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Protein Tethering into Multiscale Geometries by Covalent Subtractive Printing

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The engineering of surfaces functionalized with biological/bioactive components is important for medical and diagnostic applications, including protein arrays and biosensors,^[1] as well as fundamental life sciences studies.^[2] Spatial control at length scales of both cellular adhesive structures (e.g., focal adhesions; micrometer/sub-micrometer scale) and individual proteins (nanometer scale) has been highly sought after to produce surfaces capable of eliciting specific biological responses. A major challenge has been combining features with micrometer and nanometer dimensions onto one sample while maintaining a protein-resistant background that is stable for extended periods under cell culture conditions. Sample production must also be high-throughput and low cost in order to be broadly applicable and cost- and time-effective. Traditional microcontact printing has been successful in producing micrometer-scale patterns for biological studies quickly and inexpensively but faces limitations when approaching sub-micrometer resolution of patterns due to the diffraction limit of light, which affects the fabrication of molds prepared using photolithography, and the instability of elastomer stamp materials.^[3] Scanning probe-based techniques (i.e., dippen lithography), which control protein placement by depositing or scraping off molecules using a cantilever, have enabled access to the regime with features of the size of tens of nanometers.^[4] While patterns approaching single proteins produced with the mentioned techniques are desirable for a variety of applications, limitations in the writing areas achievable with standard equipment makes high-throughput sample production challenging. Colloidal lithography with diblock copolymers has provided control over the nanometer-scale spacing between ligands for studies of cell adhesion^[5] and apoptosis,^[6] but the pattern geometries are currently limited to spacings predetermined by micelle chemistry. In this communication, we introduce a technique with the capacity to produce multi-length-scale patterns of bioactive proteins that are covalently immobilized onto an activated surface and surrounded by a protein adsorption-resistant background. Patterns of the cell adhesive protein fibronectin were printed on a nonadhesive background to produce arrays of single cells where adhesion is constrained to the region of tethered protein. The applicability of the technique to biological studies is demonstrated by producing arrays of adherent cells on

which focal adhesion size and spatial arrangement are modulated according to the geometry of the adhesive region.

Patterns of proteins were directly immobilized by covalent tethering onto surfaces presenting mixed self-assembled monolayers of alkanethiols using a modified version of subtractive contact printing.^[7] Details of the method are shown in Figure 1. This method is also consistent with earlier work related to chemical reactions taking place on the surface of monolayers using reactive surface groups and inks.^[8,9] A protein pattern was produced on a flat elastomer by first adsorbing a uniform layer of protein from solution and then completing a contact-and-release step with a template (Figure 1A). The resulting pattern on the elastomer mirrors the recessed pattern on the template. Next, gold-coated substrates were incubated overnight in a mixed solution of alkanethiols terminated with either tri(ethylene glycol) (EG₃) or carboxylic acid–hexa(ethylene glycol) (COOH-EG₆) (Figure 1B). Before the printing step, *N*-hydroxysuccinimidyl (NHS) esters were generated from the carboxylic acid groups of the EG₆-COOH alkanethiol using NHS/EDC chemistry (where EDC is 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide). To transfer the protein pattern to the final substrate, a contact-and-release step was completed between the elastomer and the substrate (Figure 1C). Active NHS esters on the surface couple to available amine groups on the protein. All steps from activation through printing were completed in less than 45 min to avoid incomplete immobilization due to inactivation of the NHS esters. After printing, unreacted NHS esters were quenched by incubating substrates in bovine serum albumin (BSA).

Patterns of the cell adhesion protein fibronectin were produced to demonstrate the ability of the technique to immobilize proteins in controlled geometries while maintaining protein activity. Arrays of circular regions (20 μm in diameter) with a repeat spacing of 75 μm (Figure 1D) were patterned using a template produced using electron beam lithography. A complete array was printed across a 25 mm diameter coverslip in one stamping step using a 30 mm × 30 mm elastomer and equivalent template area.

