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Orthogonal Patterning of Multiple Biomolecules Using an Organic Fluorinated Resist and Imprint Lithography

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Abstract

The ability to spatially deposit multiple biomolecules onto a single surface with high-resolution while retaining biomolecule stability and integrity is critical to the development of micro- and nanoscale bio-devices. While conventional lithographic patterning methods are attractive for this application, they typically require the use of UV exposure and/or harsh solvents and imaging materials, which may be damaging to fragile biomolecules. Here, we report the development of a new patterning process based on a fluorinated patterning material that is soluble in hydrofluoroether solvents, which we show to be benign to biomolecules, including proteins and DNA. We demonstrate the implementation of these materials into an orthogonal processing system for patterning multi-biomolecule arrays by imprint lithography at room temperature. We further showcase this method's capacity for fabricating patterns of receptor-specific ligands for fundamental cell studies.

Keywords

Imprint lithography; Fluorinated resist; Hydrofluoroether solvents; Multi-protein patterning; Orthogonal processing; Patterning biomolecules

INTRODUCTION

Tremendous advances have recently been made in the field of bioelectronics. Biosensors, biological microelectromechanical systems (bio-MEMs), and microfluidic devices are revolutionizing biological and medical research.^{1–6} Similarly, the development of biomolecular diagnostic arrays and regenerative therapies are enabling unprecedented studies in fundamental biology and applications in tissue engineering, respectively.^{7–11} The

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SUPPORTING INFORMATION AVAILABLE:

This includes details of control tests performed for the DNA Compatibility Assay to confirm binding specificities, Differential Scanning Calorimetry (DSC) data of the ImR and a Scanning Electron Micrograph of the resist sidewall after patterning.

This material is available free of charge via the Internet at <http://pubs.acs.org>.

ability to spatially deposit multiple biomolecules with high-resolution onto a single surface is integral to the development of these bio-devices.^{12, 13}

Consequently, multi-component patterning of biomolecules has become an active area of research. Patterning techniques for single components have been extensively developed;^{12, 14} however, multi-component patterning introduces unique challenges, which have yet to be satisfactorily addressed. Importantly, multi-component protein patterning requires depositing distinct regions of biomolecules onto a single substrate while retaining biomolecule integrity and avoiding nonspecific binding.¹¹

Techniques using photolithography,^{15, 16} soft-lithography,¹⁷ dip-pen lithography,^{18, 19} electron beam lithography,²⁰ stereomask lithography,²¹ and spot-arraying²² have all been explored and represent the principal advances in the field of multi-component protein patterning. Photolithography is a mature patterning technique that is promising for biomolecule applications,^{23, 24} as it allows for high-resolution, precise alignment, and is a high-throughput process. The greatest disadvantage of photolithography is that it traditionally requires harsh photoresists and developers as well as applications of heat, which often lead to denaturation of delicate biomolecules. Many clever strategies have been developed to circumvent these requirements;^{25–30} however, these strategies are necessarily specialized and lack universal applicability. Soft-lithography is an inexpensive and parallel patterning process, which also has the capacity to produce three-dimensional structures. Yet, due to the elastomeric nature of the stamps, mechanical deformation necessarily occurs, which can also result in pattern deformation. Furthermore, soft-lithography is limited by a lack of registration capabilities.³¹ Dip-pen lithography can precisely place nanoscale arrays of proteins; though, by nature, this technique can also be prohibitively time-consuming.³² Similarly, electron beam lithography can produce patterns with high-resolution; however, this method is extremely expensive and difficult to scale up.²⁰ Stereomask lithography, while relatively new, is hindered by low resolution and high production costs as well.²¹ Though spot-arraying techniques, including ink-jet printing, can be cost-effective, the resolution is too low to be practical for many applications.³¹

Recently, we published our preparation and study of both fluorinated photoresists and light-emitting materials, which are processable in fluorinated hydrofluoroether (HFE) solvents, for application to very clean organic electronic device patterning.^{33–35} Similar to biomolecule patterning, organic electronic materials can be damaged by the harsh photoresists and solvents required by photolithography. We showed that these highly-fluorinated solvents and resist materials are completely benign to organic electronic materials such that they can be used for photolithographic patterning of these delicate materials.³⁶ HFEs have been shown to be environmentally-friendly, non-flammable, and non-toxic, and they have been used previously in protein research.³⁷ Furthermore, as process solvents, HFEs are recyclable to enable a sustainable processing system.

Here, we show that HFE solvents, along with a patternable fluorinated resist polymer that we have developed, are benign to biomolecules and can serve in a processing system for patterning biomolecules by imprint lithography. We show that proteins and DNA may be directly patterned by this benign lithography technique. In this way, high-resolution and high-throughput biomolecule patterning can be accomplished with precise alignment capabilities in a reproducible lithographic process.

We have selected imprint lithography to demonstrate the lithographic patterning properties of this biocompatible processing system. Imprint lithography has recently received significant attention as a patterning technique with the potential to assume a key role in the manufacture of nanoscale structures for electronic, optical, biological, and energy

applications.^{38, 39} Because of its capacity to produce patterns as small as 10 nm over large areas, with high-reproducibility and high-throughput capacity, imprint lithography has also found applications in protein patterning.⁴⁰ One particular advantage of this approach is that the proteins are not exposed to the denaturing effects of UV radiation as found with most photolithography. However, like most photolithography, imprint lithography also normally requires harsh resists and resist removal steps. We replace the traditional harsh processing system with a biocompatible one and demonstrate the lithographic patterning of DNA and multi-protein arrays. In addition, we show that this resist and patterning technique can be used to pattern protein arrays for fundamental studies with cells.

