Pluronic F127 and 1% BSA. Substrates were then rinsed three times in water and placed into a cell culture dish (100 mm $\times$ 20 mm, Corning, NY). Cells were fluorescently labeled using Alexa Fluor 488 carboxylic acid succinimidyl ester (Invitrogen) and seeded onto substrates in PBS at a concentration of 5×10^6 cells/mL and incubated at 4 $^{\circ}\text{C}$ for 2 hrs. The cell-containing solution was aspirated off and the substrates were rinsed in PBS. The resulting patterns of cells were visualized using a

fluorescence microscope (Leica DM 6000 Upright Microscope, Leica Microsystems, Bannockburn, IL).

The 3T3 Swiss Albino fibroblast cell line (ATCC) was cultured in Dulbecco's Modified Eagle Medium supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% fetal calf serum. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. Near confluent cultures were passaged by treatment with a solution of 0.05% Trypsin/0.53 mM EDTA (Invitrogen). FN was photopatterned onto BP-PEG-modified substrates as described above. Substrates were rinsed in PBS containing 0.2% Pluronic F-127 and 1% BSA and soaked overnight in the same solution. Substrates were rinsed three times in water and placed into a cell culture dish. Cells were seeded onto the substrates in serum-free media at a concentration of 2.5×10^6 cells/mL and incubated at 37 °C for 10 min. Cells that remained in the solution were aspirated off and the substrates were rinsed in PBS to reveal the resultant patterns. Slides were immersed in serum containing media overnight, rinsed, fixed in 3.2% paraformaldehyde, permeated in 1% Triton X-100 and rinsed in 1% BSA in PBS. Cell nuclei were stained with 1:1000 dilution of DAPI (Invitrogen) and cell actin was stained with a 1:500 dilution of rhodamine phalloidin (TRITC, Invitrogen) in 1% BSA with PBS. Slides were rinsed in PBS and a glass coverslip was affixed for cell imaging using a Fluorescence Apotome Microscope (Carl Zeiss Microimaging).

Results and Discussion

We have developed a straight-forward and versatile technique for generating photopatterned biomolecular substrates that are suitable for probing receptor-ligand interactions and for biointerface studies. Briefly, BP-modified substrates are assembled into a flow chamber and exposed to UV light in the presence of a biomolecular solution to generate patterns or gradients (Scheme 1). We demonstrate that this technique is compatible with both planar and corrugated substrates. The resultant patterns are visualized by recognition with a fluorescently labeled antibody, a native ligand, or by selective cell adhesion.

Characterization of BP-Modified Substrates

BP-modified substrates were prepared and characterized after each step by water contact angles (Table 1). Freshly piranha-cleaned substrates were extremely hydrophilic, giving an unmeasurable contact angles, consistent with the literature.⁵⁵ Following vapor phase silanization, the water contact angle increased to 44.8 (±1.9)°, indicating an increase in hydrophobicity due to the addition of the monolayer onto the glass substrate.^{20,56} Subsequent attachment of BP further increased the hydrophobicity of the surface, giving a water contact angle of 53.4 (±1.1)°. After protein conjugation to the BP-modified surface, a ~20° decrease in contact angle is observed.²³ To generate BP-PEG-modified substrates, aldehyde-functionalized substrates are reacted with a PEG linkers that has a terminal carboxylic acid moiety, resulting in a decrease in contact angle to 32.1 (±4.2)°.⁵⁷ Subsequent conjugation of BP to the PEG-modified substrates resulted in an increase in the contact angle to 41.8 (±6.1)°. Given the significant changes in the relative hydrophobicity measured during each step of this procedure, water contact angle measurements were routinely used to verify the success of each chemical modification step.

In order to confirm that the derivatization procedure did not lead to significant changes in substrate topography, we monitored the surface roughness following each chemical and biochemical functionalization step using atomic force microscopy (Table 1). Through the entire procedure of chemical attachment of BP and subsequent photoimmobilization of a representative protein, the root mean squared (rms) roughness of the substrates increased from 352 pm for the clean glass surface to 775 pm for the protein-modified substrate,

revealing only sub-nm changes in surface topography.⁵⁸ AFM images can be found in the Supplementary Information (SI).

Characterization of Photoimmobilized Biomolecular Patterns

We demonstrated the generation of photopatterned substrates presenting a single protein, ConA-biotin (Fig. 1a) visualized with its fluorescent binding partner. Exploiting BP and its ability to be re-excited in the presence of other biomolecules, we extended this approach to photoimmobilize three unique biomolecules: FN, P-selectin and mannan onto the same substrate (Fig. 1b). P-selectin and FN are proteins, whereas mannan is a carbohydrate (polymannose). These biomolecules were photoimmobilized via sequential exposures and incubated with a solution composed of their respective fluorescently labeled binding partners, revealing overlapping patterns of 100 μm stripes with 400 μm spacing.

