Directed Immobilization of Protein-Coated Nanospheres to Nanometer-Scale Patterns Fabricated by Electron Beam Lithography of Poly(ethylene glycol) Self-Assembled Monolayers

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Controlling the spatial organization of biomolecules on solid supports with high resolution is important for a wide range of scientific and technological problems. Here we report a study of electron beam lithography (EBL) patterning of a self-assembled monolayer (SAM) of the amide-containing poly(ethylene glycol) (PEG) thiol CH3 O(CH2 CH2 O)17-NHCO(CH2)2 SH on Au and demonstrate the patterning of biomolecular features with dimensions approaching 40 nm. The electron dose dependence of feature size and pattern resolution is studied in detail by atomic force microscopy (AFM), which reveals two distinct patterning mechanisms. At low doses, the pattern formation occurs by SAM ablation in a self-developing process where the feature size is directly dose-dependent. At higher doses, electron beam-induced deposition of material, so-called contamination writing, is seen in the ablated areas of the SAM. The balance between these two mechanisms is shown to depend on the geometry of the pattern. The patterned SAMs were backfilled with fluorescent 40-nm spheres coated with NeutrAvidin. These protein-coated spheres adhered to exposed areas in the SAM with high selectivity. This direct writing approach for patterning bioactive surfaces is a fast and efficient way to produce patterns with a resolution approaching that of single proteins.

Introduction

The controlled attachment of biomaterials such as DNA, protein, and cells onto solid supports on the micro- and nanometer scales is of interest in a wide range of applications such as the development of ultrasmall biosensors,1,2 benefiting from well-defined active areas, increased control over the density of receptor elements, and controlled reactivity of the receptor elements. Furthermore, these nanopatterned materials provide templates for tissue engineering3 and control of the chemical environment in cell-based experiments, including control of neuronal adhesion by cell adhesive regions4 as well as screening tools for diagnostics and drug discovery.5,6

Protein patterning has previously been achieved with various techniques7 such as microarray technology,8 photolithography,9 microcontact printing,9 imprint lithography,10 scanning probes,11 selective adhesion to surfaces with chemically different self-assembled monolayer (SAM) coatings,12 and template patterns fabricated with energetic beams of X-rays and electrons.13 These patterning methods can be divided into two categories: methods based on lithographic pattern transformation, where a pattern on a mask or stamp is transferred to a surface in a one-process step, and methods based on direct writing, where a pattern is transferred to a surface in a sequential fashion. The former method is essentially a copying process, with exceptional capacity for high-throughput production. The latter uses a much slower method for pattern transfer but has significantly greater flexibility in terms of modifying the pattern to be produced, which is particularly useful in a research and development setting. In particular, it does not require the time-consuming step of producing a mask or stamp.

Electron beam lithography (EBL) is a direct writing method and despite its name is strictly speaking not a lithographic method of patterning. EBL is capable of high speed and high resolution because of the very fine focus of the electron beam (~2 nm). As a primary patterning tool, EBL has a large dynamic range, efficiently writing both large and small structures by automatically changing exposure modes. The speed with which the beam can be deflected gives very high writing speeds when the exposed material has a high sensitivity to electron radiation. For research and prototyping purposes, the capabilities of this tool can be exploited in situations where direct writing of patterns is desired.

Protein patterning procedures based on electron irradiation and EBL have previously been reported. Electrochemical patterning of SAMs has been achieved by locally modifying the SAM’s tail-group functionality with electron irradiation by converting nitro groups to amino groups. The patterns were then backfilled with dyes containing carboxylic groups for binding

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to the amino groups.\textsuperscript{14,15} Harnett et al.\textsuperscript{16,17} have been using SAMs for chemical patterning of bioactive templates, where exposed areas differ chemically from unexposed areas. Patterning is either due to the destruction of the functional group at the terminus of the molecules in the SAM, thereby preventing subsequent binding of a target molecule to the exposed area, or to backfilling an exposed area with a chemically active molecule in an otherwise inert SAM, thereby creating local binding sites. This EBL-based patterning method was further developed by Senaratne et al.,\textsuperscript{18} where a poly(ethylene glycol) (PEG)\textsuperscript{19}--modified silicon dioxide surface was used to minimize the nonspecific adsorption of protein.