EXPERIMENTAL SECTION

MATERIALS

Benzotrifluoride (TFT); 3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-heptadecafluorodecyl methacrylate; 3-aminopropyl (trimethoxysilane) (APTMS); and 3-aminopropyl (triethoxysilane) (APTES) were purchased from Sigma Aldrich (St. Louis, MO). Due to the loss of the alkoxy groups APTMS and APTES result in surfaces of similar chemistry. Azobisisobutyronitrile (AIBN) was purchased from Sigma Aldrich and recrystallized from CHCl_3 . 3MTM NovecTM Engineered Fluid Hydrofluoroether (HFE) 7200 and HFE 7500 were purchased from 3MTM USA (St. Paul, MN).

Bovine serum albumin (BSA), biotin-BSA, and streptavidin labeled with horseradish peroxidase (streptavidin-HRP) were purchased from Sigma Aldrich. DNP-BSA was prepared from BSA conjugated to 2,4-dinitrophenyl (DNP), (Invitrogen, Carlsbad, CA), with approximately 20 DNP per BSA. Cy3 and Cy5 fluorescent dyes were purchased from GE Healthcare Life Sciences (Piscataway, NJ) and conjugated to DNP-BSA, with a stoichiometry of 2–3 molecules of each dye per DNP-BSA. Mouse monoclonal anti-DNP Immunoglobulin E (IgE) was purified as described by Subramanian et al.⁴¹ The IgE was then modified with AlexaFluor 488 (A488), purchased from Invitrogen, according to the labeling kit instructions. Streptavidin-AlexaFluor 568 (streptavidin-A568) and streptavidin-AlexaFluor 546 (streptavidin-A546) were purchased from Invitrogen and used as received. The 20 mer DNA probe 5'-CTGAACGGTAGCATCTTGGA-3' construct with biotin at its 5'-terminus and the 20-mer target DNA 5'-TCCAAGATGCTACCGTTCAG-3' construct with A488 at its 5'-terminus were purchased from Integrated DNA Technologies (San Diego, CA). Immunopure streptavidin and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) peroxidase substrate were purchased from Pierce (Rockford, IL). Mouse monoclonal antibodies (Mab clones AP003S and BP005S) against prostate specific antigen (PSA) were purchased from Scripps Laboratories (San Diego, CA). Free-PSA calibrators in human serum were obtained from CIS Bio-International (Bagnols-sur-Cèze, France). Mouse monoclonal anti-PSA antibody (clone BP005S) conjugated to biotin was prepared according to a published method.⁴² MaxiSorp polystyrene 96 well-plates were purchased from Nunc (Roskilde, Denmark).

METHODS

Synthesis and Characterization of Imprint Resist (ImR)

To a 25 cm³ Schlenk tube, 7.00 g (13.15 mmol) of 3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10-heptadecafluorodecyl methacrylate was added followed by 0.07 g (0.43 mmol) AIBN and 7 mL TFT. The tube was then sealed and degassed by three freeze-thaw cycles in liquid N₂ under reduced pressure. The reaction was stirred at 75 °C for 12 h under a N₂ atmosphere. The solution was then precipitated in hexanes and dried under reduced pressure to give a colorless solid, yielding 6.5 g (93 % of theoretical yield). Characterization of ImR: IR: ν = 1732, 1194, 114, 704, 655, 559 cm⁻¹; ¹H NMR (400 MHz, CDCl₃:CFCl₃ (v/v)): δ = 4.25

(br s, 2 H, CH_2CF_2), 2.49 (m, 2H, $\text{CH}_2\text{CH}_2\text{CF}_2$), 1.67-0.80 ppm (m, 5 H); T_d (TGA) = 168.92 °C; M_n = 611,000; M_w/M_n = 1.2. GPC measurement was performed by Asahi Glass Company according to the published method.⁴³

Lithographic Evaluation

The lithographic properties of ImR, using silicon wafers, were investigated using a Nanonex NX-2500 nanoimprint lithography tool. The resist films were spun-cast from a solution of polymer (0.05 g) in HFE 7500 (1.0 g) at 2000 rpm. The resulting films had a thickness of *ca.* 200 nm. Before imprint, the film was exposed to a 5 sec oxygen plasma clean to improve the adhesion to the template. Films were imprinted at 300 psi and 25 °C for 3 min using a fused silica stencil with *ca.* 600 nm feature relief. The imprint stencil was prepared through standard photolithographic technique⁴⁴ on a fused silica mask with feature sizes ranging from 1 μm to 100 μm .

Streptavidin-Biotin Binding Biocompatibility Assay

The effect of ImR and HFE solvents on streptavidin-biotin interactions was investigated. Streptavidin solutions of final concentrations 1 $\mu\text{g/mL}$, 2 $\mu\text{g/mL}$, and 5 $\mu\text{g/mL}$ were prepared in carbonate buffer (43 mM NaHCO_3 , 7 mM Na_2CO_3 , 0.05 % (w/v) NaN_3 , pH 9.2). These solutions were deposited into three separate sets of microtitration wells and incubated for 1 h at room temperature (RT) for protein adsorption. Then the supernatants were decanted, and the wells were washed twice with a 10 mM Tris-HCl solution (washing solution, pH 8.25) before refilling with 100 mM NaHCO_3 (pH 8.5) supplemented with 10 mg/mL BSA for 1 h at RT to minimize nonspecific binding. Finally, wells were rinsed with washing solution (pH 8.25) and followed by two rinses with distilled water before adding test samples.

