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Fabrication of Protein Dot Arrays via Particle Lithography

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Abstract

The ability to pattern a surface with proteins on both the nanometer and micrometer scale has attracted considerable interest due to its applications in the fields of biomaterials, biosensors, and cell adhesion. Here we describe a simple particle lithography technique to fabricate substrates with hexagonally patterned dots of protein surrounded by a protein-repellant layer of poly(ethylene glycol) (PEG). Using this bottom-up approach, dot arrays of three different proteins (fibrinogen, P-selectin, and human serum albumin) were fabricated. The size of the protein dots (450 nm - 1.1 μm) was independent of the protein immobilized, but could be varied by changing the size of the latex spheres (diameter = 2 - 10 μm) utilized in assembling the lithographic bead monolayer. These results suggest that this technique can be extended to other biomolecules and will be useful in applications where arrays of protein dots are desired.

INTRODUCTION

The fabrication of micro- and nano-scale patterns of proteins has applications in a number of fields. In biology, protein patterning has been used to investigate the role of protein spatial presentation on cell adhesion¹, chemotaxis², cell proliferation³, and cell apoptosis⁴. In the field of biosensors, patterning has been utilized to fabricate multianalyte immunosensor arrays⁵, protein microarrays for bacterial detection⁶, patterned DNA microarrays^{7, 8}, and to align neuronal cell growth on microelectronic devices⁹. Finally, protein patterned substrates have been utilized for the growth of cardiomyocyte cultures for myocardial repair¹⁰, the growth of osteoblast cells for bone tissue engineered constructs¹¹, and for growing co-cultures of hepatocytes and fibroblasts¹².

Current methods of protein patterning include both “top-down techniques” such as microcontact printing^{1, 13, 14}, microfluidic patterning¹⁵⁻¹⁸, photolithography^{19, 20}, imprint lithography²¹, and E-beam lithography²²⁻²⁴; and “bottom-up techniques” such as block copolymer micelle nanolithography²⁵⁻²⁷ and particle lithography²⁸⁻³⁰. Important parameters in choosing one method over another often include: size of pattern, shape of pattern, pattern fidelity and long range order, processing time, necessity of specialized equipment, and cost. The reader is referred to the reviews of Voros et al.³¹, and Christman et al.³², for an in-depth analysis of the specific advantages and disadvantages of these methods.

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Particle lithography is an attractive technique for protein patterning due to its simplicity, low-cost, and versatility. This technique has been widely used in a number of areas to generate nanometer to micrometer sized patterns with a variety of materials, such as inorganics^{33, 34}, metals³⁵⁻³⁸, and polymers³⁹⁻⁴¹. However, in the area of protein patterning, particle lithography is a relatively new method (Table 1). In an initial study by Garino et al²⁸, surfaces with periodic protein nanostructures were prepared by mixing latex spheres with a specific protein followed by deposition on flat surfaces. Upon sphere removal, a honeycomb protein pattern was obtained. This technique has been extended to the fabrication of honeycomb patterns of protein mixtures (Protein A & BSA)⁴² and protein rings⁴³. Similar honeycomb structures have been obtained by our group by an alternative procedure in which incubation of the protein solution occurs only after the sphere monolayer is formed⁴⁰. Alternatively, particle lithographic methods have been developed to fabricate patterns of protein dots^{29, 30, 44-46}.

A persistent challenge in fabricating useful patterns via particle lithography for both protein and non-protein applications is to create patterns with high fidelity and long-range order. In response to this challenge, considerable research has been performed and a diverse set of strategies, such as spin coating^{41, 47}, confined convective assembly⁴⁸, physical confinement⁴⁹, template assisted assembly^{50, 51}, chemically patterned surfaces⁵²⁻⁵⁴, patterned wettability^{55, 56}, Langmuir-Blodgett technique^{57, 58}, and electrodeposition^{59, 60} have been developed. Excellent reviews of these colloidal techniques are available^{61, 62}. Despite the fact that these techniques are available, most of the studies that employ particle lithography to pattern proteins have relied on simple solvent evaporation (see Table 1). As will be discussed below, we have employed the technique of patterned wettability to produce large areas of well-ordered microspheres and subsequent protein dot patterns.