Determining the site density of ligands as a function of UV exposure time allows substrates to be tailored to present specific concentrations of ligands for subsequent applications. Substrates were patterned to present uniform ConA-biotin, and the resulting F.I. data were plotted as a function of exposure time (Fig. 2a). Site density was quantified by incubating substrates with a saturating concentration of [¹²⁵I]-labeled streptavidin. Calibration plots were made using known concentrations of [¹²⁵I]-labeled streptavidin (see S.I.) and the resulting radioactivity (CPM) for each known concentration of [¹²⁵I]-labeled streptavidin was plotted as CPM vs. number of molecules (Fig. 2b). Using the calibration plot, and knowing the analysis area, the site density (molecules/ μm^2) was determined for each exposure time. Since fluorescence measurements are considerably more convenient to perform on substrates compared to radioimmunoassays, site density was plotted against the F.I. for each time point to establish a correlation to convert from fluorescence to loading density (Fig. 3). Increasing the exposure time results in higher densities of immobilized ligands on the substrate up to ~200 molecules/ μm^2 under conditions utilized in this study. Quantification of protein deposition is essential for future applications in biointerface science that require precise tuning and characterization of the underlying substrate.

Applications of BP photopatterning to corrugated substrates

Many applications of biomolecularly functionalized substrates require immobilization onto nonconformal surfaces,^{16,59,60} since topographical cues play a critically important role in influencing cell behavior *in vivo*.^{61,62} Since our photochemical patterning scheme does not require conformal physical contact with the substrate or precise control over fluid mixing, which would be disturbed by topography, we sought to extend the BP immobilization approach to corrugated surfaces. As a test case, we chemically etched a stripe pattern into glass microscope slides prior to BP functionalization and photopatterning of ConA-biotin (Fig. 4). Using a combination of photolithography and wet etching, we created glass substrates that had 100 μm wide channels etched to a depth of 35 μm , with 400 μm between channels (Fig. 4c). BP conjugation and ConA-biotin photopatterning were carried out on the etched substrates in precisely the same manner as planar substrates. Imaging with a confocal fluorescence microscope allowed visualization of both the side- (Fig. 4A) and top-view (Fig. 4b) of the resulting photopatterned substrate, where the photopatterning was carried out with the same photomask used to generate the corrugated substrate, but with a 90° rotation. Notably, a line trace of fluorescence intensity as a function of position across the substrate, shown in Figure 4d (path of line trace indicated in Fig. 4b) indicates that the protein is photoimmobilized to a similar extent across the patterned substrate, irrespective of topography.

The ability to spatially pattern biomolecules with precision on surfaces with topographical discontinuities would be very difficult with many techniques that require precise laminar

flow conditions. This proof-of-principle demonstration of patterning onto corrugated surfaces shows the potential of this methodology to incorporate biomolecular species onto topographically complex substrates that are not amenable to printing or flow-based methods. This feature will be important in designing experiments to probe the competing or synergistic effects of biochemical and topographical cues on processes such as directed cell migration.^{63,64}

Demonstration of photopatterned protein function: native ligand recognition and cell patterning

Common applications of substrates presenting biomolecules include recognition of native ligands and fundamental cell adhesion studies. It is therefore important to verify that the photoimmobilization process, which involves exposure to UV light ($\lambda = 350\text{--}365\text{ nm}$), does not physically damage biomolecules, rendering them unrecognizable by appropriate receptors on the surface of cells. To demonstrate the ability of photopatterned proteins to retain their specific ligand-binding properties, we generated substrates presenting P-selectin in arrays of geometric shapes (Fig. 5). P-selectin is a glycoprotein involved in leukocyte tethering on the endothelial lining of blood vessels and HL-60 cells are known to express PSGL-1, a receptor for P-selectin.¹³ We found that P-selectin is able to bind specifically to both soluble PSGL-1 (Fig. 5a) and cell surface-bound PSGL-1 expressed on HL-60 promyelocytic cells (Fig. 5b). HL-60 cells, which were fluorescently labeled for visualization purposes, specifically adhere to the square patterns of P-selectin and not to the surrounding background, thereby demonstrating the integrity of the photoimmobilized biomolecule and suggesting a general utility of the BP attachment scheme to creating cell-compatible biomaterials interfaces.

To further demonstrate cell adhesion to specific biomolecules of interest, a native ECM protein, FN, was photoimmobilized in $100\text{ }\mu\text{m}$ stripes onto a BP-PEG substrate (Fig. 6). Surface modification with PEG is well-known for rendering surfaces more resistant to non-specific binding of proteins and cells.^{65,66} 3T3 fibroblast cells express integrins that recognize the RGD motif within FN. BP-PEG-modified substrates were utilized to generate photopatterned FN substrates. Cells were seeded onto BP-PEG substrates and were found to selectively adhere to FN stripe patterns amidst a BSA-blocked PEG background. The ability to incorporate PEG molecules into our surface chemistry approach demonstrates the applicability and generality of this biomolecular patterning method to studies involving both natively non-adhesive and adhesive cells.

