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Positioning Multiple Proteins at the Nanoscale with Electron Beam Cross-Linked Functional Polymers

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Abstract

Constructing multicomponent protein structures that match the complexity of those found in Nature is essential for the next generation of medical materials. In this report, a versatile method to precisely arrange multicomponent protein nanopatterns in two-dimensional single-layer or three-dimensional multilayer formats using electron beam lithography is described. Eight arm poly(ethylene glycol)s were modified at the chain ends with either biotin, maleimide, aminooxy, or nitrilotriacetic acid. Analysis by ¹H NMR spectroscopy revealed that the reactions were efficient and that end group conversions were 91-100%. The polymers were then cross-linked onto Si surfaces using electron beams to form micron sized patterns of the functional groups. Proteins with biotin binding sites, a free cysteine, an N-terminal α -oxoamide, and a histidine tag, respectively, were then incubated with the substrate in aqueous solutions without the addition of any other reagents. By fluorescence microscopy experiments it was determined that proteins reacted site-specifically with the exposed functional groups to form protein micropatterns. Multicomponent nanoscale protein patterns were then fabricated. Different PEGs with orthogonal reactivity were sequentially patterned on the same chip. Simultaneous assembly of two different proteins from a mixture of the biomolecules formed the multicomponent two dimensional patterns. Atomic force microscopy demonstrated that nanometer sized patterns of polymer were formed and fluorescence microscopy demonstrated that side-by-side patterns of the different proteins were obtained. Moreover, multilayer PEG fabrication produced micron and nanometer sized patterns of one functional group on top of the other. Precise three-dimensional arrangements of different proteins were then realized.

INTRODUCTION

Many of the desired applications for nanopatterning necessitate that multiple, distinct proteins be conjugated to the same surface with precision. Nanometer features of one type of protein have been successfully fabricated by a number of techniques.¹ These include dip-pen nanolithography (DPN),²⁻⁴ nanografting,⁵ nanocontact printing,^{6,7} nanoimprint

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SUPPORTING INFORMATION Supporting Information Available: polymer synthesis, myoglobin modification, single channel fluorescence images. This material is available free of charge via the Internet at <http://pubs.acs.org>.

lithography,⁸ and electron beam (e-beam) lithography.⁹ Self assembly of particles,¹⁰ polymers,¹¹ and DNA¹² into ordered templates for subsequent protein or peptide attachment has also been employed. Micropatterning of multi-peptides and proteins has been accomplished by a wide variety of methods including printing, stamping, and photodeprotection.¹³⁻¹⁵ Thus far, only a few examples of nanopatterning multiple proteins on a single substrate have been reported. DPN has been effectively used to construct arrays of two different proteins by directly writing onto substrates.^{16,17} Likewise, adsorption of multiple antibodies was accomplished using sequential nanografting.¹⁸ A novel vibrational AFM mode selectively replaced areas of self assembled protein monolayers with different proteins under mild conditions in a technique called native protein nanolithography.¹⁹ A combination of an elastomeric stamp and a nanotemplate was recently reported for multicomponent protein patterns.²⁰ However, to our knowledge e-beam lithography has not been employed. Yet, this method can not only generate arbitrary protein nanopatterns of different shapes, sizes, and curvatures, but also control inter-feature spaces and locations precisely. The latter could possibly result in patterns with nanoscale inter-feature spacings for heterogeneous patterns. Because of this potential advantage, we sought to determine if e-beam lithography could be utilized and describe the first multicomponent protein nanopatterns by this technique.

Three-dimensional arrangements of multiple proteins also provide entry to a range of sophisticated applications.²¹ For example, intricate, three-dimensional shapes at the micron scale consisting of a single protein have been successfully used to guide neuronal development,²² trap bacteria,²³ and for bioelectronics.²⁴ Multi-protein structures have been created at the micron scale.²⁵ To our knowledge, constructs with nanoscale components have not yet been realized. We also utilized e-beam lithography to construct the first multicomponent, multilayer heterogeneous protein patterns that range from the micron to nanoscale.