An additional issue in the fabrication of protein dots via particle lithography is creating a background that repels protein. Because of its protein-resistant properties, most strategies have employed poly(ethylene glycol) (PEG) as the inert background via two different strategies. In the first scheme, particle lithography is employed to initially define hydrophobic regions on a surface (*e.g.* alkane thiols on gold nanodisks⁴⁵ or dodecyl phosphates on TiO₂³⁰) and then the copolymer poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) is electrostatically grafted around these regions to provide an inert and protein-repellant background. Subsequently, proteins are adsorbed to the patterned hydrophobic regions. By contrast, the second scheme involves covalently grafting a layer of poly(ethylene glycol) through silane chemistry around the beads²⁹. Upon removal of the beads, holes in the protein-repellant layer are exposed for subsequent protein deposition.

In this work, we report a novel method (Fig. 1) for fabricating periodic patterns of protein dots, 450 nm - 1.1 μ m in diameter. To create patterns with long-range order we first used the method of patterned wettability to self-assemble a monolayer of latex spheres. This sphere monolayer then served as a lithographic mask to selectively graft a nm-thin layer of poly(ethylene glycol) (PEG). A solution phase grafting procedure with methoxy-poly(ethylene glycol)-(triethoxy) silane (PEG-silane) was developed to form a siloxane-anchored self-assembled monolayer of PEG around the beads. In contrast to the method of Cai described above²⁹, the PEG layer in our procedure was grafted directly to the glass substrate instead of a previously deposited silane monolayer. A challenge in forming the PEG layer was to find a solvent that would allow for the silanization reaction but that would not dissolve the polystyrene beads. Following sphere removal, periodic patterns of holes in the protein-repellant PEG layer were exposed, and proteins selectively adsorbed onto the underlying surface in these holes. We demonstrate the versatility of this technique by fabricating dot patterns of different diameters and different proteins.

EXPERIMENTAL SECTION

Chemicals and Materials

Polystyrene latex spheres (2 μm , 5 μm , and 10 μm mean diameter) were purchased from Duke Scientific, and washed once in DI water by centrifugation before use. Silicon wafers (P type, <111>) were purchased from Wafer World, Inc (West Palm Beach, FL), while methoxy-poly(ethylene glycol)-(triethoxy)silane (mPEG-Si, MW = 5000) was purchased from LaySan Bio (Arab, AL). Poly(dimethylsiloxane) (PDMS; Sylgard-184, Dow-Corning) was purchased from Krayden (Marlborough, MA). AlexaFluor488-labeled Fibrinogen, AlexaFluor488-Bovine Serum Albumin (BSA), and an AlexaFluor 488 protein labeling kit were obtained from Invitrogen (Carlsbad, CA). The labeling kit was used to label P-selectin (R&D Research) according to the manufacturer's instructions. 1.4 nm FluoroNanogold-anti-mouse Fab'-AlexaFluor 488 and GoldEnhance EM were purchased from Nanoprobes (Yaphank, NY).

Substrate Preparation and Self-Assembled Monolayer Formation—Microscope glass slides, coverslips, or silicon wafers were sequentially cleaned in trichloroethylene, acetone, methanol, and DI water for 2 min each cycle using an ultrasonic cleaner. A small circular region of each substrate was selectively made hydrophilic by placing a cured PDMS mold containing a 4.5 mm diameter hole onto the surface (Fig. 1B) and exposing to an air plasma at 400 millitorr (Harrick Scientific Corp., Model PDC-32G, Ithaca, NY) under low power (0.010 W/cm³) for 30 seconds (Fig. 1C). After plasma treatment, the PDMS mold was removed and the substrate was left untouched for ~30 minutes (Fig. 1D). Next a drop (3 μl - 18 μl) of a washed bead suspension was deposited on the patterned region and allowed to dry overnight at 4 °C to form the monolayer of microspheres (Fig. 1E). The volume and concentration of the bead solution deposited were adjusted so that there were a sufficient number of beads to form a complete monolayer over the plasma treated area. After bead monolayer formation, substrates were heated at 80 °C for one hour in order to keep the beads anchored to the surface during washing and wet chemical modification. Finally, each substrate was gently placed in a petri dish full of DI water and allowed to soak for five minutes to remove residue from the bead suspension. Substrates were gently removed from the DI water and allowed to dry in ambient conditions for at least one hour before PEG grafting.

