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Protein Nanopatterns by Oxime Bond Formation

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Abstract

Patterning proteins at the nanoscale is important for applications in biology and medicine. As feature sizes are reduced, it is critical that immobilization strategies provide site-specific attachment of the biomolecules. In this study, oxime chemistry was exploited to conjugate proteins onto nanometer-sized features. Poly(Boc-aminooxy tetra(ethylene glycol) methacrylate) was synthesized by free radical polymerization. The polymer was patterned onto silicon wafers using an electron beam writer. Trifluoroacetic acid removal of the Boc groups provided the desired aminooxy functionality. In this manner, patterns of concentric squares and contiguous bowtie shapes were fabricated with 150–170 nm wide features. Ubiquitin modified at the N-terminus with an α -ketoamide group and N^e-levulinyl lysine modified bovine serum albumin were subsequently conjugated to the polymer nanopatterns. Protein immobilization was confirmed by fluorescence microscopy. Control studies on protected surfaces and using proteins presaturated with O-methoxyamine indicated that attachment occurred via oxime bond formation.

Keywords

Nanobiotechnology; protein patterns; site-specific conjugation; oxime chemistry

Introduction

Control over protein immobilization and patterning at the nanoscale has numerous applications in medicine ranging from diagnostic arrays to cell and tissue engineering. There are many methods to pattern proteins in this size region.¹ Among the techniques that have been employed to achieve this are dip-pen nanolithography,² nanografting,³ nanocontact printing,^{4, 5} and nanoimprint lithography.⁶ In the majority of reported cases immobilization is achieved either through physical adsorption or chemical attachment of lysine residues or carboxylic acids that are plentiful in proteins. For both of these conjugation strategies, however, there is a lack of control over protein orientation on the surface. Random attachment, as well as harsh conditions from additional reagents and non-aqueous solvents, typically leads to reductions in bioactivities of the attached proteins.⁷ As feature sizes are reduced such losses become increasingly significant. Thus, there is a considerable drive to

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Supporting Information Available: Control study images and complete reference 41. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

develop surface chemistries and patterning techniques that allow for site-specific protein immobilization under mild conditions.

Herein, we communicate a method to produce site-specific immobilized protein nanopatterns using oxime bond formation. Attachment at the nanoscale through specific sites has been previously achieved utilizing His-Tags^{8, 9} or reaction with free cysteines.¹⁰ These methods are effective; yet the proteins must be recombinantly engineered or contain native free cysteines not participating in disulfide bonds, which are rare. In an effort to explore alternative, general strategies, we examined an oxime bond immobilization strategy.¹¹ The N-terminal amine of most proteins can be selectively converted to an α -oxoamide.^{12–14} The oxo group thus formed will react with alkoxyamines chemoselectively over other functional groups found on the protein, including amines,¹⁵ to form an oxime bond in the absence of other reagents and under mild, aqueous conditions.¹⁶ Thus, this linkage provides a convenient method for anchoring proteins site-specifically through the N-terminus. Oxime bonds are also stable under physiological conditions. Finally, it has been demonstrated that immobilization via oxime bonds leads to superior bioactivities of the surface-bound proteins compared to nonspecific attachment.¹⁷

We and others have previously reported biomolecule immobilization onto surfaces via oxime bonds. DNA has been conjugated onto self assembled monolayers (SAMs)^{18–21} or glass substrates.^{19–21} Oxime chemistry has also been demonstrated for adsorption of carbohydrates onto polymer films.^{22–24} Peptide patterns for examination of peptide microarrays, as well as for controlled cell adhesion have also been fabricated.^{25–27} Proteins have been examined by several groups. Meijer showed that proteins could be immobilized onto alkoxyamine surfaces via aniline-catalyzed oxime ligation of the N-termini.¹⁷ We demonstrated that micron sized protein patterns could be produced after employing photoacid generator (PAG)-based photolithography to selectively hydrolyze acetals to aldehydes or to deprotect Boc groups to reveal aminooxy functionality.^{14, 28, 29} In addition, we demonstrated that micron sized patterns of proteins could be produced using electron beam (e-beam) lithography and aminooxy end-functionalized poly(ethylene glycol)s (PEGs).³⁰ All of the above approaches resulted in either full surface coverage or micropatterns. Yet, there is considerable interest in producing nanopatterns of biomolecules.