Patterns were prepared by cross-linking an eight arm poly(ethylene glycol) (PEG) polymer, which was modified with one of four protein-reactive moieties: biotin, maleimide, aminoxy, or nickel(II) nitrilotriacetic acid (Ni^{2+} -NTA) (Figure 1a). Each of these groups can conjugate proteins at distinct sites. Biotin is a high affinity ligand ($K_A \sim 10^{15} \text{ M}^{-1}$) for streptavidin (SAV),²⁶ and is often used as a linker between biotinylated surfaces and biotinylated proteins.³ Maleimides and free cysteines react by Michael addition. Since these residues occur in low abundance in proteins, this reaction is convenient for site-specific immobilization. Aminoxy groups conjugate proteins with reactive carbonyls, chemospecifically forming oxime bonds. Proteins are readily modified by transamination reactions providing α -oxoamides only at the N-termini²⁷ and a route for site-specific immobilization.²⁸ Ni^{2+} -NTA offers a convenient handle for immobilization of proteins due to the affinity interaction of multiple histidines with the complex.²⁹ Proteins are easily tagged with a polyhistidine sequence at either the C or N terminus using recombinant techniques. All of the chosen polymer end groups provide advantageous conditions for protein immobilization once they are cross-linked to the surface. First, proteins are captured in aqueous solutions and ambient temperatures, without requiring additional reagents or activation steps which can lead to protein denaturation. Therefore, sensitive biomolecules prone to denaturation under direct-write conditions may be utilized with this method. Second, immobilization is site-specific; physical adsorption or covalent attachment via groups that are abundant on proteins such as carboxylic acids or amines often leads to random orientation and loss of bioactivity.

E-beam lithography was employed to prepare the patterns of protein-reactive PEG hydrogels. It is known that when PEG is exposed to focused e-beams, it cross-links to itself and to Si surfaces.^{9,30,31} The process occurs through a radical mechanism, similar to solution based radical-mediated cross-linking of PEG.³²⁻³⁴ The PEG component not only

cross-links to the surface,³⁰ but is an opportune choice because it is protein resistant and therefore typically used as a nonfouling surface.³⁵⁻³⁷ This latter point is important to produce patterns that resist non-specific binding, while allowing specific protein conjugation via the chosen functionality. In this manuscript, fabrication of the polymer patterns by e-beam lithography, conjugation of proteins, and characterization by atomic force microscopy and fluorescence microscopy is described.

RESULTS AND DISCUSSION

The end groups of eight arm PEGs were modified with biotin, maleimide, aminooxy, or nitrilotriacetic acid, in most cases using commercially available starting materials (see ESI for experimental procedures). Modification was determined by analysis of the ¹H NMR spectra, and conversions of the end groups were found to be efficient (91%, 100%, 97%, and 100%, respectively). These polymers were then utilized for e-beam cross-linking reactions to form functionalized patterns.

The PEG polymer layer was formed by spin-coating a 2% (w/w) solution in either methanol (maleimide-, aminooxy-, NTA-PEG) or chloroform (biotin-PEG) onto a Si wafer, and the desired patterns were created using an e-beam lithographic system. Any uncross-linked polymer was removed by rinsing the samples with methanol and H₂O, producing patterns of functionality (Figure 1a). The technique was first demonstrated for individual end-functional groups with micron-sized features. The e-beam exposure threshold was experimentally determined for each polymer and found to be 60-110 $\mu\text{C}/\text{cm}^2$ with the exception of the maleimide PEG which was 1.1 $\mu\text{C}/\text{cm}^2$. Cross-linking of the PEG occurs by a radical mechanism,^{30,38} and thus it is possible that some of the maleimides participated in this process, lowering the required dose.

Chips were subjected to the appropriate model proteins to produce the desired biomolecule patterns. The proteins were visualized by fluorescence after antibody staining, with the exception of streptavidin which was directly labeled with a dye. Biotin-PEG micropatterns were incubated with Alexa Fluor 568 SAv (Figure 1b), bovine serum albumin (BSA), which contains one free cysteine was attached to the maleimide-PEG patterns (Figure 1c), and N-terminal α -oxoamide-modified myoglobin was immobilized to aminooxy-PEG designs (Figure 1d). The latter protein was formed by an N-terminal specific transamination reaction (see ESI for experimental details) using a recently reported procedure.²⁷ To create the Ni²⁺-NTA-PEG patterns, NTA-PEG features were first generated and then Ni²⁺ was chelated. The metal chelated surface was characterized by scanning electron microscopy (SEM), which demonstrated that the Ni²⁺-NTA-PEG was patterned as expected (Figure 1e, top). A histidine-tagged calmodulin was subsequently immobilized (Figure 1e, bottom). In every case, fluorescence was located only on the patterns (Figure 1b-e).