PEG Grafting

The bead monolayer then served as a mask to selectively graft a nm-thin layer of poly(ethylene glycol) (PEG) to the substrate. Briefly, the substrates were exposed to an air plasma at 400 millitorr for 1 minute at high power (0.027 W/cm³), immediately incubated with a mPEG-Si solution (4mM mPEG-Si in anhydrous acetonitrile), and left overnight at room temperature (Fig. 1F). For the mPEG-Si incubation, either a 400 μl droplet of the solution was deposited on the surface or the substrate was completely immersed in the liquid. We observed similar results using both techniques. The substrates were then gently placed in three consecutive petri dishes full of acetonitrile and allowed to soak for five minutes in each to remove any ungrafted mPEG-Si. Finally, the substrates were rinsed with a stream of DI water using a standard laboratory wash bottle (Nalgene #2401-0500) to further remove any ungrafted mPEG-Si and to remove the beads. The final result was a PEG-coated surface with periodic holes (Fig. 1G).

Protein Adsorption

AlexaFluor488-labeled proteins were dissolved in Hanks Balanced Salt Solution (HBSS) at concentrations of 10 to 100 $\mu\text{g}/\text{mL}$. Aliquots of a protein solution were dispensed on the PEG patterned surface and incubated for 2 hr at room temperature to allow protein to adsorb into the uncoated holes (Fig. 1H). Following incubation, the substrates were rinsed first with 25 mL HBSS using a transfer pipette and then with 50 mL DI water using a standard laboratory wash bottle to remove any unbound protein.

Immunogold Labeling

1.4 nm FluoroNanogold (FNG) was dissolved in Phosphate Buffered Saline (PBS), pH 7.4 at concentrations of 20 to 80 $\mu\text{g/ml}$. Aliquots of an FNG solution were dispensed on the PEG patterned surface and incubated for 2 hr at room temperature. Following incubation, the substrates were rinsed first with 25 mL PBS using a transfer pipette and then with 50 mL DI water using a standard laboratory wash bottle. Next, an aliquot of GoldEnhance (GE) EM was mixed according to the manufacturer's instructions using a 1:2:1:1 or 1:3:1:1 ratio of GE EM solutions A, B, C, and D, respectively. The GE EM solution was dispensed on the FNG patterned surface and allowed to incubate for 20 min at room temperature. Following incubation, the substrates were rinsed with 50 mL DI water using a standard laboratory wash bottle and then dried under a stream of nitrogen. Finally, substrates were coated with 5 nm of carbon using thermal evaporation.

Substrate Characterization

Bead monolayer formation was characterized by both optical and scanning electron microscopy (SEM). Optical images were acquired with a Zeiss Axiovert 200 inverted microscope equipped with a CoolSnap cf digital camera. SEM images of bead monolayers were obtained by sputtering the monolayers with an Au/Pd layer (5 nm) and then imaging with a Zeiss 960A scanning electron microscope under 15-kV accelerating voltage.

To characterize the coverages of bead monolayers using optical microscopy, bright field images were taken of parts of bead monolayers and then combined to form montages of complete monolayers. The areas covered by close-packed beads were measured with MetaMorph Imaging Software. We defined surface coverage as the percentage of area covered by single-layers of close-packed beads with respect to the total area of the substrate. (Small defects and areas of multiple layers of beads were considered reduced coverage).

