High-Resolution, High-Throughput, Positive-Tone Patterning of Poly(ethylene glycol) by Helium Beam Exposure through Stencil Masks

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Abstract

In this work, a collimated helium beam was used to activate a thiol-poly(ethylene glycol) (SH-PEG) monolayer on gold to selectively capture proteins in the exposed regions. Protein patterns were formed at high throughput by exposing a stencil mask placed in proximity to the PEG-coated surface to a broad beam of helium particles, followed by incubation in a protein solution. Attenuated Total Reflectance–Fourier Transform Infrared Spectroscopy (ATR–FTIR) spectra showed that SH-PEG molecules remain attached to gold after exposure to beam doses of 1.5–60 µC/cm² and incubation in PBS buffer for one hour, as evidenced by the presence of characteristic ether and methoxy peaks at 1120 cm⁻¹ and 2870 cm⁻¹, respectively. X-ray Photoelectron Spectroscopy (XPS) spectra showed that increasing beam doses destroy ether (C–O) bonds in PEG molecules as evidenced by the decrease in carbon C1s peak at 286.6 eV and increased alkyl (C–C) signal at 284.6 eV. XPS spectra also demonstrated protein capture on beam-exposed PEG regions through the appearance of a nitrogen N1s peak at 400 eV and carbon C1s peak at 288 eV binding energies, while the unexposed PEG areas remained protein-free. The characteristic activities of avidin and horseradish peroxidase were preserved after attachment on beam-exposed regions. Protein patterns created using a 35 µm mesh mask were visualized by localized formation of insoluble diformazan precipitates by alkaline phosphatase conversion of its substrate bromochloroindoyl phosphate-nitroblue tetrazolium (BCIP-NBT) and by avidin binding of biotinylated antibodies conjugated on 100 nm gold nanoparticles (AuNP). Patterns created using a mask with smaller 300 nm openings were detected by specific binding of 40 nm AuNP probes and by localized HRP-mediated deposition of silver nanoparticles. Corresponding BSA-passivated negative controls showed very few bound AuNP probes and little to no enzymatic formation of diformazan precipitates or silver nanoparticles.

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Introduction

Surface patterning of biomolecules is important in the study of cell adhesion, in tissue engineering, and in the development of diagnostics and biomedical assays such as protein nanoarrays [1,2,3,4]. Controlled attachment of biomolecules can be achieved by approaches generally categorized as “top-down” (e.g., photolithography, focused ion beam lithography, electron beam lithography, nanografting), or “bottom-up” (e.g., self-assembly of monolayers, dip-pen nanolithography, micro/nano contact printing or stamping), or combinations of these techniques. Top-down techniques manipulate an instrument to modify the bulk material to create patterns. Photolithography is a high-throughput lithographic process but its resolution is diffraction-limited below the micron-scale, and it is expensive to use for low-volume manu-
chemical functionality. Upon exposure to the electron beam, the nitro groups are converted to amines while the underlying aromatic groups are dehydrogenated and cross-linked. The amine groups were then used to attach molecules (carboxylic acid anhydrides and rhodamine dyes) to the exposed regions of the surface. Perhaps the most popular approach, however, involves the use of poly(ethylene glycol) (PEG) [9,10,11], which is well-known for its resistance to non-specific adsorption of proteins through a combination of entropic (steric stabilization) and enthalpic (hydrogen bonding) mechanisms [12,13]. Entropic passivation is favored by longer chains which can assume a greater variety of configurations [13,14,15], but even smaller ethylene oxide chains of 3–6 monomer units can resist protein adsorption as long as the molecular conformation is helical or amorphous, favoring a more stable interfacial layer of tightly-bound water [12,13,15,16,17,18].

Electron beam lithography is commonly used to create protein patterns on PEG surfaces. Hong et al. [19] observed that proteins selectively attach to electron beam-exposed regions of amine-terminated PEG spin-coated on silicon. Patterns consisting of multiple proteins also can be formed by introducing PEG bearing different functionalities (e.g., biotin, maleimide, amineoxyl or metal chelate) that are patterned over several exposures [20]. Harnett et al. [21] used low-energy electron beam exposure to destroy the amine functionality in selected regions of an amine-functionalized silane-PEG on silicon surfaces, allowing proteins to attach only on the unexposed regions (negative-tone patterning). Inert silane-PEG on silicon and thiol-PEG on gold have also been used to create a protein-resistant surface, with electron beam exposure used to remove the SAM on exposed regions and protein-reactive PEGs used to backfill the exposed regions (positive-tone patterning) [22,23].