PEG is recognized as a biocompatible molecule characterized by hydrophilicity, flexibility, high exclusion volume in water, nontoxicity, and nonimmunogenicity.\textsuperscript{20} Material in contact with biological fluids often develops biofouling as a consequence of protein adsorption and cell adhesion.\textsuperscript{21} PEG molecules can suppress protein and cell adhesion, including adhesion to surfaces that are coated with PEG in the form of a SAM.\textsuperscript{18,22--25} Proteins adhere nonspecifically to most surfaces, and the protein-repelling properties of PEG make it an important component in biotechnological systems, where the prevention of protein adsorption, as well as cell adhesion and growth, is desired.

Here, we report on the use of EBL to pattern PEG SAMs on Au surfaces. One of our long term goals is to engineer surfaces for advanced bioelectronic interfaces to neurons and other electrically active cells. With this in mind, we used patterned Au surfaces on insulating SiO\textsubscript{2}, which allows for a variety of interconnected contacts and will later allow the patterned surfaces to be addressed electrically. Granular Au (Figure 1a) or flame-annealed Au (Figure 1b) films were incubated in an ethanolic solution with a PEG thiol (Figure 1c), resulting in the formation of a PEG SAM. The PEG SAMs were grown on flame-annealed Au for characterization purposes. In particular, the flame-annealed Au is locally smooth enough for surface characterization by AFM, with a roughness significantly smaller than the thickness of the PEG SAM. However, for most applications granular Au is needed because flame annealing can produce only small monocrystalline areas (typically a few micrometers wide) and adds an extra manufacturing step. The PEG SAMs were patterned by EBL (Figure 1d) and characterized before and after exposure by contact-mode AFM in a phosphate-buffered saline (PBS) solution. AFM characterization of the EBL patterning revealed two competing types of electron-dose-dependent patterning: removal of the SAM and deposition of material. Protein-coated

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Schematic describing the patterning process. The PEG SAM (c) was grown on either granular (a) or flame-annealed (b) Au surfaces and subsequently exposed by EBL, resulting in the removal of the SAM in the exposed areas in a self-developing process. The patterned surfaces were incubated in a solution of NeutrAvidin-coated, 40-nm fluorescent polystyrene spheres suspended in PBS. The spheres immobilize in the holes of exposed patterns, creating protein patterns with ultimately only one or a few spheres at each site (e). These protein patterns have been imaged with fluorescence microscopy and AFM.}
\end{figure}

40-nm fluorescent spheres are used as a model system for patterning the written EBL patterns with protein. When a patterned chip is incubated in a solution containing protein-coated spheres, the spheres attach to the patterns where the PEG is removed (Figure 1e). The spheres allow for rapid imaging with fluorescence microscopy and are of a size that gives good contrast against the PEG background for high-resolution AFM imaging.

**Experimental Details**

**Preparation of Au Surfaces.** Au films were patterned in the shape of 20 \times 20 \mu m\textsuperscript{2} interconnected squares with a standard lift-off procedure described in detail elsewhere\textsuperscript{26} on substrates that were cut out of a 250-\mu m-thick Si wafer with a 1-\mu m-thick SiO\textsubscript{2} layer. Thermal
Flame annealing of the small 20-nm-wide Au squares produced better results than continuous Au films. In the latter case, overheating of the Au film caused smaller crystallites and a rougher surface. Our flame-annealing technique is further discussed in our earlier work.\(^\text{27}\) In addition to improved flame annealing, the patterned Au squares also serve as alignment marks for EBL and as location markers for navigating on the chip in the AFM. Such marks are necessary to find the extremely small patterns studied here.