Activity of the tethered fibronectin was verified using the HFN7.1 monoclonal antibody (Figure 1D), which binds to the cell-binding domain of fibronectin and has been used as a probe of integrin receptor binding and reporter of fibronectin activity.^[10] Immunostaining analyses revealed an array of circular regions matching the fibronectin pattern. This result indicates that protein is not denatured during the printing and tethering steps and that the central integrin receptor-binding domain of fibronectin is active in the final configuration. Uniform fibronectin distribution is demonstrated by low variation in the signal intensity across the entire array as well as within circles (line profile in Figure 1D). A recombinant fragment of fibronectin comprising the cell adhesive 7th–10th type III repeats of the molecule was also successfully printed and tethered to mixed self-assembled monolayers of alkanethiols, showing that various protein sizes are supported by this technique (results not shown). The background region between circular printed regions remained devoid of antibody, indicating that high contrast patterns are produced by the patterning technique and that the nonfouling background effectively resists protein adsorption. Taken together, these results verify that protein activity is maintained during patterning and immobilization and that the nonpatterned areas are resistant to protein adsorption.

Complex patterns of proteins with spacing and sizes varying across multiple length scales are desirable for studies of biological processes whose functionality requires coordination across different length scales. In order to demonstrate these capabilities, patterns of proteins were produced in which the pattern geometry includes feature dimensions at both micrometer and nanometer length scales. Fibronectin patterns printed to substrates coated with mixed self-assembled monolayers of alkanethiols were visualized with fibronectin-

specific polyclonal antibodies and fluorophore-conjugated secondary antibodies. Geometrical features measuring as small as several hundred nanometers were simultaneously patterned and printed with micrometer features of size $2\ \mu\text{m} \times 2\ \mu\text{m}$ (center square) (Figure 2). Spacings ranging from $100\ \mu\text{m}$ between patterns (Figure 2A) to several hundred nanometers between individual features in a cluster of four (Figure 2B) were easily achieved. High-fidelity patterns and features with uniform dimensions and protein staining were maintained across all size scales. Validation experiments demonstrating tethering of proteins and nonfouling background are presented in the Supporting Information.

To examine the ability to control protein patterning at sub-cellular length scales, we developed patterns to direct the formation of focal adhesions in adherent cells. Focal adhesions are integrin-mediated adhesive junctions between cells and extracellular matrix components that provide strong adhesive forces and signals.^[11,12] Vinculin is a structural component of focal adhesions that functions as a central regulator of cell adhesion strength.^[13–15] Using standard immunofluorescence techniques, we visualized the localization of vinculin in response to patterns of fibronectin. Vinculin staining in cells spread on nonpatterned fibronectin shows areas of high intensity at sites of vinculin localization (Figure 3A), indicating the formation of elongated focal adhesions that are typical of spread cells. Patterns of fibronectin were then used to limit vinculin localization and focal adhesion formation to defined regions. Circular patterns ($10\ \mu\text{m}$ diameter) of fibronectin supported adhesion of one cell per island (Figure 3B). Vinculin was localized on focal adhesion structures that were constrained to the adhesive region. In these micrometer-sized islands, vinculin was localized preferentially at the edge of the circular area, although punctate and elongated complexes were visible throughout the interior region. These results are in agreement with previous work that demonstrated comparable focal adhesion formation on surfaces produced by micro-patterning alkanethiols and coated with adsorbed fibronectin.^[15] In order to control focal adhesion formation, patterns were designed with different spatial arrangements of the adhesive region. Patterns with dimensions of $1\ \mu\text{m} \times 1\ \mu\text{m}$ were arrayed around an outside diameter of $10\ \mu\text{m}$, which limits adhesion to one cell per island (Figure 3B). Vinculin staining shows preferential localization of focal adhesions to the eight regions at the edge of the pattern. The patterns of vinculin recruitment shown in Figure 3B were consistent for more than 50 cells analyzed. These results demonstrate that focal adhesion formation can be controlled with high precision by modulating the geometry of the adhesive region.

In summary, we present a protein printing strategy for surface modification that generates high-resolution patterns of proteins by covalently tethering them to surfaces coated with a mixed monolayer of alkanethiols. This modified subtractive contact printing approach is used to simply and efficiently achieve pattern dimensions ranging from the micrometer to the nanometer scale. Tethering directly from a stamp to a mixed self-assembled monolayer provides a straightforward strategy for producing patterns of covalently immobilized proteins surrounded by protein-resistant background. Applicability to biological studies is demonstrated by directing focal adhesion formation of adherent cells by varying the nanometer-scale geometry of fibronectin patterns. The high-throughput and multiple-length-scale characteristics of this technique make it readily accessible to researchers across a variety of fields, including microfluidics, biomaterial functionalization, and fundamental biology studies. This technique can be easily combined with other surface engineering approaches to generate multiprotein substrates,^[7,16] aligned/anisotropic topographies or chemistries,^[17–20] dynamic characteristics,^[21] or combinatorial features to produce unique surfaces with new functionalities.