Conclusion

In this manuscript we present an extension of a previously developed biomolecularly general photopatterning methodology based on the UV exposure of BP-modified substrates in the presence of solution phase biomolecules. We characterize each step of the surface modification procedure using contact angle goniometry and atomic force microscopy, and demonstrate the extension of this methodology for creating multi-component patterns of three different biomolecules, including proteins and a carbohydrate. Furthermore, we employ a modified radioimmunoassay technique to establish the quantitative nature of biomolecular immobilization, which can be tuned by rationally adjusting the UV exposure time. We demonstrate the ability to make spatially confined protein patterns onto both planar and corrugated substrates and show that photopatterned biomolecules retain their ability to recognize their native ligands, and to be recognized by native cellular receptors. Future efforts will include the incorporation of additional biomolecules for the creation of biochemically complex *in vitro* models of cellular microenvironments, fundamental studies

of cell-materials interactions in the context of native biomolecular responses, and biomedical and tissue engineering applications.^{67–71}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. One-component and three-component patterns of biomolecules on BP-modified substrates. (A) Photoimmobilization of biotinylated ConA in the “Illinois logo” pattern, visualized with fluorescently labeled streptavidin. (B) Three-component pattern of mannan (blue, stripes running from top right to bottom left), P-selectin (red, stripes running from top right to bottom left), and Fibronectin (green, vertical stripes) photoimmobilized sequentially on BP-modified substrates with a 100 μm stripe pattern with mask rotation between exposures. Scale bar: 100 μm .

Figure 2. Protein loading is positively correlated with UV exposure time. Biotinylated ConA (5 $\mu\text{g}/\text{mL}$) was uniformly photopatterned onto BP-modified substrates for 5, 15, 30, 60, or 120 sec. The signal from subsequent (A) fluorescence analysis and (B) radioimmunoassays show the increase of signal as a function of UV exposure time ($\sim 365\text{ nm}$, $17\text{ mW}/\text{cm}^2$). Control substrates were employed to account for non-specific binding of antibodies. Data represents the average of $n=3-4$ ($\pm 95\%$ C.I.).

Figure 3.

Results from fluorescence-radioactivity correlation studies. Data from the biotinylated ConA radioimmunoassay (Fig. 4) were converted from units of CPM to site density using the linear regression from a standard curve generated from known amounts of [^{125}I]-labeled Streptavidin (see E.S.I.). Plotting fluorescence intensity (F.I.) as a function of ConA-biotin site density allows data from future fluorescence analyses to be converted to immobilized protein site density.

Figure 4. Glass substrates were etched to generate channels 100 μm wide and 35 μm deep (A and B), followed by functionalization with BP. Biotinylated ConA was photoimmobilized using the same photomask used for etching rotated 90° and imaged using a confocal microscope, demonstrating our ability to pattern down into the channels with no loss in fluorescence intensity (C and D).

Figure 5.

Native ligand recognition by photopatterned glycoprotein P-selectin. (A) P-selectin was immobilized onto BP-modified substrates in 800 μm circles. Fluorescence images were obtained by incubating substrates with PSGL-1. (B) HL-60 promyelocytes, which express PSGL-1 on their surface, selectively adhere to 500 μm squares of photoimmobilized P-selectin on BP-modified substrates, as visualized by fluorescent cell labeling and microscopy. Scale bar: 500 μm .

Figure 6.

BP-PEG-modified substrates are suitable for adhesive cell patterning on photopatterned ECM proteins. (A) A carboxy-terminated poly(ethylene glycol) linker is conjugated to aldehyde-functionalized glass slides, followed by reaction of BP with the surface using standard EDC/NHS chemistry. Contact angles track with surface hydrophobicity changes. (B) Fibronectin, a protein found in the ECM, was photopatterned to BP-PEG-modified substrates in 100 μm stripes, and imaged using a fluorescently labeled binding partner. The resulting line scan is shown in (C). (D) 3T3 mouse fibroblast cells selectively adhere to 100 μm stripes of photoimmobilized FN on BP-PEG substrates. Cell nuclei (blue) and actin (red) are fluorescently stained and visualized using fluorescence microscopy. Scale bar: 100 μm .

Scheme 1.

A schematic diagram showing the preparation of BP-modified substrates and subsequent biomolecule photoimmobilization. Glass microscope slides are cleaned, silanized and functionalized with BP. A biomolecule is introduced to the substrate followed by exposure to UV light (365 nm), resulting in the formation of new C-C bonds between the surface and the biomolecules. The resultant biomolecular patterns are visualized with a fluorescently labeled binding partner.

Table 1

Surface characterization of BP-modified substrates. Surfaces were characterized using contact angle goniometry and AFM to determine changes in hydrophobicity and surface roughness. Contact angle data represent the average of $n=9$ substrates ($\pm$ standard deviation) from two batches of slides made on the same day. AFM was done after each reaction and the RMS roughness was determined.

	Glass	Aldehyde silane	BP functionalization	Post - Protein Conjugation
Contact Angle (H ₂ O)	0°	44.8 $\pm$ 1.9°	53.4 $\pm$ 1.1°	30.2 $\pm$ 2.1°
RMS Roughness (AFM)	352 pm	915 pm	833 pm	775 pm
Chemical Structure