**PEG Thiol SAM Growth.** Prior to PEG thiol incubation, the chips and the tweezers for handling the chips were immersed in a washing solution of \(\text{H}_2\text{O}_2/\text{NH}_4\text{H}_2\text{O} 1:1.5 \text{ v/v} \) which was heated to 50 °C for 10 min to remove organic contamination. The chips were then rinsed with a stream of running deionized MilliQ water (> 18 MΩ cm) for a few minutes, followed by rinsing in ethanol to remove any remaining water. Care was taken to keep the Au films covered with solution, avoiding exposure to air while moving them between solutions. The 750 Da PEG thiol \(\text{CH}_3\text{O(}\text{CH}_2\text{CH}_2\text{O) }_{17}\text{NHCO(}\text{CH}_2\text{) }_2\text{-SH} \) (Figure 1c) was obtained from Rapp Polymere GmbH (Tübingen, Germany). The chips were incubated in 25 mL of a 20 μM ethanolic PEG solution freshly prepared from stock solution. Incubation times were typically 24 h or longer, which was sufficient for making a complete SAM.\(^\text{27}\) After incubation, chips were sonicated in ethanol for 1 min to stop incubation and to remove any PEG molecules not chemisorbed to the surface. Chips were blown dry with a stream of \(\text{N}_2\) before placing them in the vacuum of the EBL system chamber.

**EBL on PEG SAMs.** A Turnkey 150 EBL system (Raith GmbH, Dortmund, Germany) equipped with a high-precision laser interferometric stage was used to pattern the PEG SAM (Figure 1d). The acceleration voltage was 1 kV, and a 10-μm aperture with typical electron beam current of 17–18 pA was used. The pattern was written with a 5-nm step size, and the dose was controlled by the time spent at each pixel (0.1-μs resolution). Electron doses in the range 10 to 20 480 μC cm\(^{-2}\) were tested when writing lines with a nominal width of 40 nm and a pitch of 200 nm. The chamber pressure was \((2.1–2.3) \times 10^{-7} \text{ mbar}\) during exposures. In addition to the line dose test, arrays of 40-nm squares with a pitch of 200 nm were exposed with electron doses in the range of 1000 to 8000 μC cm\(^{-2}\).

**Protein-Coated Sphere Immobilization.** The exposed chips were first cleaned by sonication in MilliQ water for 20 min. Chips were then incubated for 15 h in a solution of 40-nm NeutrAvidin-coated fluorescent spheres (FluoSpheres, Molecular Probes Inc., Invitrogen, Stockholm, Sweden) in stock solution from the manufacturer (aqueous suspension containing 1% solids, 5 mM sodium azide, and 0.02% Tween) diluted 200-fold with PBS (Figure 1e). After incubation, chips were washed with MilliQ water on a shaking table for 3 min, sonicated for 3 min to remove loosely bound spheres, and then dried under a stream of \(\text{N}_2\). The chips were then immediately imaged by AFM or fluorescence microscopy.

The fluorescent spheres have the fluorochrome Bodipy FL encapsulated polystyrene sphere that prevented fluorochrome proximity to the Au surface and thereby avoided quenching of the fluorescence, making detection more efficient. The 40-nm sphere produces sharp contrast in the AFM against the flat background of the PEG on the flame-annealed Au surface.

**Fluorescence Microscopy.** The fluorescence microscope setup consists of a Nikon Eclipse E 600 with Plan Apo objectives and a Nikon B-2A filter block designed for observations of fluorescence emission in the yellow-green range. The excitation filter is a band-pass filter with a range of 450 to 490 nm, and the long-pass barrier filter (emission filter) has a cut-on wavelength of 515 nm. The filter combination employs a long-pass dichromatic mirror with a cut-on wavelength of 500 nm. The microscope is equipped with a cooled Spot CCD camera (Diagnostic Instruments, Sterling Heights, MI) for imaging. The images were captured under ambient conditions, and the typical exposure time was 5 s.