Each surface was also subjected to controls where the protein handle was consumed by pre-saturating with a compound containing the respective functional group, except in the case of Ni²⁺-NTA-PEG where no Ni²⁺ was present for the control. There was no fluorescence detected for the biotin-, aminooxy-, and Ni²⁺-NTA-PEG controls, and little fluorescence was observed on the maleimide-PEG. This demonstrated that protein immobilization was a result of the desired chemistry and not from random adsorption on the functionalized patterns. The slight fluorescence that was observed on the maleimide-PEG was eliminated when the BSA was directly labeled with the fluorophore (data not shown); it was therefore attributed to nonspecific binding of the antibodies, which may have been caused by the presence of free cysteines.³⁹ Taken together, these results indicated site-specific attachment of the proteins to the functional groups, which is important for fabrication of bioactive features. In addition, undesirable nonspecific protein adsorption outside of the patterns was

minimal (signal to noise ratios are provided in the ESI). This is important to eliminate large background signals for the application of these materials and is likely due to the low concentrations of proteins and short contact times utilized in the staining of the surface.⁵ If required for a particular application, nonspecific adsorption could be further minimized by cross-linking the star PEGs onto Si precoated with a thin, unfunctionalized PEG layer.^{9,31}

With this e-beam induced cross-linking strategy, it is feasible to pattern more than one type of 8-arm PEG on the surface. All of the described end groups interact with proteins via different mechanisms. Therefore, immobilization of multiple proteins directed by the different functional groups is possible (Figure 2a). To demonstrate this, we first patterned the biotin-PEG on Si wafers at the micron scale. After removal of uncross-linked polymer, the maleimide-PEG features were subsequently fabricated on the same chip next to the biotin ones. The samples were then incubated with a solution containing both Alexa Fluor 568 SAv and BSA, followed by antibody staining. Red fluorescence was observed at locations of the biotin-PEG patterns, while green fluorescence was observed at the maleimide-PEG patterns (fluorescent overlay, Figure 2b and c). This demonstrated that the proteins were arrayed as directed by the functional groups on the surface with good specificity (individual green and fluorescent channels are provided in the ESI). Interestingly, the proteins were arrayed simultaneously from a mixture of the biomolecules; although not critical, this result showed that sequential immobilizations may not be necessary. The results also illustrated that a variety of designs such as letters and triangles can be produced, which is an advantage of e-beam lithography.

E-beam lithography can produce high resolution nanostructures; therefore, nanoscale patterning of both proteins was next demonstrated. Concentric squares of biotin and maleimide were sequentially fabricated side-by-side. Below each, a micron sized letter indicating either maleimide (M) or biotin (B) was also fabricated to allow for easy identification of the different functional nanopatterns. Atomic force microscopy (AFM) images displayed 130 nm wide nanoscale patterns of both functional PEGs aligned next to each other (Figure 2d). The concentric squares allowed visualization by standard fluorescence. Again, SAv and BSA were simultaneously immobilized, and green fluorescence and red fluorescence on both the micron-sized letters and nanoscale patterns were observed (fluorescence overlay, Figure 2d, inset). This confirmed self-sorting of the proteins (individual green and fluorescent channels are provided in the ESI). These results demonstrated that the original functional groups directed formation of multi-component protein patterns with nanosized features.