In this study, we explored the use of helium beam proximity lithography to create micron and nanoscale patterns of proteins on grafted PEG in a high-throughput manner. Unlike focused ion beam lithography techniques, proximity printing forms its patterns by exposing a thin membrane with etched openings corresponding to the desired pattern (a “stencil mask”) to a broad beam of helium particles, which are either stopped in the opaque regions of the mask or pass through the openings. In this way, the pattern can be formed very quickly and without the need for expensive ion optics. After exposure to the beam, protein patterns were detected and visualized using three different methods chosen to be representative of common approaches: (1) formation of localized diformazan precipitates by patterned alkaline phosphatase (AP) from its substrate BCIP-NBT [24,25,26], (2) binding of gold nanoparticle probes (40 nm and 100 nm gold particles conjugated with antibodies) [27,28,29], by patterned antibodies and avidin, and (3) localized redox formation of silver deposits mediated by patterned horseradish peroxidase (HRP) [30,31,32,33].

Materials and Methods

Materials

Alkaline phosphatase from bovine intestinal mucosa (Product number P6772), goat polyclonal anti-rabbit antibodies (R2004), goat polyclonal anti-mouse antibodies (M8645), hen egg white lysozyme (L6876) and BCIP-NBT phosphatase substrate (72091) were purchased from Sigma-Aldrich (St. Louis, MO), while avidin (21121) was from Pierce Protein Research Products, Thermo Scientific (Rockford, IL). Rabbit polyclonal anti-Rickettsia antibodies were generously supplied by Dr. Juan Olano (University of Texas Medical Branch, Galveston, TX) and mouse anti-lysozyme monoclonal D1.3 antibody [34] was produced for us in hollow-fiber hybridoma culture by Biovest (Minneapolis, MN). Streptavidin-horseradish peroxidase (strept-polyHRP80, 65R-S105PHRP) conjugate from Fitzgerald Industries International (Action, MA) is a polymer consisting of 80 streptavidin molecules per conjugate and 5 HRP monomer molecules per streptavidin giving an
Helium beam exposure of PEG and surface characterization

The helium beam is generated in a saddle-field ion source that is based on the designs of Hogg [35] and Franks [36]. In this source, a plasma is ignited at low pressure when electrons are trapped along long oscillatory paths through a saddle-point in the electric potential distribution, and ions escape through a small aperture machined into one end of the source. As the ions escape, a fraction is neutralized through charge-exchange collisions with the neutral helium gas ambient and a beam of mixed atoms and ions drifts along the length of a vacuum beam line to an exposure chamber, located about 1.5 meters from the source [37]. To form patterns, a stencil mask is held in proximity to the wafer by a fixture and the wafer can be moved below the mask using an in-vacuum x-y stage to allow for step-and-repeat patterning of large surfaces. The exposure time is controlled through a computer that acts a mechanical shutter.

Patterns of beam-modified PEG (and, subsequently, proteins) on the gold surfaces were formed by casting shadows using a stencil mask in proximity to the substrate (see Figure 1). The mixed ion and atom flux was equivalent to a helium ion beam current density of about 70 nA/cm² with a beam energy of about 6.5 keV (for a source power supply setting of 10 kV at 1 mA), and three doses were tested: 30, 150 and 600 seconds (approximately 2, 10 and 45 μC/cm², respectively). After exposure, samples were stored dry at 4°C until use. Surfaces were analyzed by Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy, ATR-FTIR (Nicolet 4700 FT-IR, Thermo Scientific) before and after beam exposure, and after PBS buffer incubation. In addition, surfaces before and after incubation with protein (15 μg/mL avidin) were analyzed by X-ray Photoelectron Spectroscopy (Physical Electronics model 5700 XPS instrument) using a monochromatic Al-kα X-ray source (1486.6 eV) operated at 350 W. The analyzed area, collection solid cone and take-off angle were set at 0.8 mm diameter, 5° and 45°, respectively. Applying a low pass energy filter of 11.75 eV resulted in an energy resolution of better than 0.51 eV. All spectra were acquired at room temperature under a vacuum of 5×10⁻⁵ torr or better. A survey scan was first performed to determine the major elements present, followed by element-specific scans of at least 15 minutes per scan. Data processing was carried out using the Multipat®software package (Physical Electronics, Inc.). A Shirley background [38] subtraction routine was applied.