To one set of wells (Set 1), consisting of wells coated with 1 $\mu\text{g/mL}$, 2 $\mu\text{g/mL}$, and 5 $\mu\text{g/mL}$ streptavidin concentrations, washing solution was added. To a second set of wells (Set 2), HFE 7200 solvent was added. To the third set of wells (Set 3), a 10 % (w/v) solution of ImR dissolved in HFE 7500 was added and incubated for 2 min at RT and then decanted. Set 3 wells were then baked for 5 min at 50 °C. To remove the resist, Set 3 wells were washed in HFE 7200 four times for 3 min each while shaking, which mimics the processing conditions of ImR removal after each patterning cycle. The wells of Sets 1 and 2 remained filled with buffer and HFE solvent, respectively, for the whole duration of processing Set 3. All wells in Sets 1, 2, and 3 were finally decanted and rinsed first with washing solution (pH 8.25) and then with distilled water before testing for biotin binding capacity.

To test the binding capacity of streptavidin immobilized in the wells, 100 μL of 100 ng/mL BSA multiply conjugated with biotin in phosphate buffer (16 mM Na_2HPO_4 , 34 mM KH_2PO_4 , pH 7.0) or 100 μL of blocking solution (phosphate buffer, pH 7.0, containing 10 mg/mL BSA) were added to the wells and incubated for 30 min at RT. Following streptavidin-biotin binding, wells were rinsed four times with TWEEN washing buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05 % TWEEN20 (v/v)). To detect the bound biotin-BSA, a solution of 250 ng/mL streptavidin-HRP in blocking solution was added to all wells and incubated for 15 min at RT while shaking. Wells were washed as described above. The presence of streptavidin-HRP was determined via addition of ABTS peroxidase substrate solution and incubation for 30 min at RT while shaking. Absorption signals were measured at 405 nm on a Labsystems Multiskan RC microplate reader.

Antibody-Antigen Interaction Compatibility Assay

To investigate the effect of ImR and HFE solvents on antibody-antigen binding, a solution of 5 $\mu\text{g/mL}$ mouse monoclonal anti-prostate specific antigen (Mab-PSA) in carbonate buffer

(pH 9.2) was deposited into three separate sets of microtitration wells and incubated overnight at RT to adsorb. Wells were then washed, blocked, and processed as described above for the streptavidin-biotin binding assay. To each set of wells, 20 μ L of free-PSA calibrator solutions (0, 0.39, 0.95, 2.48, and 4.9 ng/mL) and 100 μ L of 5 μ g/mL biotinylated anti-PSA monoclonal antibody solution in Tris-HCl buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mg/mL BSA, pH 8.25) were added and incubated for 1 h at RT while shaking. Wells were then washed four times with TWEEN washing buffer. PSA, bound to the immobilized antibodies, was detected via addition of streptavidin-HRP and ABTS peroxidase substrate solution in the sequence described above for the streptavidin-biotin binding assay. Absorption was measured at 405 nm as described above.

DNA Compatibility Assay

For testing the effect of ImR and HFE solvents on the binding of complementary DNA strands, a 20-mer probe 5'-CTGAACGGTAGCATCTTGGA-3' was selected with its complementary target sequence 5'-CCAAGATGCTACCGTTCAG-3'.⁴⁵ The probe DNA contained biotin at its 5'-terminus while the target DNA was labeled with A488 at its 5'-terminus for fluorescence detection. Both constructs were purchased post-modification from Integrated DNA Technologies, Inc.

Silicon wafers were patterned with 1 μ m or 5 μ m features using the parylene lift-off method as previously described.^{28, 46} The silicon wafer chips with patterned parylene layer were first incubated with streptavidin-A568 (25 μ g/mL in phosphate buffer, pH 7.0) for 30–60 min at RT, which adsorbed to the substrate, and this was followed by rinsing with phosphate buffer (pH 7.0). Nonspecific binding was prevented by incubation with blocking solution for 15 min at RT and followed by rinsing again with phosphate buffer (pH 7.0). To assemble the ssDNA monolayer, these chips were incubated with 1 μ M biotinylated DNA probe in phosphate buffer for 30–45 min at RT, followed by rinsing three times with phosphate buffer (pH 7.0). Separate chips were then incubated with a solution of phosphate buffer (pH 7.0), HFE 7200, or ImR for 5 min at RT. In the sample with ImR, the resist was baked for 5 min at 37 °C and then removed via three separate washes with HFE 7200. To test the capacity for binding complementary DNA strands, the sample chips were rinsed three times with phosphate buffer (pH 7.0) and incubated with 0.75 μ M A488-labeled target DNA in phosphate buffer for 30 min at 37 °C followed by 15 min at RT to allow for DNA hybridization. The sample chips were then incubated with blocking solution and the parylene layer was peeled off. The remaining DNA patterned arrays were imaged using a x63 (NA of 1.4) oil immersion objective lens on a Zeiss 710 confocal microscope. A488-target DNA was excited using the 488 nm line of an argon laser. Streptavidin-A568 was excited using a 561 nm laser line. Acquired images were processed with ImageJ software.

Assay for Protein Stability over Repeated Cycles of Imprint Lithography

This assay assessed the stability of immobilized proteins undergoing multiple cycles of resist application and removal with ImR and HFE solvents. Prior to immobilizing proteins, silicon wafers were first hydrophilized for 20 min in Piranha solution (1:1 v/v concentrated H₂SO₄: 30 % H₂O₂) and then thoroughly washed with distilled water. *Caution: Piranha is a very strong oxidant and should be handled with care.* After drying the wafers under a stream of N₂, they were immersed in 2 % (v/v) aqueous APTES for 20 min at RT. Wafers were gently washed, dried with N₂, and baked for 20 min at 120 °C. Then, one wafer was incubated with phosphate buffer (pH 7.0) containing 25 μ g/mL of a biotinylated-BSA for 1 h at RT, while a second wafer was incubated with 25 μ g/mL BSA solution for blank measurements. After this step of protein adsorption, both wafers were washed with phosphate buffer (pH 7.0) and blocking solution for 1 h at RT. After rinsing with washing solution containing 150 mM NaCl (pH 8.25) and distilled water, the wafers were then dried

and a piece of each was cleaved off to be kept as a control. The remaining wafers were spun-cast with ImR, baked for 5 min at 50 °C, and developed with HFE 7200 to remove the resist.

