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A Direct Biophotolithographic Method for Generating Substrates with Multiple Overlapping Biomolecular Patterns and Gradients

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We describe an approach to generate multicomponent surface-immobilized patterns and gradients based upon the photochemically controlled covalent coupling of solution-phase biomolecules to benzophenone-modified substrates. Gradients were simply achieved by continuously varying the exposure to non-damaging UV light across the surface with the gradient profile controlled by biomolecule concentration and the spatial and temporal illumination of the surface. Sequential exposure of the same surface in the presence of different biomolecules resulted in overlapping patterns and gradients of proteins and carbohydrates. Finally, we preliminarily demonstrate that the resulting surfaces are suitable for generating model substrates to probe cell-substrate interactions.

Introduction

Cellular adhesive environments encountered *in vivo* are enormously complex and dynamic, and the density and spatial distribution of many distinct biomolecules significantly impacts the response of cells. To better understand the nature of these complex interfacial recognition events and subsequent processes, scientists have utilized expertise in molecular synthesis, design, and control to influence the behavior of cells,¹ resulting in well-defined surfaces presenting biologically relevant ligands at distinct spatial locations.² Particularly challenging to fabricate are surface-immobilized gradients in which the density of a specific biomolecule is spatially varied in a continuous (rather than digital) manner. However, *in vivo*, many biomolecules are involved in defining adhesive environments. Thus, a further challenge exists in generating multicomponent, overlapping surface gradients wherein the surface density of multiple biomolecules is spatially controlled.

Biomolecular gradients can be formed by first generating chemical gradients on surfaces, via chemical or photochemical modification of surface functional groups^{3,4} or manipulation of self-assembled monolayers^{5–7} followed by conjugation of biomolecules via various chemical functionalities. Alternatively, biomolecular gradients can be created in solution by controlled mixing in microfluidic networks followed by direct transfer to a uniform surface.^{8–11} Surface gradients generated via these approaches have proven to be exceedingly valuable in investigating biological processes such as cell polarity,⁸ migration and metastasis,^{7,9,12} wound healing,¹³ and neuronal guidance.^{10,11} However, many of these enabling approaches require complicated microfluidic designs, have limits on the number of components incorporated, or require specific and often non-native chemical functionalities, which can limit their general applicability to different classes of biomolecules (peptides, proteins, carbohydrates, etc.).

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Supporting Information. Fluorescence images of large-scale patterns and gradients, line scans for replicate P-selectin gradients, MALDI results for UV-exposed proteins, demonstration of photoimmobilization on BP-PEG substrates, and details on control cell experiments are provided in the SI. This material is available free of charge via the Internet at <http://pubs.acs.org>.

In this report, we describe a direct, biomolecularly universal method for creating overlapping, multicomponent patterns and surface gradients. Our approach, outlined in Figure 1, involves the spatially selective photochemical activation of benzophenone (BP) monolayers in the presence of a solution-phase biomolecule. Upon illumination with 365 nm light, BP undergoes an $n \rightarrow \pi^*$ transition to form a transient diradical that can covalently attach proximal biomolecules to the surface via insertion into a C–H bond.^{14–16} Excited BPs which fail to undergo C–H insertion relax back to the ground state whereby they can be re-excited in the presence of a different biomolecule solution, allowing for the creation of overlapping patterns and gradients. Because of the general reactivity of benzophenone towards biomolecularly ubiquitous C–H bonds, this method represents a universal approach for direct surface bioconjugation. Previous reports utilizing BP-functionalized molecules indicate that the attachment chemistry is applicable to substrate modification,^{17–19} but this approach is susceptible to crosslinking in solution and multilayer formation because the reaction is not confined to the surface. BP-modified surfaces have been utilized to attach non-biological polymers to surfaces,^{20–24} and relevant to this study are reports of biomolecule immobilization.^{25–30} In this letter we describe the first application of BP photochemistry to generate *multicomponent* surface-immobilized biomolecular patterns and gradients that can be tailored to model complex physiological microenvironments.