In this report, generation of aminooxy-functionalized polymer nanopatterns by e-beam lithography and conjugation of an N-terminal α -ketoamide-modified protein and N^ε-levulinyl lysine modified protein are described. We had previously shown that nanopatterns of end-functionalized poly(ethylene glycol)s (PEGs) could produce nanopatterns of proteins.^{30, 31} In an effort to increase the density of aminooxy groups, we explored conjugation of a polymer with aminooxy groups at every repeat unit. In order to achieve this, we exploited a brush polymer with aminooxy-functionalized PEG graft chains. Synthesis of the polymer and characterization of the final protein patterns is disclosed.

Experimental Section

Materials

Boc-aminooxy tetra(ethylene glycol) methacrylate was synthesized using a previously reported method.¹⁴ All other reagents were purchased from Acros and utilized as received, unless otherwise indicated.

Instrumentation

¹H NMR was acquired on a Bruker ARX 500 MHz spectrometer. Infrared spectroscopy was recorded by a Perkin-Elmer SpectrumOne Fourier Transfer IR (FTIR). Gel permeation chromatography (GPC) was conducted on a Shimadzu HPLC system equipped with a

refractive index detector RID-10A and two Polymer Laboratories PLgel 5 μ m mixed D columns (with guard column). Near-monodisperse poly(methyl methacrylate) (PMMA) standards (Polymer Laboratories) were employed for calibration. DMF with 0.1 M LiBr at 40 °C was used as a solvent (flow rate: 0.6 mL/min). UV-Vis spectroscopy analysis was performed with a BioMate 5 Spectrophotometer (Thermo Spectronic Instruments).

Synthesis of Poly(Boc-aminooxy tetra(ethylene glycol) methacrylate) (1)

In a Schlenk tube, 2,2'-azobisisobutyronitrile (AIBN, 0.5 mg, 0.0029 mmol), Boc-aminooxy tetra(ethylene glycol) methacrylate (104 μ L, 0.29 mmol) and toluene (105 μ L) were subjected to three freeze-pump-thaw cycles prior to placing in an oil bath at 75 °C. The reaction was allowed to proceed under argon for 1 h and the resulting polymer was isolated by precipitation into cold ether. ^1H NMR (500 MHz, MeOH- d_4) $^{\text{TM}}$ ppm: 4.37 (bs, 2H, NH-O-CH $_2$ -CO $_2$), 4.28 (bm, 2H, NH-O-CH $_2$ -CO $_2$ -CH $_2$), 4.07 (ms, 2H, CO $_2$ -CH $_2$ -CH $_2$ O), 3.70 - 3.61 (bm, 12H, CH $_2$ -O-CH $_2$), 1.93 - 1.81 (bm, 2H, (CH $_2$ polymer backbone), 1.42 (s, 9H, C-(CH $_3$) $_3$), 1.02-0.87 (bm, 3H, CH $_3$ polymer backbone). FTIR: 3297 (w), 2880 (m), 1725 (s), 1454 (m), 1392 (w), 1368 (m), 1350 (w), 1270 (m), 1246 (m), 1199 (m), 1160 (s), 1107 (s), 1039 (m), 980 (m), 853 (m), 775 (m), 749 (w) cm^{-1} . Number-average molecular weight (Mn) = 102,600 Da and polydispersity index (PDI) = 2.59

Protein modification

Bovine serum albumin (BSA) was modified to contain N $^{\epsilon}$ -levulinyl lysine residues using a previously described procedure.³²

Ubiquitin (Ub, Sigma) was subjected to a transamination reaction utilizing pyridoxal-5-phosphate (PLP) by a previously reported procedure¹³ which installs an N-terminal α -ketoamide. Ub (50 μ M) was dissolved in phosphate buffer (25 mM, pH 6.5) and then PLP (10 mM) was added. The mixture was stirred for 24 hr at 37 °C. The PLP was removed by centrifugal filtration (5000 MW cutoff). Protein modification was confirmed by incubating modified Ub with an excess of Alexa Fluor 488 hydroxylamine for 1 hr in the dark. After removing excess hydroxylamine by extensive centrifugal filtration (5000 MW cutoff), the modified Ub displayed an absorbance peak at 495 nm (λ_{max}). Unmodified Ub did not absorb in this region, indicating the PLP modification had installed a ketone.