**AFM Studies in PBS.** The surfaces were analyzed using a MultiMode AFM system with a J scanner, a fluid cell, and the NanoScope IV controller (Veeco Metrology Group, Digital Instruments, Santa Barbara, CA). The samples were imaged by contact mode AFM in a PBS solution at pH 7.4 using oxide-sharpened...
silicon nitride probes (Olympus OTR4, Veeco Metrology Group, Santa Barbara, CA) with a nominal tip radius of curvature of 15 nm and a spring constant of ~0.08 N/m. The samples were imaged in the low-force regime, typically ~0.1 nN between tip and sample, calculated from the nominal spring constant value and the measured cantilever bending. Prior to imaging, the cantilever was allowed to find its equilibrium, which took about 30 min after contact with the room-temperature PBS. The raw data was processed with the NanoScope IV software by a third-order plane fit and first-order flattening.

**Results**

**Au Film Preparation and PEG SAM Characteristics.** Immersion of the flame-annealed Au films (Figure 2a and b) into the ethanolic PEG thiol solution for 24 h or longer resulted in the spontaneous formation of a SAM on the Au. The SAM covers the ethanolic PEG thiol solution for 24 h or longer resulted in the spontaneous formation of a SAM on the Au. The SAM is rougher in appearance with a nodular substructure (Figure 2e). This change in roughness is quantified by measuring the rms roughness, which increases by a factor of 2.4 from 0.57 to 1.38 nm after vacuum treatment and rehydration, respectively. Although AFM cannot discern the cause of this change in roughness, we note that both before and after vacuum treatment the PEG SAM is a soft layer that is easily destroyed when in contact with the AFM probe if the imaging force is too high (≥0.1 nN).

**EBL Exposures on PEG SAM.** For high-resolution EBL, it is necessary to work around the disruptive influence of sample charging with the electron beam. Exposing isolated Au squares on a SiO2 surface to 1 kV electrons results in charge accumulation in the Au and a local electric field distorting the electron beam. The interconnected Au squares were therefore connected to a 2 × 2 mm² Au pad that was grounded to the EBL system stage with a clamping pin.

Electron beam exposure on the PEG SAM results in removal of the PEG in a self-developing process (Figure 3). The amount of removed material in the SAM increases with increasing electron dose. The removal of material is seen as a decrease in monolayer thickness as well as a widening of the exposed features as the exposure dose increases (Figure 3). The widening of the exposed features is ascribed to electrons backscattered from the bulk material. The dependence of feature width, α, on electron dose, D, shows a power-law dependence, and the data can be fit to the empirical formula (dashed lines in Figure 4) |ω| = ω₀Dα, with ω₀ = 1.34 nm and α = 0.212 (D expressed in μC cm⁻²) for 40-nm-wide line exposures and ω₀ = 0.69 nm and α = 0.332 for 40-nm square exposures (Figure 4).

The response of the PEG to irradiation can be classified into three types based on the depth of the exposed features (Figure 3c and d): Type I, for doses between 10 and 80 μC cm⁻², is underexposed with only partial removal of the SAM. Type II, with doses in the range 160 to 500 μC cm⁻², is used for the complete removal of the exposed SAM. Type III, with electron doses >500 μC cm⁻², has a bump at the bottom of each line that increases in height as the dose increases (one such bump is marked with ↑ in Figure 3b). This bump indicates the deposition of material, a well-known phenomenon in electron microscopy and EBL, often called contamination writing.

Thus, our AFM studies revealed that EBL on SAMs is a balance between two competing phenomena: the destruction of the SAM, creating openings in the PEG layer, and the deposition of material, which reduces the feature depth. This competition can be seen by plotting the depth of the written features versus electron dose, which is shown in a semilog plot (Figure 5). Two straight dashed lines have been fit to the data points from which we can interpolate an optimal dose of ~170 μC cm⁻² for 40-nm lines, where there is maximal removal of the PEG SAM and minimal deposition of material.