To characterize the PEG layer formed, small pieces (1 cm $\times$ 1 cm) of silicon wafers with a native oxide were modified with a uniform PEG layer by the methods described for the glass substrates. Ellipsometric measurements of the PEG layer film thickness were made using a Gaertner L-117C manual ellipsometer (Gaertner Scientific Corp., Chicago, IL) with a 632.8 nm He-Ne laser at an angle of incidence of 70°. All film thicknesses were determined using the Gaertner Ellipsometry Measurement Program (GEMP) in the one-layer and two-layer mode. We measured PEG layer thicknesses in two ways. Initially, we modeled the two layers (PEG layer on a native oxide layer on silicon) as a one-layer system, based on the fact that the index of refraction for PEG ($n_{\text{PEG}} = 1.46$)⁶³ and that for silicon dioxide are very close ($n_{\text{SiO}_2} = 1.46$). Finally, we used better optical data for a native silicon dioxide layer based on the work of Subramanian et al.⁶⁴ as part of a two-layer system. The results in both cases were within statistical error. In the latter analysis, we determined the index of refraction and thickness of the native oxide layer by iteration, using the usual parameters for the silicon substrate ($n = 3.85 + 0.02i$), and the index of refraction versus thickness curve reported by Subramanian et al.⁶⁴. Doing this we determined our native oxide thickness was typically 2.9 nm with $n = 2.43$ (cf. with a thickness of 3.2 nm assuming an $n = 1.46$). Using these properties for our native oxide, we used the GEMP program in the two-layer mode to determine the PEG layer thickness. For all measurements of PEG film thickness we measured the sample in at least two different locations on at least two identically prepared films and these were compared to an out-of-the-box sample from the same silicon wafer prepared and measured with the PEG samples.

Characterization of the protein patterned substrates was done by fluorescent imaging with a fluorescent microscope (Zeiss Axiovert 200) and analyzed with MetaMorph Imaging Software. Substrates patterned with FluoroNanogold were imaged with a Zeiss 960A scanning electron microscope (SEM) with 20-kV accelerating voltage using a back-scattered electron detector.

Calculations and Statistics

Values are presented as mean $\pm$ standard error of the mean unless otherwise specified, and statistical significance was assessed when appropriate by a Student's t-test for paired data, with $P < 0.05$ considered as statistically significant.

Results and Discussion

Periodic patterns of protein dots were fabricated on glass or silicon wafers by a three step procedure (Fig. 1): 1. Formation of a bead monolayer, 2. Grafting of a protein-resistant PEG layer, and 3. Selective adsorption of protein. A variety of complementary microscopy techniques were employed to characterize the structures produced and optimize the process.

Bead monolayer formation

A persistent challenge in the use of particle lithography is the ability to create large areas of hexagonally packed, defect free, bead monolayers. Thus, we investigated whether surfaces with patterned wettability^{55, 56} would result in the formation of large continuous areas of close-packed and ordered bead monolayers. A circular area of glass or silicon substrates was selectively treated with an air plasma via a PDMS mold to create a distinct region of increased hydrophilicity or wettability (Fig. 1A-1D). Aqueous suspensions ($\sim 0.05\%$ V/V) of latex beads were dispensed onto the plasma cleaned substrates. As a control, suspensions of latex beads were also dispensed onto substrates that had not been plasma cleaned. As solvent evaporated from the colloidal suspensions, attractive capillary forces between the beads developed and caused them to self-assemble into closely packed and ordered monolayers. In both cases (Figs. 2A & 2B), imperfections in bead ordering (e.g. point defects, dislocation, and grain boundaries) were observed. On substrates that were not exposed to the plasma cleaning process ("unpatterned substrates") the bead monolayer was not confined to a specific area, and the monolayer formed included large areas absent of any beads (Fig. 2B). However, on plasma cleaned substrates ("patterned substrates") the monolayer of beads was observed to preferentially nucleate at the edge of the plasma-treated region, resulting in a distinct boundary (Fig. 2A) of regions with and without beads. Furthermore, patterned substrates exhibited a lower concentration of defects and an increased surface coverage of bead monolayers as compared to substrates that were not plasma cleaned. For example, patterned surfaces had bead surface coverages $>84\%$ with $5\ \mu\text{m}$ and $10\ \mu\text{m}$ diameter beads whereas untreated glass surfaces exhibited surface coverages of 60% and 38% , respectively. Figure 2C-2E shows images of periodic arrays formed on patterned substrates with $2\ \mu\text{m}$, $5\ \mu\text{m}$, and $10\ \mu\text{m}$ diameter beads. Due to the improved surface coverage subsequent studies were performed with patterned substrates.