Results and Discussion

We first demonstrated the ability to create single-component biomolecular patterns of the biotinylated lectin, Concanavalin A (ConA-biotin) on BP-modified glass substrates by illuminating the surface through a 500 μm square photomask pattern followed by visualization with a fluorescently labeled binding partner, as shown in Figure 2a. By sequentially exposing the same substrate in the presence of two different biomolecules, overlapping, multicomponent patterns were generated, as observed with two spectrally distinct fluorescently labeled binding partners (Figure 2b). First, a BP surface was illuminated through the photomask in the presence of mannan, followed by rotation of the mask $\sim 45^\circ$ and exposure in the presence of P-selectin. To our knowledge, the resulting two component substrate is the first example of overlapping patterns of carbohydrates *and* proteins on the same substrate.

Following these initial demonstrations, we wondered whether exposure to 365 nm UV light would damage the biomolecules, rendering them useless for modeling adhesive environments. This concern is relieved by the fact that both of the biomolecules patterned in Figure 2b are visualized by structure-specific recognition elements. Furthermore, MALDI-MS analysis on proteins after illumination showed no evidence of protein fragmentation (see SI).

Based upon the digital patterning results shown above, we reasoned that we could generate gradients of biomolecules on the surface by continuously varying the spatial exposure across the surface during illumination. In addition to the spatial exposure to UV light, the relative amount of surface attachment can also be controlled by adjusting the concentration of solution-phase biomolecule. Figure 3a demonstrates both of these levels of control in the fabrication of one component continuous surface gradients of ConA-biotin by positioning a programmable shutter in between the light source and the substrate. Excitation and linear movement of the shutter were initiated simultaneously so that a gradient in light exposure was created across the substrate. Gradient profiles at three different ConA-biotin concentrations show the relationship between solution-phase biomolecule concentration and the slope of the resulting gradient. We have established that gradient fabrication is reproducible under a given set of exposure conditions and that multiple compositionally distinct large-scale overlapping patterns can be generated on the same substrate (see SI).

Similarly to the two-component patterning experiments above, parallel overlapping gradients of P-selectin (red) and mannan (green) were fabricated by sequential exposures with 180° shutter reorientation, as shown in Figure 3b. We also were able to generate two-component perpendicular gradients by rotating the shutter by 90°, as shown for mannan and P-selectin in Figure 4. Notably, the photochemical approach allows immobilized biomolecule concentration to be varied along the direction of flow. A diagonal line drawn from bottom left to top right across Figure 4a gives an identical profile to that of Figure 3b, while the diagonal from top left to bottom right shows both components varied together from high to low concentration. We feel as though the simplicity and versatility provided by the BP-patterning methodology will allow facile construction of complex, overlapping gradient substrates that will find utility in future studies of multiparametric cell-surface interactions.

To preliminarily demonstrate the potential of photochemically patterned surfaces for use in studies of cell adhesion, we investigated the interaction of HL 60 promyelocytic leukemia cells with photochemically generated patterns and gradients of P-selectin. P-selectin is a glycoprotein involved in leukocyte tethering on the endothelial lining of blood vessels.³¹ HL 60 promyelocytes express its ligand, P-selectin glycoprotein ligand-1,³² and are known to adhere to and roll on immobilized P-selectin.^{32–34} As shown in Figure 5a, the number of immobilized cells correlates with the amount of immobilized P-selectin and degree of HL 60 attachment as the number of cells decreases from left to right down a P-selectin gradient. Control experiments showing no cell adhesion were carried out on non-specific protein surfaces as well as on surfaces presenting no protein ligands (see SI). As a further demonstration of the utility of photochemically generated biomolecular gradients as cell adhesive models, we investigated the rolling behavior of HL 60 cells as they were flowed perpendicularly over a P-selectin surface gradient (Figure 5b). The observed range of rolling velocities and the correlation between adhesion and the amount of immobilized P-selectin is consistent with previous reports.^{32,35} HL 60 cells were not observed to adhere to or roll on surfaces of photoimmobilized non-specific proteins or plain BP-modified substrates.