The 40-nm square exposures show the same optimal dose behavior as the lines, but shifted toward higher doses, with an optimal dose of ~2000 μC cm⁻². This shift toward a higher dose for the squares is consistent with the well-known proximity effect in EBL. The proximity effect is due to the fact that a large part of the exposure actually comes from backscattered electrons, which penetrate well into the substrate, are reflected back, and are ultimately captured in the SAM. These backscattered electrons have a much wider range than the forward-scattered electrons, resulting in a large accumulated dose for patterns that have many exposed pixels in close proximity to one another. Thus, large areas (with many exposed pixels) require a smaller dose to reach the threshold for exposure than small features (with fewer exposed pixels).

**Protein Adhesion to Patterns.** Protein adhesion to EBL patterns on both granular Au and flame-annealed Au was examined. For these experiments, we use protein-coated fluorescent spheres as a model. Direct protein adsorption onto the Au could not be detected using fluorescence because of Au-induced quenching. The granular surfaces were first covered with a PEG SAM and patterned by EBL in large rectangular areas 2 × 5 μm² as well as lines and squares with nominal widths of 500, 250, 100, and 50 nm with doses varying from 100 to 2000 μC cm⁻². These patterned surfaces were then immersed in a solution with protein-coated fluorescent spheres and imaged by fluorescence microscopy. The spheres are found to adhere to exposed areas, and a clear dependence on the electron dose for the adhesion of the spheres can be seen (Figure 6).

The smallest electron doses needed for sphere adhesion versus nominal feature size for lines and squares were investigated (Figure 7). Here we find that lines require a smaller dose than squares and that wider features require a smaller dose, consistent with the proximity effect. For 50-nm squares, electron doses at the maximum tested (2000 μC cm⁻²) appear to be insufficient for sphere adhesion when using fluorescence imaging, with only occasional or no adhesion of spheres. Fluorescence microscopy was used to demonstrate sphere adhesion to the EBL patterns in the PEG SAM on granular Au. We are not able to resolve single spheres at the binding sites, and for this reason, we developed methods to image the exposed areas with AFM.

The fluorescence microscopy demonstrated that the spheres adhered to the patterns, but to study the adhesion process in more detail, we used AFM to visualize patterns produced on flame-annealed Au surfaces. AFM provides lateral resolution good enough for single-sphere detection on a PEG SAM background. AFM imaging of spheres adsorbed to the line and square patterns described above (Figure 3) reveals individual 40-nm spheres bound to the patterns (Figure 8). We note that sphere binding is not present in the lines with doses of 10 and 20 μC cm⁻² whereas the remaining lines with doses of 40–20 480 μC cm⁻²

bind spheres. We also find that the 40-nm square array exposed at 2000 \( \mu \text{C cm}^{-2} \) efficiently binds spheres to every exposed site (Figure 8b). In some cases, we can bind a single sphere, and in other cases, two spheres. With further refinement of this technique, we believe that individual objects (proteins, spheres, etc.) with dimensions down to roughly 30 nm can be placed on the surface with complete control of the desired pattern.

**Discussion**

**Mechanisms for EBL Patterning of SAMs.** The field-emission electron microscope has an electron beam diameter of a few nanometers when it is well focused.\(^{29}\) However, the size of the feature written with EBL is a function of the limitations of the resist and the spatial extent of the scattered electrons, which increase the exposed volume in the polymer layer. Aliphatic SAMs (e.g., alkane thiol (AT) and octadecyl siloxane (ODS)) and aromatic SAMs (e.g., biphenyl thiol (BPT) and hydroxybiphenyl siloxane (HBP)) have been patterned by EBL, and a variety of exposure mechanisms have been identified.\(^{14,15,30-37}\)

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The result of the exposure of a SAM to electron irradiation can be divided into two main categories of patterning based on whether the molecule is aliphatic or aromatic: EBL is destructive to the molecules in the group of aliphatic SAMs, and aromatic SAMs were strengthened and became more rigid.35,37 The damage to aliphatic SAMs from electron exposure has been characterized with AT as a model system. Both the alkyl chains and the substrate-thiolate bonds at the S-Au interfaces are affected, and the most significant damage in the AT films occurs in the early stages of irradiation. The most noticeable processes when irradiating an AT SAM with low-energy electrons is the induced dissociation of C-H, C-C, C-S, and substrate-thiol bonds. The main processes are the complete breakdown of the orientational order in the initially well-ordered film, partial dehydrogenation, and desorption of large fragments of the film, resulting in a decrease of its thickness.38,39 EBL exposure of aromatic SAMs is characterized by cross linking of the aromatic