Grafting of a protein-resistant PEG layer

Following bead monolayer formation, a protein repellent background of PEG was created around the beads by chemically grafting methoxy-poly(ethylene glycol)-(triethoxy)silane to the surface. Subsequent removal of the bead monolayer uncovered periodic holes in the PEG layer. It should be noted that covalently grafting the PEG-silane to the glass in solution around polystyrene beads was not trivial. Since the original work of Sagiv and coworkers on the formation of SAMs from siloxane compounds^{65, 66} subsequent research has shown that the formation of organosilane SAMs depends upon a number of key parameters: water content, solvent, temperature, and deposition time⁶⁷. Furthermore the reactivity of the silane depends upon its type (e.g. chlorosilanes, alkoxysilanes) and functionality (e.g. mono-, di-, or trifunctional silanes). Normally silanes are grafted to glass in organic solvents such as toluene to eliminate the problems of multi-layer formation or polymerization in the bulk solvent phase in the presence of large amounts of water. However organic solvents such as toluene will dissolve the beads. Therefore the number of solvents that allow for PEG monolayer formation

but do not dissolve the beads is limited. After trying a number of different solvents, acetonitrile was chosen. To confirm the successful grafting of the PEG layer, we performed ellipsometry on both bulk silicon and silicon modified with a PEG-Si film. Ellipsometry of the bare silicon provided the refractive index and thickness of the native silicon dioxide layer which we used to subsequently determine the PEG layer thickness. The oxide thickness was measured to be 2.9 nm, and the PEG layer thickness was 0.5 ± 0.2 nm. This value is smaller than previous measurements of the thickness (1.6 - 1.7 nm) of a dry PEG layer (MW = 5000) using Ellipsometry and atomic force microscopy (AFM)⁶⁸. We hypothesize that this difference in thickness is related to variations in the grafting density of PEG.

Protein Patterned Substrates

The final step of the process was the selective adsorption of protein into the wells of the patterned PEG substrate. Figure 3 shows fluorescent images of surfaces that were patterned with PEG via 2 μm , 5 μm , and 10 μm diameter beads and subsequently incubated with solutions of fluorescent fibrinogen. Well-resolved dots of protein in a hexagonal pattern were observed. As the size of the bead increased, both the size of the protein dots formed (450 nm to 1050 nm) and the center-to-center spacing between dots increased. To demonstrate the versatility of this technique we fabricated patterned substrates with two other types of cell adhesion molecules (P-selectin and Albumin) and measured the diameter of the dots formed by fluorescent microscopy. As shown in Figure 4, the size of the protein dots produced for a given bead size was constant and independent of the protein deposited.

It should be noted that the fluorescent dot measurements (450 nm) measured for the 2 μm beads were near the resolution limit of standard fluorescent microscopes (200 - 500 nm). When objects with dimensions smaller than the resolution limit are imaged they will project with dimensions of the resolution limit. Thus, in order to confirm that the fluorescent measurements of the protein dots were accurate, we patterned FluoroNanogold (FNG) on substrates made with 2 μm , 5 μm , and 10 μm diameter beads and then imaged the samples using SEM.