The resist application/removal cycle described above was repeated up to ten times. A piece was cut from each wafer following 1, 2, 4, 6, 8, and 10 cycles. All wafer pieces were then covered with 5 µg/mL streptavidin-A546 in blocking solution, and incubated for 30 min at RT. After washing with phosphate buffer (pH 7.0) containing 0.05 % (v/v) TWEEN20 and distilled water, the chips were dried with N₂ gas. The stability of the adsorbed protein to repeated resist application/removal cycles was evaluated by fluorescence intensity measurements on images (5 per sample) acquired via an Axioscope 2 Plus epifluorescence microscope facilitated with a Sony Cyber-Shot digital camera and processed with the ImagePro Plus software.

Fabrication of Protein Arrays

Patterned arrays containing one or two different proteins were fabricated as follows. ImR was spun-cast from solution in HFE 7500 onto a silicon substrate to yield a *ca.* 200 nm polymer film. The film was imprinted at room temperature as described above (see Figure 5A). A layer of APTMS was then vapor deposited using an Applied Microstructures MVD100 vapor deposition tool. The self-assembling monolayer of APTMS is used to provide reactive (-NH) groups at the wafer surface for biomolecule adhesion. Protein solutions containing 25 µg/mL of either DNP-BSA or biotin-BSA in phosphate buffered saline (PBS, pH 7.4) were prepared. The first protein solution was incubated for 1 h at RT on the wafer surface before being rinsed with additional PBS. Following deposition of the first protein, the resist was removed using washes with HFE 7200 while shaking. Onto the first patterned protein layer, another layer of ImR was spun-cast and patterns were then imprinted offset from the initial protein layer. APTMS was once again vapor deposited on the surface of the resist. The second protein solution was incubated for 1 h at RT on the wafer surface before being rinsed with PBS and removing the resist with HFE 7200.

After these two distinct steps of protein immobilization, photoresist was removed using 10 min washes with HFE 7200 while shaking. Then, to prevent nonspecific binding of fluorophores, wafer chips were incubated with PBS blocking solution (PBS, 10 mg/mL BSA, pH 7.4) for 15 min at RT followed by rinses with PBS. For detection of each protein, fluorescently labeled components (25 µg/mL in PBS) were added in separate incubations with the wafer chips for 30–45 min each at RT followed by rinsing with distilled water between each fluorophore addition. Biotin-BSA was detected with Streptavidin-A568 and DNP-BSA was detected with A488-IgE specific for DNP. Fluorescence microscopy was performed using a Zeiss 710 confocal microscope with the same lens and lasers described above for the DNA compatibility assay with A488 and A568 fluorophores. Acquired images were processed with ImageJ software.

Cell Culture

Rat basophilic leukemia (RBL-2H3) cells⁴⁷ were maintained in a monolayer culture in Minimum Essential Medium (MEM) from Invitrogen (Carlsbad, CA) supplemented with 20 % Fetal bovine serum (FBS), purchased from Atlanta Biologicals (Lawrenceville, GA), and 10 µg/mL gentamicin sulfate, purchased from Invitrogen. Cells were harvested with trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, Invitrogen, Carlsbad, CA) three to five days after passage.⁴⁸

Ligand-Mediated Cell Patterning

A layer of ImR was imprinted atop a silicon substrate. A solution of 25 µg/mL of DNP-BSA, conjugated to cyanine fluorescent dyes (Cy3-Cy5), in PBS was deposited and

incubated with the substrate for 1 h at RT for protein adsorption. The resist was removed, using washes in HFE 7200, leaving behind the patterned DNP-BSA Cy3-Cy5. RBL-2H3 cells were first sensitized by incubating for 40–60 min at 37 °C with 2–3 µg/mL A488-IgE, specific for DNP, then washed and re-suspended at a concentration of $1\text{--}2 \times 10^6$ cells per mL in buffered saline solution (BSS, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 20 mM HEPES, pH 7.4) supplemented with 1 mg/mL BSA. Sensitized cells were added to the protein patterned substrate in the center of a 35 mm Petri dish with coverglass bottom (0.16–0.19 mm; MatTek Corp., Ashland, MA), as described by Torres et al.⁴⁵ After 30–60 min of incubation at 37 °C, cells were fixed with 4 % paraformaldehyde in PBS for 20 min followed by quenching with PBS blocking solution. After fixation, the substrates were imaged using a x63 (NA of 1.4) oil immersion objective lens on a Zeiss 710 confocal microscope. A488-IgE was excited using the 488 nm line of an argon laser and Cy3-Cy5 DNP-BSA using a 561 nm laser line. Acquired images were processed with ImageJ software.

RESULTS

Imprint Resist (ImR) Is Patternable

Imprint resist (ImR) was synthesized by radical polymerization of 2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9,9-Heptadecafluorononyl methacrylate using AIBN as the radical initiator (Figure 1A). ImR was designed to be fluorinated enough to be processable in HFE solvents and also free of unnecessary functional groups that might react with biomolecules. The resulting polymer demonstrated sufficient solubility to be processable in both HFE 7200 and HFE 7500 solvents.