Pattern formation

Silicon wafers (SQI International) were cleaned by immersion in piranha solution (3:1 sulfuric acid : 30% hydrogen peroxide(aq), CAUTION: piranha solution reacts violently with organic compounds, handle with care) for 10 min. Freshly cleaned wafers were then spin-coated with a 0.25 % (w/v) solution of **1** in chloroform at 3000 rpm for 30 s. E-beam lithography was then performed with a JEOL 5910 scanning electron beam microscope. Pattern files were created in DesignCAD 2000 and used by a JC Nability lithography system (Nanometer Pattern Generation System, Ver. 9.0). Following patterning, wafers were rinsed with methanol and then MilliQ water for 10 s each.

AFM Microscopy

Height images taken with a Dimension 3100 (Digital Instruments) atomic force microscope in tapping mode (silicon cantilever, spring constant = $\sim$ 40 N/m, tip radius = < 10 nm, scan rate = 1.5 Hz).

Fluorescence visualization

All samples were visualized with fluorescence microscopy using a Zeiss Axiovert 200 fluorescent microscope equipped with an AxioCam MRm monochrome camera, and pictures

were acquired and processed using AxioVision LE 4.1. Fluorescence intensity was measured using NIH Image and signal-to-noise ratios were determined as (signal – background)/standard deviation of background.

Results and Discussion

It is well established that PEG cross-links upon exposure to electron beam irradiation to form hydrogels.^{33, 34} Focused electron beams have been used to crosslink PEG to either a plasma-generated silicon oxide,³⁵ a PEG silane,³⁶ or a piranha-cleaned native oxide of silicon.³⁰ This process is thought to occur via a radical mechanism,³⁵ and has been employed previously to immobilize proteins via a variety of functional groups.^{30, 37–39} Because the polymer is covalently attached to the surface during the process,³⁵ the features are stable during storage and upon immersion with solvents and buffer. Therefore, in this study, e-beam lithography was used to produce aminooxy-functionalized nanopatterns. A polymer was designed with side chains of aminooxys tethered to the backbone by oligo(ethylene oxide) units. The former provided the desired reactive groups. The latter was added to allow the polymer to be cross-linked to the surface and to reduce any nonspecific binding to the resulting patterns.

Poly(Boc-aminooxy tetra(ethylene glycol) methacrylate) was synthesized by conventional free radical polymerization (Figure 1). Polymerization for 1 h of a 50% (v/v) solution of Boc-aminooxy tetra(ethylene glycol) methacrylate in toluene was initiated by 1 mol% AIBN at 75 °C. The resulting polymer was analyzed by gel permeation chromatography, and found to have a number-average molecular weight of 103 kDa and a polydispersity index of 2.59.

A 0.25% (w/v) solution of the polymer in chloroform was spin-coated onto Si wafers at 3000 rpm. The polymer was site-specifically cross-linked to the native oxide of the substrate using an e-beam writer (Figure 2a; 0.1 nC/cm line dose, 30 kV accelerating voltage, ~4.5 pA beam current). One potential disadvantage of using e-beam lithography is that it is a serial technique, and thus can be slow for producing large numbers of features. The patterning times for each concentric square and contiguous bowtie demonstrated in this report were ~2 s and 1 s, respectively. Therefore, patterning large numbers of these features via e-beam lithography would be feasible for pilot studies.

After rinsing with methanol and water to remove any unreacted polymer, patterns of the Boc-protected aminooxy-terminated polymer remained. To reveal the aminooxy functionality, the patterned substrates were immersed in trifluoroacetic acid (TFA) for 30 min to remove the Boc group (Figure 2b) and then rinsed with water and dried with a stream of air. An advantage of e-beam lithography is that a wide variety of designs may be fabricated. Indeed, two different intricate patterns were targeted: concentric squares and a contiguous bowtie shape. Atomic force microscopy (AFM) images taken in tapping mode confirmed the nanoscale features (Figure 3, top) with line widths of 150–170 nm.

Protein immobilization was demonstrated on patterned substrates. The two approaches most commonly employed to install reactive carbonyl functionality were used. A levulinyl-lysine-modified bovine serum albumin (BSA, Figure 2c)⁴⁰ and a N-terminal α -ketoamide-modified ubiquitin (Ub, Figure 2d) were conjugated to the hydroxylamines. Substrates were incubated with either the modified BSA (1 mg/mL in phosphate buffered saline, PBS) or the modified Ub (50 μ M in phosphate buffer, pH 6.5) for 1 h. Bound BSA was visualized using a sheep anti-BSA antibody (20 μ g/mL in PBS, 1 hr, Immunology Consultants Laboratory) and a green fluorescent Alexa Fluor 488 anti-sheep secondary antibody (20 μ g/mL in PBS, 30 min, Invitrogen), while immobilized Ub was visualized with a mouse anti-Ub antibody

(50 $\mu\text{g/mL}$ in PBS, 1hr, Calbiochem) and an Alexa Fluor 488 anti-mouse secondary antibody (20 $\mu\text{g/mL}$ in PBS, 30 min).