Protein patterning on PEG

After helium beam exposure, surfaces were further cleaved into smaller pieces (ca. 25 mm²) to fit into micro-centrifuge tubes. Samples used for patterning were incubated with 15 μg/mL of protein solution (either avidin, streptavidin-polyHRP, goat anti-mouse antibodies or goat anti-rabbit antibodies) in PBS buffer for 1 hour at room temperature, while negative control surfaces were incubated with protein-free PBS buffer. Before pattern detection, all surfaces were immersed in 4% BSA in PBS for 1 hour to further passivate the back and edges of the gold-coated silicon chips, as well as the walls of the micro-centrifuge tube. All surfaces were washed at least three times with PBS (except where specified) between reagents.

For pattern detection by formation of alkaline phosphatase diformazan precipitate, surfaces were incubated on an orbital shaker in a solution of 2 μg/mL of biotinylated enzyme (with ca. 3 biotin molecules per enzyme molecule, as assessed by HABA assay [39]) in 100 mM diethanolamine, 100 mM NaCl, 5 mM MgCl2, pH 9.2 (DEA buffer), for 1 hour at room temperature, then
washed with DEA buffer. BCIP-NBT (0.15 mg/mL BCIP and 0.30 mg/mL NBT) substrate was then added and allowed to react for 10 minutes. The surfaces were then washed with water, dried with compressed nitrogen, and imaged using an optical microscope. For pattern testing by gold nanoparticle probes, 300 μL suspension of ca. 10⁹ gold nanoparticles/mL conjugated with antibodies (100 nm particles with biotinylated goat anti-rabbit polyclonal antibodies, or 40 nm particles with goat anti-mouse antibodies) in PBS buffer was added to the surface and allowed to incubate for at least 12 hours at room temperature, with mixing on an orbital shaker. For HRP-mediated silver staining, surfaces were incubated with streptavidin-polyHRP conjugates (20 μL spot, 10 μg/mL) for at least one hour at room temperature, and then washed thrice with PBS and twice with DI water. Surfaces were then silver-stained using the EnzMet™ staining solution, with 2 minutes incubation time each for the manufacturer’s reagents Detect A, B and C (20 μL each). Finally, surfaces were washed thrice with water, dried with compressed nitrogen and imaged by scanning electron microscopy (Zeiss/LEO 1525 Field Emission SEM).

Figure 4. Formation of diformazan precipitates on protein-PEG patterns (35 μm mesh). Top row images show a schematic diagram (not to scale) of helium beam-patterned PEG incubated with avidin (A) or BSA (D) followed by addition of biotinylated alkaline phosphatase (AP) and substrate BCIP-NBT. Optical microscopic images below the diagram show the protected (light) and irradiated (dark) regions which display the specific patterned capture of biotinylated AP enzymes for avidin-incubated surface (B) and no pattern for BSA-incubated surface (E). Panels C and F show higher magnifications of images in B and E, respectively.

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Results and Discussion

As shown in Figure 2, ATR spectra confirmed the presence of SH-PEG assembled on gold surfaces before helium beam exposure, after beam exposure and after post-exposure incubation in PBS buffer for one hour. Peaks near 2870 cm⁻¹ and 1120 cm⁻¹ were observed, corresponding to the C–H stretch of methoxy and C–O stretch of ether groups, respectively [40]. We found that our MW 5000 SH-PEG SAM monolayers at least partially survived 6.5 kV helium beam exposures with doses of 1.5–60 μC/cm². It is notable that previous researchers [22,23] found that monolayers of smaller PEG molecules (MW: 290 and 750) were damaged by doses of 5–80 μC/cm² and completely removed by doses over 160 μC/cm² when using 1 kV electrons.