To demonstrate its capacity for patterning, ImR was tested using imprint lithography (Figures 1B–F). During imprint lithography, a pattern is “stamped” into the resist using high pressure, rather than UV-exposure techniques required by traditional photolithography, which cause biomolecule damage. A solution of ImR in HFE 7500 was spun-cast onto a wafer surface, forming a thin film. Several parameters were varied and tested to identify the optimal set of imprint processing conditions, including temperature, pressure, imprint time, resist thickness, and surface treatments of both the stamp and substrate. Optimal results were achieved by imprinting at 25 °C and 300 psi for 3 min. Since this temperature is well below ImR’s glass-transition temperature or melting temperature, the patterns are not formed by the traditional process of resist flowing to take the shape of the template’s features. Rather, we hypothesize that patterns are formed by the resist adhering to the template more strongly than the substrate. The ImR is then pulled off with the template during imprinting. We suspect that this is facilitated by the formation of cracks in the resist at the edge of imprint features due to shear forces present between the imprinted, compressed regions and the non-imprinted regions. See the Supporting Information for Differential Scanning Calorimetry data and a Scanning Electron Micrograph of the imprinted ImR side wall profile. Square features ranging in size from 1 µm to 100 µm were obtained using a silica template with corresponding features and *ca.* 200 nm relief, as shown in Figures 1B–F.

ImR Is Compatible with Streptavidin Patterning and with PSA Monoclonal Antibody Patterning

We examined the capacity of ImR to pattern binding-active proteins. Specifically, a streptavidin-biotin assay was employed to determine the effect of ImR and HFE solvents on binding between immobilized streptavidin and a biotinylated ligand. For the assay, three sets of samples were evaluated (see Materials & Methods), and ImR and HFE solvents were deposited onto adsorbed streptavidin so as to mimic the processing steps required for imprint patterning. The capacity of streptavidin to bind biotin-BSA following exposure to HFE 7200

or ImR in HFE 7500 was tested and compared to a control set which received only buffer (Figure 2A). The amount of biotin bound in each well was detected using HRP-streptavidin conjugate and a chromogenic peroxidase substrate (H_2O_2 /ABTS) to produce measurable signal.

As shown in Figure 2A, the immobilized streptavidin was not affected by the resist application/removal procedure. At each streptavidin concentration, the optical density (OD) values for those samples treated by ImR application/removal (open triangles) are within standard deviation of the respective control samples treated only with washing solution (open squares), which suggests that the binding capacity, and thus the structure, of the immobilized streptavidin was not significantly altered by the addition and removal of ImR. However, it should be noted that samples treated solely with HFE solvent (open circles) produced a slight decrease (5–18 %) in the binding capacity of immobilized streptavidin. This effect could be ascribed to the fact that the solvent remained in contact with the streptavidin in Set 2 wells for the whole duration of the resist application, bake, and removal required for Set 3 wells (a total time of approximately 20 min). Whereas, in the case of ImR treated samples (Set 3 wells), this resist layer may have played a protective role against the solvent effect seen in the Set 2 wells. OD values from control wells incubated with non-biotinylated-BSA (blanks) are shown in Figure 2A as closed circles. In addition, separate blanks involving wells treated with HFE 7200 or ImR and non-biotinylated-BSA were tested (data not shown) and the OD values obtained from these trials were not statistically different from the blank values shown.

The capacity of PSA monoclonal antibody to bind PSA following exposure to HFE solvents and ImR was investigated. Similar to the streptavidin-biotin binding assay described above, HFE 7200 or ImR in HFE 7500 was placed onto immobilized anti-PSA antibody under conditions that mimic lithographic processing conditions. Following this, a sandwich type assay involving the addition of both PSA standards and biotinylated anti-PSA antibody was performed. The presence of biotinylated anti-PSA antibody was then determined using addition of streptavidin-HRP and incubation with ABTS peroxidase substrate. These results were compared with those from the control samples, which did not receive HFE solvent and/or ImR applications (Figure 2B). At low PSA standard concentrations ($< 1 \text{ ng/mL}$), the absorbance values for the control (open squares), HFE 7200 treated (open circles), and ImR treated (open triangles) samples are similar. However, at higher PSA standard concentrations (2.48 and 4.9 ng/mL), both the HFE 7200 and resist treated samples show a similar slight, yet significant, decrease in absorbance values as compared to samples incubated with washing solution only (control). In addition, blanks comprising wells treated with non-biotinylated anti-PSA antibody were tested and confirmed binding specificity for each experimental condition. These values were subtracted from their corresponding preparation condition – washing solution (control), HFE 7200, or ImR in Figure 2B. Thus the signals obtained from each experimental set are indeed due to the specific binding of biotin or PSA.

ImR Is Compatible with Immobilized Oligonucleotides

A DNA hybridization assay was performed to determine the effects of ImR and HFE solvents on patterned single-stranded DNA. A 20-mer DNA probe and its complementary target sequence were selected for this assay because they anneal stably at RT.⁴⁵ Additionally, the DNA probe was synthesized with biotin at the 5'-terminus to serve as a linker. For fluorescence detection, the target complementary DNA strand was labeled with A488 at the 5'-terminus.

Square arrays, formed using the parylene lift-off patterning method,^{28, 46} were chosen to pattern the initial streptavidin protein layer. To confirm uniform adsorption of the protein to

the silicon wafer surface, the streptavidin was conjugated to A568 for visualization. The biotinylated DNA probe was incubated with the immobilized streptavidin. Samples then underwent treatment with control buffer, HFE solvent, or ImR application and removal so as to mimic lithographic processing techniques. Following treatment, the complementary ssDNA target-A488 was added to the biotin-DNA probe by first relaxing the DNA strands at 37 °C and then allowing them to hybridize at RT.