Though largely beyond the scope of this letter, we would like to briefly address the issue of non-specific cell adhesion. HL 60 cells are suspension cultured cells derived from peripheral leukocytes and do not require elaborate chemical passivation to reduce non-specific adsorption onto our surface. To generate substrates that will be more compatible with adhesive cell types, we have successfully incorporated poly(ethylene glycol) tethers into the backbone of BP monolayers and demonstrated photoimmobilization of biomolecules onto substrates which show reduced non-specific protein adsorption (see SI).

Conclusion

We have demonstrated the ability to create multicomponent, overlapping biomolecular patterns and continuously varying surface gradients via a photochemically controlled C–H bond insertion reaction. An alternative to conventional gradient generation methods, this simple surface modification approach requires only a relatively inexpensive UV light source and translation stage, provides molecularly general direct covalent attachment, and allows patterning of multiple biomolecules into complex geometric patterns and gradients, including perpendicular gradients, on account of its independence from direction of solution flow. Future work will focus on the incorporation of a more extensive range of biomolecules which define *in vivo* adhesive environments and the application of these substrates to fundamental studies of multicomponent cell-surface interactions.

Experimental Section

Preparation of BP-modified Substrates

Glass microscope slides were cleaned with Piranha solution³⁶ (4:1 (v:v) concentrated H₂SO₄ : 30% H₂O₂), rinsed with water and absolute ethanol and baked at 120 °C for 1 hour, followed by chemical vapor deposition of 4-(triethoxysilyl) butyl aldehyde (United Chemical Technologies) for 2.5 hours at reduced pressure. Slides were cured at 120 °C for 1 hour, soaked in absolute ethanol for 15 minutes, and incubated in a solution of 20 mM 4-Benzoyl benzylamine hydrochloride (Matrix Scientific) and 200 mM NaCNBH₃ in 4:1 DMF:MeOH for 4 hours at room temperature. Slides were subsequently immersed in aldehyde-blocking buffer (0.1 M Tris, 200 mM ethanolamine, pH 7.0) for 1 hour at room temperature, rinsed with water, DMF, MeOH, and EtOH, dried under a stream of nitrogen, and stored under vacuum in the dark until use.

Generation of Biomolecular Patterns and Gradients

Protein and carbohydrate solutions were freshly diluted from concentrated stocks. Stock solutions of biotinylated lectin Concanavalin A (ConA-biotin, Vector Laboratories) and recombinant glycoprotein human P-selectin (R&D Systems) were prepared by resuspending the lyophilized biomolecule in the manufacturer's recommended buffer solution to a concentration of 1 mg/mL. Aliquots were stored at -20 °C and diluted in their respective buffers immediately prior to use. A stock solution of polysaccharide mannan (from *Saccharomyces cerevisiae*, Sigma) was prepared by resuspending the lyophilized powder to a final concentration of 20 mg/mL in purified water.

BP-modified glass substrates were assembled within a rectangular parallel-plate flow chamber (GlycoTech). Biomolecule solutions were flowed through at a rate of 10 µL/min, as steady flow was found to result in more consistent and reproducible substrate patterning. Illumination at 3 mW/cm² was performed through the back of the substrate with a CF2000 UV LED source (365 nm, Clearstone Technologies). For generation of biomolecular patterns, illumination was performed through a photolithographically generated chromium-coated glass mask with 500 µm square features spaced by 500 µm. An opaque shutter connected to a computer-controlled translation stage (ThorLabs Opto DC Driver) was utilized for gradient generation. The stage was programmed to move at a velocity of 0.1 mm/sec for protein immobilization and 0.02 mm/sec for carbohydrate immobilization, with total light exposure determined by the size of the desired gradient. Following illumination, substrates were immersed in an appropriate rinse solution, and sonicated on ice. The following rinse solutions were utilized: for ConA-biotin, 0.5% Tween 20 in HEPES buffer; for mannan, 0.5 mg/mL sodium dodecyl sulfate in Dulbecco's PBS buffer (Sigma); and for P-selectin, 0.5% Tween 20 and 1% BSA in Dulbecco's PBS buffer with Ca²⁺ and Mg²⁺ (Sigma).