Patterning multiple proteins in multiple layers is also possible, because PEG can be induced to crosslink to itself.^{9,31} To demonstrate this, micron and nanosize features of aminooxy-PEG were patterned on top of a micron sized biotin-PEG pattern (Figure 3a). This was accomplished by cross-linking the biotin-PEG, washing, aligning, and then cross-linking the aminooxy-PEG. Using this approach, four 1 μm^2 features and "UCLA" characters with 250 nm line widths of aminooxy-PEG on top of 5 $\times$ 5 μm biotin-PEG patterns were fabricated. AFM images taken in tapping mode revealed that the underlying biotin-PEG was not ablated, but rather the aminooxy-PEG was cross-linked on top of the original patterns, creating 3D topographical structures (Figure 3c-d). To confirm protein immobilization on these multilayer patterns, the samples were incubated with a solution of Alexa Fluor 488 SAv and N-terminal α -oxoamide myoglobin, followed by Alexa Fluor 633 antibody staining. Although with conventional fluorescence microscopy the nanoscale "UCLA" could not be resolved, the 1 μm patterns were observed. Fluorescent image overlay of a three-by-three grid of this multilayer pattern (Figure 3b & 3c; individual channels provided in the ESI) indicated that the green fluorescent SAv had immobilized to the bottom biotin-PEG pattern and that myoglobin was localized to the top aminooxy patterns.

To further investigate the scope of this methodology, micron and nanometer sized features of maleimide-PEG were patterned on top of a micron sized biotin-PEG pattern (Figure 4a). Using this approach, four 1 μm , sixteen 500 nm, and sixteen 250 nm² features of maleimide-PEG on top of 5 μm biotin-PEG patterns were fabricated. AFM images taken in tapping mode again revealed that the underlying biotin-PEG was not ablated, and that the maleimide-PEG was cross-linked on top of the original patterns creating topographical structures (Figure 4b-d). To confirm protein immobilization on these multilayer patterns, the samples were incubated with a solution of Alexa Fluor 568 SAv and BSA. Again, although the nanoscale features could not be resolved, fluorescent image overlay (inset of Figure 4b) of the 1 μm^2 pattern indicated that the red fluorescent SAv had immobilized to the bottom biotin-PEG pattern and that BSA was localized to the top maleimide patterns. These results suggested that other patterns and functional groups can be achieved utilizing this methodology.

Finally a tri-pattern with both maleimide- and aminooxy-PEG on top of biotin-PEG was fabricated. The maleimide and aminooxy were purposely cross-linked using doses that achieved different heights³⁰ for easy visual identification. AFM images revealed that this additional combination of functionalized PEGs could also be cross-linked into three-dimensional patterns. Figure 5 displays four 1 $\times$ 1 μm patterns of both maleimide- and aminooxy-PEG on top of a 5 $\times$ 5 μm biotin-PEG patterns. The surface was incubated with Alexa Fluor 350 SAv, Alexa Fluor 555 BSA, and α -oxoamide myoglobin, followed by Alexa Fluor 488 antibody staining. Remarkably, fluorescence (image shown in inset, Figure 5b) revealed that all three proteins had been patterned.

CONCLUSIONS

We report a versatile approach to nanopattern multiple proteins using e-beam lithography that provides ready access to both single layer and multilayer formats. Cross-linking of PEG-based polymers with protein-reactive end-groups provides a tool for generating features in a variety of designs. Proteins react site-specifically under mild, aqueous conditions. Side-by-side patterns of PEG hydrogels with reactive groups direct binding of different proteins from a mixture of the biomolecules. There exists many handles and ligands for attachment of proteins to polymers,⁴⁰ and this approach should be directly applicable to immobilize additional combinations of proteins. Furthermore, this technique is powerful for multiplexed biomolecules in three-dimensional multilayer formats. This offers exciting possibilities to construct topographically interesting biomolecular systems for site-isolation enzyme cascades⁴¹ and “nanoscale factories,”²¹ in addition to generating materials that match complex structures found in Nature such as protein-signaling assemblies and viral capsids.⁴² Also, it is known that both micron and nanometer chemical and topographical cues are critical for cell adhesion.^{11,22,43-45} The methodology described here is useful for generating surfaces where both chemistry and topography is spatially controlled. Thus, it can be envisioned that by patterning extracellular matrix-derived signals, the surfaces will be useful to study the intricate relation between subcellular chemical and topographical cues that can lead to differences in adhesion, proliferation, protein expression or differentiation. This information would be valuable for understanding how to control cell behavior and for creating the next generation of biomaterials. We are currently exploring these possibilities.