The polymer lines were generated in close proximity so that binding could be visualized by standard fluorescence microscopy. The features and spaces between the nanopatterns were below the resolution of fluorescence microscope, such that the details of the nanopatterns were not resolved in the images. Therefore, adjusting the line interspacing of the nanopatterns effectively controls the observed strength of the fluorescence signal by controlling the density of aminooxy functional groups, and thus immobilized proteins, within the visible features. Fluorescence microscopy images did confirm binding of modified BSA (Figure 3a, bottom) and modified Ub (Figure 3b, bottom) to the aminooxy patterns. Some background fluorescence was observed on substrates, however the patterns were significantly brighter with signal-to-noise ratios of 22:1 and 11:1, respectively. However, if complete removal of the background was required for a particular application, passivation may be achieved by first immobilizing a PEG silane on the surface.³¹⁻³⁶ Control studies were conducted to confirm immobilization via oxime bond formation. When the substrates were not immersed in acid to deprotect the polymer, no binding of the proteins to the polymer pads was observed (Figure S1). Furthermore, fluorescent patterns were not observed when the proteins were first saturated with O-methoxyamine hydrochloride to consume the reactive carbonyls (Figure S1).

Taken together these results indicate that protein binding occurred via oxime bond formation of the protein with aminooxy groups on the nanopatterned substrate. Since many proteins, both native and recombinant, can be modified to contain either an aldehyde or ketone,^{12-14, 41} this approach should be generally applicable to pattern a wide variety of proteins at the nanoscale. Moreover, such modifications can be performed in a site-specific manner, which is key to be able to maintain full protein bioactivity.¹⁷ Thus, this methodology may be employed to site-specifically anchor proteins that retain their bioactivity at the nanoscale; this is exceedingly important as feature sizes decrease and fewer proteins are immobilized on each pattern. Such technology could have significant implications in the design of biosensors, where target proteins might not naturally contain the appropriate functionality for site-specific immobilization. Finally, since e-beam lithography easily produces patterns of different designs and sizes, this approach should be applicable for arbitrary-designed pattern formation for a wide range of applications.

Conclusions

We demonstrated that nanoscale patterns of proteins could be fabricated using electron beam lithography. Specifically, poly(Boc-aminooxy tetra(ethylene glycol) methacrylate) was synthesized by standard free radical polymerization. The polymer was crosslinked to the piranha cleaned, native oxide of Si using e-beam lithography and then deprotected with acid to reveal aminooxy groups for immobilization of proteins with reactive carbonyls. Two different approaches for protein immobilization were demonstrated. First, BSA modified at lysine residues with levulinate groups to anchor via ketone functionality was demonstrated. Second, N-terminally transaminated ubiquitin was immobilized via the α -ketoamide group. Polymer feature sizes were determined by AFM to be between 150 and 170 nm. Immunochemical staining revealed that the proteins had conjugated to the nanosized patterns.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Synthesis of poly(Boc-aminooxy tetra(ethylene glycol) methacrylate). The polymer was prepared by free-radical polymerization of Boc-aminooxy tetra(ethylene glycol) methacrylate in toluene using AIBN at 75 °C.

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Figure 3.

Electron beam (e-beam) crosslinked nanopatterns of poly(Boc-aminoxy tetra(ethylene glycol) methacrylate). A layer of the polymer is spin-coated onto Si substrates and then site-specifically crosslinked to the native oxide using an e-beam writer. Top images, a–b. Nanoscale patterns of the polymer are visible in atomic force microscope images. Lower image, a. Fluorescence image of two 5 micron sized squares each containing concentric square nanopatterns after immobilizing a levulinyl-lysine-modified bovine serum albumin (BSA) through an oxime bond. The bound protein was visualized using a sheep anti-BSA antibody and a green fluorescent anti-sheep secondary antibody. Lower image, b. Fluorescence image of two 5 micron wide bowtie patterns containing contiguous nanolines as seen in the top image after immobilizing an N-terminal α -ketoamide-modified ubiquitin (Ub), also through an oxime bond. Surface bound Ub was visualized using a mouse anti-Ub antibody and a green fluorescent anti-mouse secondary antibody.