Fluorescence Imaging of Biomolecular Patterns and Gradients

Substrates were blocked with 1% BSA/HEPES overnight followed by incubation with fluorescently labeled binding partners and visualization with a fluorescence slide scanner (GenePix 4000B, MDS Analytical Technologies) to obtain fluorescence intensity line scans. For visualization of immobilized ConA-biotin, substrates were incubated for 1 hour in a solution of Streptavidin-AlexaFluor647 conjugate (0.05 µg/mL, Invitrogen) in 1% BSA/HEPES. For substrates with two-component mannan and P-selectin immobilization, slides were incubated for 1 hour in a solution of ConA-biotin (0.1 µg/mL), Streptavidin-Cy3 conjugate (0.05 µg/mL, Invitrogen), mouse anti-human P-selectin (0.5 µg/mL, R&D Systems), and AlexaFluor647-conjugated rabbit anti-mouse IgG (1 µg/mL, Invitrogen) in 1% BSA/HEPES.

Cell Adhesion and Rolling Studies

HL 60 cells (gift from the Hergenrother group at UIUC) were cultured in RPMI 1640 supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL) and 10% fetal bovine serum (SCS Cell Media Facility, UIUC). Three millimeter photopatterned P-selectin gradient substrates were prepared as described, rinsed and sonicated for 30 minutes in 0.2% Pluronic F127 (Sigma) in PBS, and blocked overnight in 0.2% Pluronic F127 in 1% BSA/PBS. Cells were seeded onto substrates at 5×10^6 cells/mL in PBS and incubated at 4 °C for 2 hours. Non-adhered cells were aspirated off after gentle rinsing with PBS and the resulting cell gradients were imaged using an optical microscope (Zeiss 40C Invertiskop, Carl Zeiss Incorporated).

For HL 60 cell rolling studies, a 6 mm P-selectin gradient was prepared as described and assembled within the parallel plate flow chamber. Cells were resuspended in cell media at 5×10^5 cells/mL and flowed through the chamber at 50 µL/min, yielding an applied shear stress of 0.24 dynes/cm².³⁷ Videos of cell rolling were recorded with a digital camera through the optical microscope and the velocities of rolling cells were determined with ImageJ (National Institutes of Health).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic outlining the generation of multi-component biomolecular surface patterns and gradients via sequential exposures of benzophenone-modified substrates in the presence of different biomolecules.

Figure 2.

Demonstration of direct photoimmobilization of proteins and carbohydrates on benzophenone-modified glass substrates: (a) biotinylated Concanavalin A; (b) overlapping patterns of glycoprotein P-selectin (red) and polysaccharide mannan (green). Dashed lines indicate areas in fluorescence line scans. F.I., Fluorescence Intensity. Scale bars: 500 μm .

Figure 3. Demonstration of control over gradient profile and biomolecular generality of the photochemical approach: (a) ConA-biotin gradient slope can be controlled by varying the biomolecular concentration during photoimmobilization; (b) a single substrate displaying two overlapping gradients of P-selectin (red) and mannan (green). F.I., Fluorescence Intensity. Scale bar: 500 μm .

Figure 4.

(a) Fluorescence image and line scans of overlapping gradients of mannan (green) and P-selectin (red), parallel and perpendicular to the direction of solution flow, respectively. Dashed lines indicate areas in fluorescence line scans. (b) Illustration of perpendicular gradients which present different ratios of two components in various regions.

Figure 5.

(a) Optical micrograph of HL 60 cells seeded onto a 3 mm surface gradient of P-selectin overlaid with the corresponding line scan showing the gradient in the number of immobilized cells. (b) Plot of HL 60 cell rolling velocity versus position on a 6 mm P-selectin gradient. Wall shear stress was 0.24 dynes/cm². Error bars represent the 95% confidence intervals determined for n= 44, 44, 46, 40, 45, 39, and 49 cells for positions, 0 through 6 mm, respectively.