EXPERIMENTAL SECTION

MATERIALS

Si chips were cleaned by exposure to piranha (3:1 H₂SO₄:H₂O₂, CAUTION). The chips were then rinsed with Millipore-H₂O and stored in Millipore-H₂O. Right before spin coating, Si wafers were further cleaned by rinsing with acetone, methanol, isopropanol, and

Millipore-H₂O for ~10 sec each and then dried with a stream of air. For multicomponent PEGs, gold features were fabricated on the Si chips prior to PEG spin coating and writing. Chips were cleaned in piranha, and patterned via standard photolithography procedures. Then, 10 nm of titanium followed by 300 nm of gold was deposited on the chips by metal evaporation, and patterns were developed by lift-off. This resulted in gold squares and lines which were used in later steps solely as reference markers to align the e-beam patterns to a fixed location on the chip in order to ensure alignment precision when patterning multiple PEGs.

METHODS

Samples were visualized with fluorescence microscopy using a Zeiss Axiovert 200 fluorescent microscope equipped with an AxioCam MRm monochrome camera or a Zeiss Axiovert 200M equipped with a Hamamatsu C4742-95 monochrome camera, and pictures were acquired and processed using AxioVision LE 4.1. The 3D images and the nanoscale side-by-side patterns were taken with a 40X objective. AFM images were collected on dry samples using a Dimension 3100 (Digital Instruments) in tapping mode (silicon cantilever, spring constant = ~ 40 N/m, tip radius = < 10 nm, scan rate = 1.5 Hz) and processed and analyzed using NanoScope IIIa Ver. 5.30r1 (Digital Instruments). SEM samples were mounted to the sample holder using carbon tape, and imaged with no additional processing on a JEOL JSM-6700F FE-SEM. An accelerating voltage of 10 kV, a probe current of 10 pA, and a working distance of 8 mm were used.

PATTERN FORMATION

The basic scheme for creating all of the end-functionalized polymer patterns was as follows: A 2% (w/w) PEG solution in either methanol (maleimide-, aminooxy-, NTA-PEG) or chloroform⁴⁶ (biotin-PEG) was spin-coated onto a cleaned Si chip at 3000 RPM for 20 sec. The chips were then used directly without any drying or baking steps. Specific patterns of polymer were cross-linked to the native oxide of the Si using a JC Nabity e-beam lithographic system (Nanometer Pattern Generation System, Ver. 9.0) modified from a JEOL 5910 scanning electron microscope. An accelerating voltage of 30kV was used, with a beam current of ~4.5pA. Biotin-PEG micropatterns were generated using a minimum e-beam dose of 110 $\mu\text{C}/\text{cm}^2$. Maleimide-PEG micropatterns were generated using a minimum e-beam dose of 1.1 $\mu\text{C}/\text{cm}^2$. Aminooxy-PEG micropatterns were generated using a minimum e-beam dose of 60 $\mu\text{C}/\text{cm}^2$. NTA-PEG micropatterns were generated using an e-beam dose of 90 $\mu\text{C}/\text{cm}^2$. The biotin-PEG nanopatterns were generated with a line dose of 3 nC/cm, the aminooxy-PEG nanopatterns were prepared with a minimum line dose of 0.5 nC/cm, whereas the maleimide-PEG nanopatterns were produced with a minimum line dose of 0.03 nC/cm. After exposure, any uncross-linked polymer was removed by rinsing the samples in methanol for 5-10 sec, followed by rinsing in dH₂O for 5-10 sec, and drying with a stream of N₂. Ni²⁺-NTA-PEG was generated by dipping the NTA-PEG patterns in a solution of nickel(II) chloride hexahydrate (200 mg/mL in Milli-Q H₂O) for 20 min, rinsing with Milli-Q H₂O, and drying with a stream of air. For side-by-side patterns and multilayer patterns, biotin features were first generated by the process described above. After the uncross-linked biotin-PEG was removed by rinsing with methanol, a layer of the second aminooxy-PEG or maleimide-PEG was spin-coated onto the same chip. The chips were then aligned utilizing gold features that were pre-fabricated on the chip, and the second PEG patterns were cross-linked as described above either next to or on top of the original biotin-PEG patterns. A wash step removed any unreacted PEG. For the tri-patterned chip aminooxy-PEG was cross-linked, followed by maleimide-PEG.