The radiation chemistry of electron and ion/atom beams on PEG and alkanethiols would be expected to be very similar, mainly involving hydrogen abstraction of the PEG hydrocarbons [41] and dissociation of C–H, C–C, C–S and substrate–SH bonds [23,34]. Hydrogen abstraction and bond dissociation form radicals, which eventually cause cross-linking within the polymer [42,43], affect hydrogen bonding and disrupt the highly organized interface between water and PEG. Our results are consistent with a similar effect of helium beam exposure. Cross-linking of PEG was observed on helium beam exposed surfaces as evidenced by the water insolubility of exposed spin-coated thiol-PEG on silicon (unexposed thiol-PEG film on silicon is soluble). Furthermore, disruption of PEG chains was confirmed by XPS, as shown in Figure 3. As presented in Figure 3A, prominent carbon (Cls) peaks were observed at 284.6 eV and 286.6 eV binding energies, which correspond to alkyl and ether C1s bonding states, respectively [44,45]. As the helium beam dose was increased, the C1s ether peak at 286.6 eV decreased while the alkyl peak at 284.6 eV increased, indicating the destruction of ether bonds and formation of more alkyl bonds in the PEG molecules. A decrease in the oxygen (O1s) signal (532 eV binding energy) also was observed as the beam dose was increased. Helium beam exposure renders the grafted PEG on the surface less hydrophilic by a decrease in C–O ether and an increase in C–C alkyl functionalities, and thus potentially more amenable to protein adsorption. There also is literature evidence that electron [9] or ion (argon) beam [10,11] exposure can create carbonyl functionalities (which could be charged carboxylate or protein amine-aldehyde) in PEG samples. A relatively small number of these functional groups could alter the local protein-capture properties of the PRG surface, while being difficult to detect against the background of much-more-numerous ether and alkyl functionalities.

Elemental XPS spectra of exposed and unexposed samples incubated with avidin are shown in Figures 3D, 3E and 3F for carbon (Cls), oxygen (O1s) and nitrogen (N1s) signals, respectively. In Figure 3D, aside from the observed decrease in the Cls peak at 286.6 eV and increase in the Cls peak at 284.6 eV discussed previously, the Cls peak at 288 eV, a characteristic binding energy of amide Cls [46], appeared as the helium beam dose was increased, confirming that avidin attaches to the beam-exposed surfaces. This is further supported by the increase in the nitrogen N1s signal (400 eV) with increasing beam dose, as shown in Figure 3F. Unlike in Figure 3B, the differences in oxygen signal intensities in Figure 3E were not distinct, particularly in the unexposed and exposed (30 sec and 150 sec) samples. This might be due to the added attenuation length for photoelectrons in XPS provided by the avidin layer (there is little nonspecific adsorption of avidin on unexposed PEG as shown by the nitrogen signal in Figure 3F). The oxygen signal intensities for unexposed and exposed (30 sec and 150 sec) PEG samples are similar because the avidin layer somewhat overshadows the small oxygen signal differences observed in Figure 3B. However, since more avidin molecules were found to attach on the exposed (600 sec) sample, its oxygen signal is distinctly different from the latter samples.

Among the helium beam doses tested, we chose to use the 150 second exposure time for further work as a balance between protein attachment effectiveness and processing throughput. Patterning of proteins using a 35 μm mesh is presented in Figures 4 and 5, wherein attachment of proteins on beam-exposed regions is indicated by enzymatic formation of localized diformazan precipitates and also by binding of gold nanoparticle probes. As shown in Figure 4, the helium beam was able to pattern avidin over a large surface area (25 mm², with 2.3 mm² shown in Figure 4). The darkened regions indicate the formation of...
and create protein patterns on the order of hundreds of nanometers. Corresponding negative controls showed very few bound gold nanoparticles and little to no formation of silver nanoparticles, as BSA was able effectively to passivate the beam-exposed regions.

**Summary**

We have demonstrated that helium beam exposure through a stencil mask in proximity to the substrate can be used for the massively-parallel creation of micro- and nano-scale protein patterns on PEG-grafted surfaces. Proteins captured on irradiated PEG regions were shown to retain their functionality, as the patterned avidin could bind biotinylated molecules and patterned HRP conjugates were able to produce silver nanoparticles. Protein attachment on helium beam-exposed PEG may be due to hydrophobic interactions, electrostatic interactions, formation of reactive oxidized products, or a combination of these mechanisms. Further studies will be needed to establish the governing mechanism(s) and also to maximize processing throughput.

**Author Contributions**

Conceived and designed the experiments: EEC PR RCW. Performed the experiments: EEC AN TS. Analyzed the data: EEC SK KK PR RCW. Contributed reagents/materials/analysis tools: EEC SK KK GES PR RCW. Wrote the paper: EEC PR RCW.

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