As visualized with fluorescence microscopy, neither treatment with HFE solvent nor ImR impeded the capacity of the target DNA-A488 strand to bind to the complementary DNA probe (Figure 3). Expression of the DNA A488 target strand mirrors the expression of the underlying streptavidin-A568 with similar abundance whether treating with control buffer, HFE solvent, or ImR. This suggests that the ssDNA is not significantly degraded by the HFE solvent and ImR materials or by the treatment conditions, as DNA hybridization occurs readily. It should be noted, however, that the physical application and removal of ImR may partially remove the adsorbed streptavidin-ssDNA complex, as evidenced by the heterogeneous appearance of both probe and target DNAs in Figure 3. Covalent binding between the surface and the initial streptavidin layer may prevent this loss of protein-ssDNA as we have observed in other experiments (data not shown). Additional controls were performed to confirm binding specificities and to rule out bleed through from green to red channels (Supplementary Figure 1). In particular, when Cy3-Cy5 BSA was adsorbed to the wafer surface in place of streptavidin-A568, neither the biotin-DNA probe nor the complementary target DNA-A488 bound to the patterns (Suppl. Figure 1D). Similarly, omission of the biotin-DNA probe prevented binding of the complementary target DNA-A488 to the adsorbed streptavidin proteins (Suppl. Figure 1C).

Repeated Imprint Lithography Cycles Do Not Adversely Affect Previously Deposited Proteins

Further tests were performed to determine the potential of using ImR and imprint lithography for sequentially patterning multiple biomolecules. Biotin-BSA solution was adsorbed onto a clean, aminosilanized silicon wafer surface. ImR was then applied and removed with HFE solvents multiple times to mimic lithographic patterning techniques. A small piece was cut off from the wafer at 0, 1, 2, 4, 6, 8, and 10 cycles of resist addition and removal. These ten cycles serve to imitate the processing effects of imprinting ten subsequent resist layers onto the first biomolecule deposited. The chips were then incubated with streptavidin-A546 for detection of the protein remaining on the chip surface. The stability of protein adsorption after repeated resist application and removal cycles was evaluated by fluorescence intensity measurements. As shown in Figure 4, the relative fluorescence intensity of streptavidin-A546 bound to biotin-BSA (white columns) did not vary significantly over the course of ten resist application and removal cycles as compared to the original surfaces (at 0 cycles). A series of negative control wafer chips involving adsorption of non-biotinylated BSA (blank; black columns) in place of biotin-BSA was also performed and tested in parallel. These blank chips result in considerably lower signal values compared to those coated with biotin-BSA, further validating that the fluorescent signal measured was due to specific streptavidin-biotin binding.

ImR Can Be Used for Orthogonal Patterning of Multiple Proteins

To demonstrate the potential of ImR and imprint lithography for multi-component biomolecule patterning, protein arrays containing one or two different proteins were fabricated (Figure 5). Clean silicon wafers coated with ImR were patterned using the imprint lithography technique described in the Methods section and Figure 5A. Following aminosilanization of the patterned surface, the first protein solution was allowed to adsorb across the entire patterned area (Figures 5B–C). Lift-off was performed by washing the

wafer repeatedly with HFE solvent, which allowed for removal of the resist while retaining the adsorbed patterned protein. For multi-protein patterning, a new layer of ImR was then applied to coat the entire wafer. New patterns were imprinted offset by 50–100 μm from the original protein patterned underneath. After deposition of a second protein sample, the resist was removed.

To demonstrate multi-protein patterning with ImR and HFE solvent, DNP-BSA and biotin-BSA proteins were patterned in sequential layers onto silicon wafer surfaces with feature sizes ranging from 1 μm to 100 μm . Following both rounds of protein deposition, separate solutions of fluorescently labeled (anti-DNP) IgE and streptavidin were added to bind specifically to their respective ligands. Patterns were then imaged with fluorescence microscopy. Example images of single protein patterns and two-protein arrays are depicted in Figures 5B–E. It should be noted that small amounts of residual resist occasionally remained on the wafer following final resist removal, and this appeared as small spots between the features and/or flaps adjacent to feature edges. These spots typically occur on or close to the locations of our alignment marks on the imprint stencil, suggesting that repeated imprinting directly onto these closely spaced marks can sometimes make it difficult to remove all of the resist. Because our imprint technique does not rely on the traditional process of resist flowing around the template's features, we attribute the formation of flaps around the edges of some features to the development of cracks at the edge of the imprint feature causing formation of misshapen features. Additionally, the competition between shear forces both above and below the resist during imprint processing may cause adjacent regions of resist, such as flaps, to remain. As marked by white arrows in Figures 5B–E, some labeling fluorophores can bind nonspecifically to residual resist and appear as small spots (Figure 5D) and/or flaps on feature edges (Figures 5B & 5E) among the protein patterns.

Aside from traditional multi-protein patterns, where multiple proteins are deposited as arrays with distinct spacing between each feature (Figure 5D), our patterning technique also allows fabrication of multi-protein patterns with features of one protein located within features of a different protein (Figure 5E). Previous multi-protein patterning efforts have focused on two-protein patterns wherein one protein is deposited with discrete features and the second protein is used to fill in the surrounding background.²⁵ Our approach differs from this backfill technique by providing the ability to adhere new layers of proteins within already existing layers and in a spatially defined manner. While the second round of imprinting did remove deposited biotin-BSA proteins directly below template features to allow for deposition of DNP-BSA, the remaining biotin-BSA layer remains unaffected. Addition of fluorophores after all imprinting had taken place reveals the specificity of the second protein deposition to discrete features and further demonstrates that ImR successfully protects previously adhered protein layers. This technique makes possible the ability to deposit a large number of protein layers and thus fabricate much more complicated arrays.

ImR Is Compatible with Ligand-Mediated Cell Patterning

Further tests were performed to demonstrate the capabilities of this resist and patterning method with ligand-mediated cell patterning. Previous studies have shown that RBL cells sensitized with anti-DNP IgE display parallel clustering of receptor-bound IgE above patterns of DNP ligands.^{28, 46} We examined the capacity of ImR to pattern DNP-labeled proteins such that cells retain their capacity to interact with these proteins. A single protein array of Cy3-Cy5 labeled DNP-BSA was patterned onto an aminosilanized wafer surface with ImR as described in the Methods section. RBL-2H3 mast cells, sensitized with A488-IgE specific for DNP, were allowed to settle onto the wafer surface for several minutes before undergoing fixation and imaging. As shown by Figure 6, the A488-IgE-receptor complexes on these cells cluster over the patterned DNP-ligands, consistent with previous

studies. These results indicate that any residual polymeric resist or HFE solvents left behind on the patterned silicon surface do not prevent cell adhesion or mobility of the cell's surface proteins. Thus, our processing conditions for imprint patterning are also biocompatible with ligand-mediated cell patterning, which points to its use in fabricating multi-protein patterns for fundamental studies of protein interactions on cells.