FNG is composed of an antibody Fab' fragment covalently bound to a 1.4 nm gold particle, which is smaller than the resolution limit (~ 50 nm) of the SEM we used. In order to make the patterned FNG visible by SEM imaging, we used GoldEnhance EM to increase the size of the gold particles. To prevent the substrates from charging in the SEM, we coated them with a thin (5 nm) layer of carbon. The final result was patterned dots of gold particles on a carbon background. The large atomic number difference between the gold particles and the carbon background allowed us to use backscattered electron (BSE) imaging to view the FNG dots. Figure 5 shows BSE images of substrates that were patterned with FNG via 10 μm , 5 μm , and 2 μm diameter beads. The diameters of the dots on substrates made with 10 μm , 5 μm , and 2 μm beads were $1200 \text{ nm} \pm 300 \text{ nm}$, $640 \pm 60 \text{ nm}$, and $450 \pm 50 \text{ nm}$, respectively. These measurements agree quite well with those made by fluorescent microscopy. In addition, the absence of gold particles between the dots provides additional evidence of the ability of the PEG regions of the substrate to prevent protein adsorption.

In order to corroborate that the protein selectively adsorbed in the patterned PEG holes and not in the PEG background, we exposed fluorescent fibrinogen dots formed using 10 μm beads to fluorescent light until they were completely photobleached. Figure 6 shows the fluorescent intensity (arbitrary units) of a linescan across three dots with time. Although the fluorescent intensity inside of the dots decreases to zero in about ten minutes, the baseline intensity between the dots remains constant. This confirms the protein resistant properties of the PEG grafted between the protein dots.

Effect of Bead Monolayer Cleaning

In the course of our study we found that cleaning the bead monolayer in DI water was essential for the formation of consistent protein dots. Figure 7A-D shows representative fluorescent images of protein patterns formed on substrates that were not thoroughly cleaned prior to PEG grafting. At the periphery (Fig. 7E, Region A) of bead monolayer patterns that were not cleaned prior to Si-PEG grafting, dot patterns with interconnecting lines (Fig. 7A) were frequently observed. In addition a 2-fold variation in the protein dot diameters was often observed, with the dot size gradually decreasing and becoming uniform as one moves from the periphery towards the center of the pattern (Fig. 7B). Towards the center of the pattern (Fig. 7E, Region B) we observed a haze of protein (Fig. 7C) and in the center of the pattern (Fig. 7E, Region C) we observed smaller dots with uniform diameters (Fig. 7D).

The presence of the interconnecting fluorescent lines suggested that something on the surface was preventing complete grafting of the PEG layer around the beads. We hypothesized that the most likely cause was residual surfactant from the bead suspension being deposited on the surface during evaporation of the liquid^{69, 70}. To test this hypothesis we performed phase contrast imaging of the bead monolayers formed after water evaporation and prior to cleaning (Fig. 7C). The different shading in region A compared to regions B & C appeared to support our hypothesis. Furthermore, subsequent cleaning of the same bead monolayer by soaking in DI water for five minutes and then allowing the bead monolayer to air dry for at least one hour resulted in the removal of this ring (Fig. 7D) and produced the uniform dot patterns observed in Figure 3.

Conclusions

A simple method for fabricating protein patterned substrates with nanometer to micrometer scale dimensions was developed. The method includes selectively grafting a protein-repellant layer of poly(ethylene glycol) in the holes of a self-assembled monolayer of latex spheres, removal of the spheres, and selective adsorption of the protein. The size of the protein dots formed ranged from 450 nm to 1.1 μm , and was dependent upon the size of the spheres utilized. The versatility of this technique was demonstrated by patterning surfaces with 3 different proteins (Fibrinogen, P-selectin, and Albumin) as well as with antibody coated FluoroNanogold. These results suggest that this is a generic patterning technique that could be extended to a number of proteins. We anticipate that smaller diameter dots (~50 nm) of protein can be created via this technique by using commercially available latex spheres with diameters of 100 nm.

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Figure 1. Schematic of the protein patterning procedure

Step 1: A circular, hydrophilic region is created on a glass slide (A - D). Step 2: A drop of a bead suspension is deposited on the hydrophilic surface, and as the solvent evaporates a monolayer of spheres is formed (D - E). Step 3: The monolayer of spheres then serves as a mask to selectively graft a layer of poly(ethylene glycol) to the glass substrate through silane chemistry (E - F). Step 4: The sphere monolayer is removed exposing regions of bare glass surrounded by PEG, and protein is then adsorbed to these bare glass regions (F-H).