![Figure 4](image1.png)
**Figure 4.** Plot of the electron dose dependence of the feature width for lines and squares with a nominal width of 40 nm. The exposed features widen as the electron dose increases. The widening is ascribed to the proximity effect originating in SAM exposure by backscattered electrons. For the line exposures (open points), each data point is an average of 3 measurements of widths at half-maximum from height profiles, with each measurement being an average over 50 adjacent horizontal scan line profiles similar to those presented in Figure 3. Each data point for the nominally 40-nm square exposures (solid points) is an average of the half-width of 16 squares, with each measurement being an average over ~20 adjacent scan lines. Error bars for the data points are ±3 nm.

![Figure 5](image2.png)
**Figure 5.** Plot of feature depth versus electron dose for line and square pattern exposures. The two competing phenomena, SAM removal and contamination writing, are responsible for the observed minima. The feature depths are measured from the plateau of the unexposed SAM to the feature height at the center of the hole in section profiles similar to those in Figure 3. Error bars for data points are ±0.5 nm.

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![Figure 6](image3.png)
**Figure 6.** (a) Protein-coated FluoSphere patterns assembled onto granular Au. Fluorescence images show the line and square exposures having nominal widths of 500, 250, 100, and 50 nm (from bottom to top) and were exposed with doses of 100, 200, 300, 400,...,2000 μC cm⁻² from left to right. Fluorescence is observed from the EBL-exposed patterns, demonstrating sphere adhesion. For the 500-, 250-, and 50-nm lines, sphere adhesion is observed at electron doses of 100, 200, 300, and 400 μC cm⁻² and higher, respectively. Occasional sphere adhesion at lower electron doses is disregarded. The 500-, 250-, and 100-nm squares bind spheres at electron doses of 400, 800, and 1600 μC cm⁻² and higher, respectively. The 50-nm squares did not bind any spheres in the dose range tested. (b) Detail of the region marked with a white frame with electron doses of 1700, 1800, 1900, and 2000 μC cm⁻² from left to right. The 50-nm squares are underexposed in the micrograph.
Figure 7. Dependence of the critical electron dose for sphere binding on the nominal feature width of line and square exposures. The data points were extracted from the fluorescence microscopy and AFM (40-nm features) figures. The critical dose is defined as the lowest electron dose tested that gave rise to sphere binding. The error bars indicate the doses tested below and above the critical dose.

Limitations of Feature Size. Our observations of feature widening as the electron dose increases are consistent with the interpretation that backscattered and secondary electrons contribute to the exposure of the SAM. Because of the long-range nature of these electrons, the minimum possible feature size is not limited only by the focus of the electron beam. For a given SAM thickness, a certain critical dose is required to remove the PEG, exposing the Au beneath. Above this dose, the effect of secondary and backscattered electrons increases, with no improvement in the directly exposed area. Thus, for the highest possible pattern resolution, it is essential to find this critical dose so as to minimize the effect of backscattered and secondary electrons. The selection of specific molecules for patterns may also be important. For example, with a shorter PEG molecule or a thinner SAM, a smaller dose would be required for removal, and the influence of electron scattering would be diminished. The feature size is further limited by the edge roughness; our AFM images show that the edge roughness is comparable to the granularity of the PEG background (Figure 3). A shorter PEG molecule should result in smaller edge roughness. A recent study also shows that shorter PEGs can prevent the adhesion of protein.25 Herrwerth et al.45 give a good review of the protein-repelling property of PEG, which is still open to interpretation.