DISCUSSION

Previously, we synthesized fluorinated resists and light-emitting materials and showed that they can be processed in HFE solvent to fabricate organic electronic devices.^{33–36} Sarkari et al.³⁷ also have shown these HFEs to be applicable to the patterning of proteins, whose delicate structures can easily be damaged with traditional lithography solvents and techniques. Based on the favorable results obtained in these previous studies, the idea of benign lithographic processing was extended more generally to biomolecule patterning. We chose a resist structure, which would be fluorinated enough to be processable in HFE solvents and which was also free of unnecessary functional groups that might interact with biomolecules. We synthesized ImR (Figure 1A) as a potential resist for this application. Upon further testing, ImR also proved to be soluble in HFEs.

We selected imprint lithography to demonstrate the lithographic patterning properties of this processing system, because of its capacity to create reproducible patterns of small features over a large area and its previous application to protein patterning.⁴⁰ While the use of UV radiation is not required for imprint lithography, this technique typically uses harsh resists and developers, much like photolithography methods, which can easily damage and denature biomolecules. Here, we replace the traditional harsh processing conditions with benign fluorinated ImR and with unreactive HFEs as processing solvents.

Traditional imprint lithography requires a moldable resist, such that it “flows” into the recesses of the imprint stamp during imprinting and releases from the stamp afterwards. Imprinting occurs through applied pressure of the stamp and simultaneous heating of the resist. Typically, samples are significantly heated to temperatures above the glass transition temperature (T_g) of the resist material. However, for biomolecule patterning applications, significant heating would damage and denature the proteins and DNA. Therefore, we developed an imprinting process that requires no heating and can be conducted in its entirety at room temperature. As this process does not rely on the flow of the resist, large features and small features can be formed with ease, both adjacent to and placed within one another.

Additionally, we investigated different surface treatments for both the stamp and substrate, including (1H,1H,2H,2H-perfluorooctyl)trichlorosilane (FOTS), oxygen plasma treatment, Piranha cleaning, and using untreated silicon with a native oxide. Traditionally, patterns are stamped into the imprint resist layer to give a degree of feature relief (Figure 5A). The resist is then anisotropically etched to remove the remaining resist residue in the thinner regions to produce patterns, which are clean to the substrate. Our low-temperature process results in practically clean removal of the resist from the substrate without the need for an additional ‘descum’ etch. Samples of ImR post-imprinting are shown in Figures 1B–G. Both ImR and HFEs were demonstrated to be benign to biomolecules in that they did not significantly deteriorate protein and/or DNA binding capacities (Figures 2–3). Varying concentrations of streptavidin, a robust protein widely used for immobilization of biomolecules,⁴⁹ were liberally exposed to ImR and HFE solvents under conditions which imitate lithographic processing. The capacity of streptavidin, post ImR exposure, to bind biotin was experimentally measured and found to be similar to that of streptavidin without any exposure to resist or solvent (Figure 2A). While longer time exposure to HFE solvent appeared to produce a small decrease in the binding capacity of the immobilized

streptavidin, use of ImR and HFE solvents on streptavidin under our fabrication conditions did not significantly alter the capacity of the protein to bind to the biotin moiety. Similarly, we have shown the ability to hybridize complementary DNA strands following treatment with ImR and HFE solvents (Figure 3), indicating that the probe DNA strand was not significantly altered by the treatment conditions. Together, these results show that both ImR and HFE solvents are compatible with biomolecule patterning.

As another sensitive test for binding capacity dependent on protein structure, a similar assay was performed wherein an anti-PSA antibody was exposed to HFE solvent and/or ImR (Figure 2B). At the higher PSA concentrations, both the solvent and resist treated samples produce a small decrease in absorbance values as compared to the buffer controls. These results indicate that the anti-PSA binding region is more vulnerable to resist application/removal processing as compared to streptavidin, consistent with a more complicated and sensitive binding structure. While streptavidin binds biotin via non-covalent interactions deep within the β -barrel structure,⁵⁰ anti-PSA binding sites contact PSA epitopes at four to five individual segments of the protein structure.⁵¹ Thus, the binding site of streptavidin is both more protected and requires fewer interactions with tertiary protein structure as compared to anti-PSA antibodies.

A major advantage of our imprint patterning technique is its capacity for multi-component patterning. The ImR resist and HFE solvents are inherently benign to biomolecules, and we found that these imprinting materials can be applied at least 10 times without adversely affecting the underlying biotinylated protein layers to bind streptavidin (Figure 4). Thus, biomolecules deposited prior to resist application are not significantly damaged by undergoing additional rounds of patterning. We directly demonstrated our technique's capacity for fabricating multi-protein patterns (Figures 5B–E). To detect for each distinct protein, we relied on bio-specific interactions: biotin binding to streptavidin and DNP binding to anti-DNP IgE. We further showed that patterns can be fabricated with multi-protein components in separate feature arrays or as features within other features. With this technique, it should be possible to fabricate much more complicated multi-component biomolecule arrays for a variety of applications.