Experimental Section

Cells and Reagents

NIH 3T3 fibroblasts (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% newborn calf serum (HyClone, Logan, UT) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). Cell culture reagents, including human plasma fibronectin and Dulbecco's phosphate-buffered saline (DPBS), were purchased from Invitrogen (Carlsbad, CA). Antibodies against vinculin (clone VIN-11-5, Sigma-Aldrich) were used for immunostaining. The receptor-mimetic antibody against human fibronectin (HFN7.1) was acquired from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Poly(dimethylsiloxane) (PDMS) elastomer and curing agent (Sylgard 184) were produced by Dow Corning (Midland, MI). ZEP520A was purchased from Zeon Chemicals (Tokyo, Japan). Tri(ethylene glycol)-terminated alkanethiol ($\text{HS}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_3-\text{OH}$; EG₃) and carboxylic acid-terminated alkanethiol ($\text{HS}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6-\text{OCH}_2-\text{COOH}$; EG₆-COOH) were purchased from ProChimia Surfaces (Sopot, Poland). Peptide tethering reagents *N*-hydroxysuccinimide (NHS) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) were obtained from Sigma-Aldrich (St. Louis, MO). 2-(*N*-Morpho)-ethane sulfonic acid was purchased from Sigma-Aldrich (St. Louis, MO).

Monolayer Preparation

Self-assembled monolayers of alkanethiols on gold were used to present anchoring groups for covalent immobilization of fibronectin within a nonfouling background. Surfaces of mixed self-assembled monolayer were prepared using tri(ethylene glycol)-terminated alkanethiol ($\text{HS}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_3-\text{OH}$; EG₃) and carboxylic acid-terminated alkanethiol ($\text{HS}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6-\text{OCH}_2-\text{COOH}$; EG₆-COOH). Gold-coated substrates were prepared by sequential deposition of titanium (100 Å) and gold (200 Å) films using an electron beam evaporator (Thermionics Laboratories, Hayward, CA, 2×10^{-6} Torr, 1 Å s^{-1}) onto clean 25 mm diameter glass coverslips (Bellco Glass, Vineland, NJ). Mixed monolayer surfaces were prepared by immersing gold-coated samples in a mixed solution of EG₃ + EG₆-COOH thiols (100 parts EG₃ to one part EG₆-COOH, 1.0 mM final concentration in 200 proof ethanol) overnight in untreated polystyrene dishes with a nitrogen cap and sealed with Parafilm. Several ratios of EG₃ to EG₆-COOH were tested to determine an optimal balance between providing sufficient sites for tethering protein during printing while maintaining a protein-resistant background. After being washed in ethanol for 15 min, twice in deionized water, and MES buffer (0.1 M 2-(*N*-morpho)-ethane sulfonic acid and 0.5 M NaCl in deionized water, pH 6.0), mixed monolayer surfaces were incubated in activation buffer (200 mM EDC and 100 mM NHS in MES buffer) for 25 min. Substrates were rinsed twice with deionized water. Excess liquid was removed by applying a stream of N₂ for approximately 5 s. Following activation of the surface, subtractive contact printing^[7] was used to produce patterns of fibronectin as described below.

Nanotemplate Fabrication and Subtractive Contact Printing

Subtractive contact printing was used to produce micrometer- and nanometer-scale patterns of fibronectin. High-resolution nanotemplates were produced using electron-beam lithography. Silicon wafers (4 in., ca. 10 cm) were spin-coated with resist ZEP520A at 5000 RPM for 60 s followed by a post-baking at 180 °C for 120 s. The ZEP resist was exposed in a JEOL JBX-9300FS electron-beam lithography system, developed in amyl acetate for 120 s, immersed in isopropyl alcohol for 1 min, and blown dry under a stream of N₂. The ZEP pattern was transferred into the silicon substrate using a STS ICP Standard Oxide Etcher.