PATTERN VISUALIZATION

Micron-Sized Patterns—Individual biotin-, maleimide-, aminooxy, and Ni^{2+} -NTA-PEG patterns were incubated with Alexa Fluor 568 SAV (5 $\mu\text{g/mL}$ in phosphate buffered saline, PBS), BSA (10 $\mu\text{g/mL}$ in PBS), α -oxoamide-myoglobin (10 $\mu\text{g/mL}$ in phosphate buffer, 25 mM, pH 6.5), and His-tagged calmodulin (5 $\mu\text{g/mL}$ in PBS), respectively for 1 h. With the exception of SAV, bound proteins were visualized by staining with a primary antibody for 1 hr in PBS followed by a secondary antibody for 30 min in PBS. A concentration of 20 $\mu\text{g/mL}$ was used for all secondary antibodies. BSA was labeled with a sheep anti-BSA antibody (20 $\mu\text{g/mL}$) and an Alexa Fluor 488 anti-sheep secondary antibody. Myoglobin was stained with a goat anti-myoglobin (equine myocardium) antibody (1:50 dilution) and then an Alexa Fluor 488 anti-goat secondary antibody. A mouse anti-calmodulin antibody (200 $\mu\text{g/mL}$) and an Alexa Fluor 488 anti-mouse secondary antibody were used to label calmodulin. Samples were rinsed after each incubation step with PBS for 10 sec. As controls, SAV, BSA, and α -oxoamide-myoglobin were incubated with an excess of biotin, maleimide, and O-methoxyamine hydrochloride, respectively, for 1 h prior to addition to the patterned substrates. As a control for nickel Ni^{2+} -NTA-PEG, NTA-PEG samples were incubated with calmodulin in the absence of Ni^{2+} .

Side-by-Side Patterns—Substrates were incubated with a solution containing both Alexa Fluor 568 SAV (5 $\mu\text{g/mL}$) and BSA (10 $\mu\text{g/mL}$) in PBS for 1 h. BSA was stained with sheep anti-BSA antibody and Alexa Fluor 488 anti-sheep secondary antibody as described above.

Aminooxy on Biotin Staining—The substrate was incubated with a solution containing Alexa Fluor 568 SAV (5 $\mu\text{g/mL}$) in PBS for 1 h. In PBS pH 6.5, α -oxoamide myoglobin (10 $\mu\text{g/mL}$) was incubated with the aminooxy patterns for 1 h. The myoglobin was stained with goat anti-myoglobin (equine myocardium) antibody (10 $\mu\text{g/mL}$) followed by Alexa Fluor 633 anti-goat secondary antibody (1 $\mu\text{g/mL}$) as described above.

Maleimide on Biotin Staining—Same as above for the side-by-side patterns.

Aminooxy and Maleimide on Biotin—The substrate was incubated with a solution containing α -oxoamide myoglobin (10 $\mu\text{g/mL}$), Alexa Fluor 555 BSA (10 $\mu\text{g/mL}$), and Alexa Fluor 350 SAV (10 $\mu\text{g/mL}$) in SuperBlock Blocking Buffer (pH 6.2) for 1 h. The chips were then washed with SuperBlock Blocking Buffer and the myoglobin was stained with goat anti-myoglobin (equine myocardium) antibody (10 $\mu\text{g/mL}$) and Alexa Fluor 488 anti-goat secondary antibody (5 $\mu\text{g/mL}$) as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Electron-beam crosslinking of end-functionalized 8-arm PEG polymers for protein patterning. a. The 8-arm PEGs were spin-coated onto Si wafers and cross-linked to the native oxide using e-beam lithography to generate specific patterns. Each PEG was end-functionalized with one of four protein-reactive handles (biotin, maleimide, aminooxy, or NTA). b. Alexa Fluor 568 SAV attached to biotin-PEG patterns through the biotin-SAV interaction. The bottom middle two features are 1 μm and 750 nm features repeated many times to make up the overall rectangle and square, respectively c. BSA was immobilized to maleimide-PEG patterns through the sulfhydryl group of the free cysteine in the BSA. d. Myoglobin that was modified with pyridoxal-5-phosphate to contain a N-terminal α -oxoamide group was attached to aminooxy-PEG patterns. The reactive carbonyl at the N-terminus of the modified myoglobin bound to the aminooxy functionality through an oxime bond. e. Histidine-tagged calmodulin was immobilized to Ni^{2+} -NTA-PEG patterns through the nickel-histidine affinity interaction. The original Ni^{2+} -NTA patterns, generated by exposure of the NTA-PEG patterns to nickel(II) chloride hexahydrate, were observed by SEM (top grey scale image). All proteins, with the exception of SAV, were labeled with the appropriate antibodies prior to visualization by fluorescence microscopy. Protein structure representations were obtained from the PDB (1SWA, 2BX8, 1WLA, 1CFD). Scale bar = 20 μm .