Figure 2. Bead Monolayers

Optical image montage of monolayer formed by 10 μm beads (A) following the plasma cleaning procedure and (B) without plasma cleaning. SEM images of (C) 2 μm , (D) 5 μm , and (E) 10 μm bead monolayers. (A-B) are both montages composed of several smaller images. The square corner features in (A) are an artifact of piecing together the montage. In (A-B), white areas contain no beads. The gray and black areas contain beads that are close-packed but contain the defects shown in (C-E). The larger grain boundary defects are visible in the close-packed areas of (A-B) as lines.

Figure 3. Arrays of protein dots fabricated via particle lithography

Fluorescent images of surfaces patterned with fluorescent fibrinogen via particle lithography with (A) 2 μm spheres; (B) 5 μm spheres; (C) 10 μm spheres. The fluorescent line intensity (arbitrary units) graphs to the right of each image correspond to the dashed lines in each image.

Figure 4. Size of protein dots is dependent upon the diameter of the latex sphere utilized
Average diameter of the protein dots created for each bead size with three different proteins. Dot diameters of protein patterns were measured from fluorescent images using MetaMorph Image Analysis software. Scanning Electron Microscopy (SEM) imaging was performed to measure the diameters of the dot patterns of FLuoroNanogold. For each condition a total 3 different slides and (80-340) dots were measured. A line to guide the eye is shown.

Figure 5. Arrays of FluoroNanogold particles fabricated via particle lithography
SEM images of surfaces patterned with FluoroNanogold particles by particle lithography with (A) 10 μm spheres, (B) 5 μm spheres, and (C) 2 μm spheres.

Figure 6. Photobleaching of protein patterns

Line scans of the fluorescent intensity of three protein dots fabricated via 10 μm beads were measured as a function of time.

Figure 7. Effect of latex sphere monolayer washing

(A-D) Fluorescent images of unwashed substrates patterned with fluorescent fibrinogen via 10 μm beads. Phase contrast image of a 10 μm bead monolayer (E) prior to cleaning and (F) after cleaning with a five minute soak in DI water.

Table 1
Comparison of Particle Lithography Methods of Protein Patterning

Pattern Type	Method of Monolayer formation	Background/Grafting Method	Pattern Size	Protein Type(s)	Ref
Dots	Spin Coating	PEO Plasma polymerization	50 - 200 nm	BSA Human IgG	46
Dots	Computer-driven dip coating	PLL-g-PEG Electrostatic	0.1 - 2.0 μ m	Streptavidin	30
Dots/rings	Solvent evaporation	PEG-Silane Liquid or vapor phase grafting	60-120 nm	Lysozyme	29
Dot	Electrostatic Solvent evaporation	PLL-g-PEG Electrostatic	124 nm	Ferritin laminin	45
Dots	Solvent evaporation	PVA Photocrosslinking	490 nm	GFP	44
Dots	Patterned wettability	PEG-silane Liquid phase grafting	0.45 - 1.2 μ m	BSA Fibrinogen P-selectin	This work
Honeycomb Rings	Solvent evaporation	NA	0.2 - 0.8 μ m	BSA Rabbit IgG	28
Honeycomb Rings	Solvent evaporation	NA	0.2 - 0.5 μ m	SpA BSA	42
Honeycomb Rings	Spin Coating	NA	0.16 - 2.3 μ m	BSA Fibrinogen Anti-mouse IgG	40
Rings	Solvent evaporation	NA	0.20 - 0.36 μ m	Ferritin Apoferritin BSA Rabbit IgG	43

PLL-g-PEG - poly(lysine)-graft-poly(ethylene glycol); PVA - poly(vinylalcohol); PEO - Poly(ethylene oxide); GFP - Green Fluorescent Protein; NA - not applicable - BSA - Bovine Serum Albumin; IgG - immunoglobulin.; SpA - Staphylococcal protein A