Adhesion to EBL Patterns. In our studies, the spheres were affixed to the exposed areas with unspecific binding of the sphere to the surface. We note that the spheres adhered equally well to the exposed Au surface (type II) as to the surface affected by contamination writing (type III), as can be seen by comparing Figures 3 and 8. Because the contaminated surface is hydrophobic, it should bind either protein or the polystyrene spheres. The binding energy between spheres and patterned PEG has not been quantified, although we have observed that spheres are not removed after up to 1 h of washing with water or PBS using ultrasound. Another possible binding mechanism between Au and protein is via thiols in the protein, which may be happening when completely removing the PEG (type II response). An alternative approach to the placement of colloids (protein, sphere, etc.) on the exposed sites is to backfill with a thiolate having an appropriate linking chemistry between the Au surface and the desired colloid. In this case, it is critical to avoid contamination writing, using doses to achieve type II response. This type of linking chemistry approach was the design used by Harnett et al.,17 who demonstrated that protein-coated spheres bind to EBL-exposed areas of AT SAMs.

The binding of FluoSpheres to the regions with contamination material (type III patterning) suggests that contamination writing might itself be used to create nanoscale self-assembly to templates. Before PEG SAM incubation, contamination writing could be used to pattern the clean Au surface, masking the substrate to deposited on silicon shows signals for carbon, oxygen, and silicon.42,44 This is consistent with a plasticlike material, although it does not say a great deal about the form of the material. Evidence regarding the composition of electron beam-deposited material comes from Akama et al.,42 who examined transmission electron microscope diffraction patterns from contamination writing material and concluded that it was amorphous, which again is consistent with a plasticlike material. We would note that contamination writing material gives weak backscattering in an SEM, indicating low density and low atomic number. Taken together, these arguments support the idea that the electron beam material is a plasticlike material that is composed of cross-linked hydrocarbons and thus is likely hydrophobic in character.

Several groups have exploited the contamination writing process to construct nanostructures40 or for nanolithography.41 Other groups routinely use this process to construct electron beam-deposited AFM tips with high aspect ratios.42–44 Although the chemistry of the deposited material is not well understood, in part because of difficulties in producing sufficient quantities for analysis, Auger spectroscopy of electron beam material...
the subsequent assembly of a PEG SAM and thereby creating small hydrophobic patterns in the PEG SAM. The hydrophobic sites could then be used to bind proteins.

When comparing the sphere binding on the two types of Au surfaces, we note that the EBL patterns in the PEG SAM on granular Au (Figure 6) and flame-annealed Au (Figure 8) appear to bind spheres equally well. Hence, what is learned from the flame-annealed surfaces can be extended to the granular surface.

**PEG SAM Sphere Repulsion.** We note that our freshly prepared PEG SAMs and vacuum-treated, rehydrated PEG SAMs appear to repel NeutrAvidin-coated spheres equally well, although they have a distinctly different appearance in the AFM. We have not noted any difference in the ability to repel FluoSpheres between hydrated and rehydrated PEG SAM. However, in both SAMs the AFM images reveal small voids and imperfections that could be the reason that occasionally unwanted spheres bind to unexposed regions. Nevertheless, we observe a much stronger affinity to the patterned areas than to the unexposed areas, but at this point, we do not have any quantitative data on the sphere affinity to the various surfaces.

**Conclusions**

We report a study of high-resolution EBL patterning on PEG SAMs on both granular and flame-annealed Au and demonstrate the patterning of biomolecular features with dimensions approaching 40 nm. Two distinct patterning mechanisms are demonstrated when exposing the PEG SAM with EBL: At low doses, pattern formation occurs by SAM ablation in a self-developing process where the feature size is directly dose-dependent. At higher doses, electron beam-induced deposition of material, so-called contamination writing, is seen in the ablated process where the feature size is indirectly dose-dependent. At higher doses, electron beam-induced deposition of material, so-called contamination writing, is seen in the ablated areas of the SAM. The balance between these two mechanisms is shown to depend on the geometry of the pattern. We have also demonstrated that the NeutrAvidin-coated FluoSpheres bind the EBL patterns and that the PEG SAM effectively prevents sphere adhesion on both granular and flame-annealed Au.

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