One such application, the capacity to fabricate patterns biocompatible with cells, can be valuable for tissue engineering applications⁹ as well as fundamental cell studies. To this end, we investigated cellular interactions with imprinted surfaces. As demonstrated with RBL-2H3 mast cells and DNP-BSA protein, which was patterned using ImR and HFE solvents (Figure 6), we found that the cells showed no signs of abnormal morphology or cytotoxicity. Furthermore, the cells exhibited the expected response of (anti-DNP) IgE-receptor clustering over the DNP patterns. These results point to the use of this ImR and patterning method in fabricating multi-protein patterns for investigations of more complicated cellular mechanisms, such as cellular responses to multiple, spatially defined stimuli.

Although our patterning technique advantageously addresses many of the challenges of biomolecule patterning with traditional lithography methods, including biocompatibility, high costs, and long production times, the full potential has not yet been reached. We were able to pattern features with dimensions as small as 1 μm , whereas tens of nanometers should be possible with available imprint lithography technology. Our current technique was limited both by the feature dimensions of our stencil and our characterization capability. In particular, the diffraction limit and the high reflectivity of silicon wafers can make it difficult to discern submicron features with fluorescence microscopy. However, fabrication of smaller features and on silicon and glass surfaces would allow for broader applications of this patterning method. We found that alignment marks, while necessary for multi-

component patterning, allowed stray depositions: As more layers of ImR were applied and imprinted in the alignment areas, it became more difficult to remove completely the resist following each imprint cycle in these areas. Repeated imprinting on top of the same alignment marks may alter the shear forces of the silicon surface in those areas, providing a stronger bind between the silicon and the ImR. Further evidence of shear forces affecting our pattern resolution includes the formation of residual resist flaps and spots among the features. Additional improvements in this technique will focus on both higher resolution and higher fidelity multi-component patterns.

CONCLUSIONS

We described an approach for multi-biomolecule patterning based on a highly-fluorinated polymer resist (ImR) that is processable in HFE solvents. We showed ImR to be patternable by imprint lithography at room temperature, in a UV exposure free process, producing feature sizes down to 1 μm . Both ImR and HFE solvents are largely benign to biomolecules, including proteins and DNA, and suitable for use in cell studies. We further used this methodology to pattern single-protein and two-protein arrays of DNP-BSA and biotin-BSA, demonstrating its potential for multi-component patterning. In addition, we showed that the surface immobilized biomolecules can undergo at least 10 cycles of lithographic processing with negligible effects. Thus, this versatile method makes possible the fabrication of arrays with multiple, spatially defined biomolecules, while avoiding many of the problems associated with more traditional lithographic approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Preparation and patterning of ImR

(A) Synthesis of ImR. ImR (shown in gray) patterned by imprint lithography leads to exposure of the underlying bare silicon wafer surface (white) with: (B) 100 μm features, (C) 50 μm features, (D) 10 μm features, (E) 5 μm features, (F) 2 μm features, and (G) 1 μm features.

Figure 2. Effect of HFE solvent and ImR application/removal onto the binding capacity of immobilized proteins

Wells coated with (A) streptavidin or (B) anti-PSA antibody were incubated with washing solution (control set; closed squares), with HFE 7200 (open circles), or were subjected to ImR application and removal (open triangles). (A) Streptavidin's capacity to bind its biotin ligand was then tested by applying a sandwich type assay for biotinylated-BSA as described in the Methods section. Concentrations of streptavidin solutions to coat wells are indicated. Blank values obtained using coated control wells, not incubated with biotinylated-BSA, are shown (closed circles). Data represents the mean of four separate trials. (B) Detection of free-PSA after application of a sandwich type assay, described in the Methods section, to the wells coated with anti-PSA antibody. Concentrations of free PSA incubated with the coated wells are indicated. Blank signal values have been subtracted from the values of all three treatment conditions. Data represents the mean of three separate trials. All error bars correspond to $\pm$ SD.

Figure 3. Effect of HFE 7200 and ImR application/removal on the binding of complementary strands of DNA

A biotinylated DNA probe was immobilized onto the wafer surface by binding to adsorbed streptavidin-A568. To determine stability of the probe DNA, a complementary target DNA-A488 strand was then added and detected using fluorescence microscopy. Prior to incubation with A488 target DNA strand, the surfaces were treated with control buffer (phosphate buffer, pH 7.0), with HFE solvent, or ImR application and removal. Scale bars represent 10 μm .

Figure 4. Effect of repeated lithography cycles on deposited protein

Fluorescence intensity values obtained from aminosilanized Si surfaces coated with BSA (black columns) or biotinylated-BSA (white columns) and subjected to repeated ImR application and removal cycles before being incubated with streptavidin-A546. The fluorescence values are expressed as percentages of the control sample value at 0 cycles. Data represents the mean of five separate experiments. Error bars denote SD.

Figure 5. Preparing multi-protein arrays. (A)

Schematic for patterning a multi-protein array by imprint lithography using ImR and HFE solvent. **(B–E)** Fluorescence microscopy images of single and two-protein patterns prepared in this manner. **(B)** 100 μm square of patterned biotin-BSA labeled with streptavidin-A546. **(C)** 20 μm squares of patterned DNP-BSA labeled with (anti-DNP) A488-IgE. **(D)** A two-protein array of separate 20 μm squares and **(E)** a two-protein array with 10 μm square features within a 100 μm square feature. White arrows highlight spots of residual resist and/or formation of flaps along feature edges. For multi-protein arrays **(D & E)**, images of each fluorophore were taken on separate channels in the microscope and then merged to produce the images provided.

Figure 6. Cells interacting with an ImR patterned surface

Fluorescence microscopy images from different experiments performed under the same conditions in which RBL-2H3 were cells sensitized with A488-IgE (green) and incubated with patterned Cy3-Cy5 DNP-BSA (red). These proteins were patterned into $1 - 5 \mu\text{m}$ features using ImR and imprint lithography techniques. Images are representative of 4 individual experiments. Scale bars represent $10 \mu\text{m}$.