Figure 2.

Dual protein patterning. a. Biotin-PEG and maleimide-PEG are cross-linked next to each other using e-beam lithography. BSA, which conjugates to the maleimide-PEG, and SAV, which binds to the biotin-PEG, are attached simultaneously from the same solution. BSA is visualized using the appropriate antibodies. b-c. Fluorescence overlay demonstrating attachment of the two proteins to microscale patterns (Scale bar = 10 μm). d. Nanoscale patterns of the two polymers are visible in the height image taken with an atomic force microscope in tapping mode. Concentric squares of maleimide-PEG and biotin-PEG with line widths of 130 nm are visible. M and B are written below the patterns to denote the respective PEGs. Inset displays fluorescence overlay of the nanoscale patterns with attached BSA and SAV.

Figure 3.

Multilayer, multicomponent biostructures. a. Schematic showing that biotin-PEG is first cross-linked to the native oxide of a Si wafer. Aminoxy-PEG patterns are then cross-linked on top of the original biotin-PEG patterns. b-c. Four $1 \times 1 \text{ micron}^2$ aminoxy-PEG patterns are cross-linked on top of base $5 \times 5 \text{ micron}^2$ biotin-PEG patterns. b. Fluorescence after incubating a 3×3 sample with green fluorescent SAV, which binds to the biotin-PEG, and red fluorescent antibody stained N-terminal α -oxoamide myoglobin, which binds to the aminoxy patterns. Inset in c is one of the features in b. c-d. Atomic force microscope images are three-dimensional representations of the height images acquired in tapping mode. d. Aminoxy “UCLA” pattern with 250 nm wide lines on a $5 \times 5 \text{ micron}^2$ biotin-PEG pattern base.

Figure 4.

Additional multilayer, multicomponent biostructures. a. Schematic showing that biotin-PEG is first cross-linked to the native oxide of a Si wafer. Maleimide-PEG patterns are then cross-linked on top of the original biotin-PEG patterns. b-d. Atomic force microscope images were acquired in tapping mode. Top images are the three-dimensional representations of the height images, while the bottom graphs are the height profiles. All images contain a base 5×5 micron² biotin-PEG pattern. b. Four 1 micron wide, c, sixteen 500 nm wide, and d, sixteen 250 nm wide maleimide-PEG patterns are cross-linked on top of the biotin-PEG. Inset in b displays fluorescence after incubating the sample with red fluorescent SAv, which binds to the biotin-PEG, and green fluorescent antibody stained BSA, which binds to the maleimide-PEG patterns.

Figure 5.

Tri-component biostructures. a. Schematic showing that 5 micron wide biotin-PEG is first cross-linked to the native oxide of a Si wafer, and two 1 micron wide maleimide and two 1 micron wide aminoxy PEG patterns are then cross-linked on top of the original biotin-PEG patterns. b. Atomic force microscope image of aminoxy- and maleimide-biotin pattern provided as a three-dimensional representation of the height images. Inset in b is the overlay image of a blue fluorescent SAV on the biotin-PEG, a green fluorescent antibody stained α -oxoamide myoglobin, and a red fluorescent BSA.