Details of the subtractive contact printing have been published previously.^[7] In brief, cured PDMS was cut into 30 mm × 30 mm square flat stamps, cleaned, and pre-stamped. The side of the elastomer that was in contact with the polystyrene dish was inked with 800 μL fibronectin solution (100 $\mu\text{g mL}^{-1}$ in DPBS) for 30 min at room temperature. Elastomers were then rinsed and excess liquid was removed with a stream of N_2 . Proteins on homogeneously inked elastomers were removed in selected areas by bringing the elastomers into contact with clean silicon nanotemplates for 15 s. The protein patterns were transferred from the elastomers to alkanethiol-coated 25 mm diameter glass coverslips using a 30 s printing step. Intimate contact between the elastomer and the nanotemplate/substrate was achieved after placing the elastomer on the nanotemplate/substrate by applying a slight pressure with tweezers. Nonadhesive areas were then blocked by incubating coverslips in 0.1% heat denatured BSA for 30 min. Finally, substrates were incubated for 2 h in DPBS to elute loosely adsorbed proteins.

Activity of Tethered Fibronectin

The biological activity of printed patterns of fibronectin was evaluated using antibodies specific to fibronectin binding domains. We previously demonstrated that the HFN7.1 monoclonal antibody specific for the integrin binding domain behaves as a receptor-mimetic antibody and its binding to immobilized fibronectin is predictive of cell adhesion activity.^[10] Substrates prepared by subtractive contact printing were treated with HFN7.1 (10 $\mu\text{g mL}^{-1}$) diluted in blocking buffer for 1 h at 37 °C. Primary antibodies were visualized using AlexaFluor 488- and 555-conjugated secondary antibodies (anti-rabbit IgG, anti-mouse IgG; 5 $\mu\text{g mL}^{-1}$) diluted in blocking buffer for 1 h at 37 °C. Images were captured using a Nikon Eclipse E400 fluorescence microscope and ImagePro image acquisition software.

Cell Seeding and Focal Adhesion Staining

Fibronectin conjugated to AlexaFluor 555 was used to visualize patterns of printed protein. In order to leave free primary amines on fibronectin for tethering to mixed self-assembled monolayers, a ratio of 25:1 w/w of fibronectin to amine-reactive AlexaFluor 555 succinimidyl ester was used in the reaction. Cells were seeded on patterned substrates at 235 cells per mm^2 in DMEM supplemented with 10% newborn calf serum (NCS). For visualization of focal adhesions, cells were extracted in cytoskeleton buffer (0.5% Triton X-100 in 50 mM NaCl, 150 mM sucrose, 3 mM MgCl_2 , 20 $\mu\text{g mL}^{-1}$ aprotinin, 1 $\mu\text{g mL}^{-1}$ leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF), 50 mM Tris (tris(hydroxymethyl)aminomethane), pH 6) for 10 min to remove membrane and soluble cytoskeletal components. Extracted cells were fixed in cold formaldehyde (3.7% in DPBS) for 5 min, blocked in blocking buffer (5% goat serum in DPBS) for 1 h, and incubated with primary antibody (anti-vinculin 1:125 dilution) diluted in blocking buffer for 1 h at 37 °C. Primary antibodies were visualized using AlexaFluor 488-conjugated secondary antibodies (anti-rabbit IgG 1:200 dilution) diluted in blocking buffer for 1 h at 37 °C. Images were captured using a Nikon Eclipse E400 fluorescence microscope and ImagePro Plus image acquisition software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Printing strategy for direct covalent immobilization of protein patterns to surfaces coated with mixed monolayers of alkanethiols. A) A surface coated with mixed alkanethiols is activated using NHS/EDC chemistry. B) Subtractive contact printing generates protein patterns on a flat elastomer. C) Upon contact and release, protein patterns tether and transfer to the final substrate. D) Proteins remain active during printing and tethering as shown in this fluorescence microscopy image, which reveals receptor mimetic antibody bound to micropatterned circles of tethered fibronectin.

Figure 2. Micrometer- and nanometer-scale patterns with various geometries are produced with high contrast. A) Arrays of eight squares (edge length 1000 nm) around a center square (2 μm) were produced over large areas (490 mm^2). B) Nanopattern features as small as 250 nm were achieved.

Figure 3. Focal adhesion (vinculin) formation on fibronectin patterns. A) Localization of the protein vinculin to focal adhesions occurs in cells adherent to surfaces printed with nonpatterned fibronectin. B) Variations in the geometry of fibronectin patterns elicit changes in the adhesive responses of adherent cells.