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PAPER

Dual Click reactions to micropattern proteins

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In the study described in this report orthogonal Click reactions were utilized to immobilize two different proteins on surfaces side-by-side and in multilayer constructs. Alkyne- and azide-functionalized poly(ethylene glycol) hydrogel features were fabricated. Copper-catalyzed Huisgen 1,3 dipolar cycloaddition and oxime chemistry were employed to conjugate an azide-functionalized ubiquitin and oxoamide-modified myoglobin, respectively. Multicomponent patterning was verified by fluorescence imaging.

Introduction

Reactions such as the Cu(I) catalyzed Huisgen 1,3-dipolar cycloaddition[1,2] and oxime bond formation[3] have been increasingly used by researchers interested in melding biological and synthetic molecules. So-called Click reactions are known to proceed with high selectivity in benign solvents, tolerate a wide range of functional groups, and produce products in high-yields.[4,5] A number of studies have utilized Huisgen 1,3-dipolar cycloaddition or oxime chemistry to create conjugates of biomolecules such as sugars, DNA, proteins and even cells.[6–13] Herein, we report application of multiple Click reactions to produce protein microarrays on surfaces. The proteins were immobilized either side-by-side or in multilayer constructs using both oxime chemistry and copper-catalyzed cycloaddition. To our knowledge this is the first time dual Click reactions have been used for this purpose.

Oxime chemistry, the reaction between *O*-hydroxylamines and ketones or aldehydes, has been shown to be effective for protein conjugation to surfaces.[14–26] For example, we prepared nanometre and micron-sized patterns by immobilizing α-oxoamide- or levulinate-modified proteins onto patterns of aminooxy groups[20,25] Francis conjugated antibodies using oxime chemistry. Meijer employed aniline-catalyzed oxime bond formation to ligate proteins to surfaces.[26] Importantly, their results showed that this method of site selective conjugation results in proteins with significantly higher activities than non-specific coupling using amidation chemistry. Retention of bioactivity of proteins on surfaces is critical for application of these materials, and typically activities are greatly reduced for proteins where the immobilization chemistry is not site specific.

Huisgen 1,3-dipolar cycloaddition reactions have also been utilized to anchor biomolecules to surfaces.[27] Azide functionalized peptides have been immobilized on alkynyl self assembled monolayers *via* Cu catalyzed triazole formation.[28] Microwave assisted azide/alkyne Click reactions have been reported to produce carbohydrate/oligonucleotide chips.[29] Green fluorescent protein labeled with an azide at the C-terminus was immobilized on alkyne functionalized agarose beads.[30] In another report, 200 nm patterns of poly(propargyl methacrylate) were produced by e-beam lithography.[31] An azide-functionalized coumarin dye was used as a model system to Click to the patterns. The authors then demonstrated immobilization of an azido-biotin *via* triazole formation. These patterns were visualized with streptavidin-coated quantum dots.

Although proteins do not naturally contain the required groups for oxime or copper catalyzed Click reactions, the required moieties are readily added by a variety of methods. Introduction of azides and alkynes into proteins has been accomplished by recombinant[32–36] and chemical techniques.[37–39] For example, recently, the diazo transfer reagent, imidazole-1-sulfonyl azide hydrochloride[40] was shown to successfully place azides at lysine side chains and the N-termini of horseradish peroxidase and the red fluorescent protein DsRed.[39] We employed this strategy to produce azide-functionalized ubiquitin for reaction with alkyne-functionalized surfaces. Aminooxy groups or reactive carbonyls have also been incorporated into proteins.[41] In this work, we exploited a transamination reaction in particular that places α-oxoamide groups at the N-termini of proteins to modify myoglobin for reaction with *O*-hydroxylamine features.

Micron-sized features containing aminooxy or alkyne groups were fabricated utilizing electron-beam (e-beam) lithography. This system is ideal for biomolecule patterning because PEG is known to be protein resistant, which is important in producing patterns that minimize non-specific binding.[42–44] Furthermore, different patterns and shapes, precise alignments and three dimensional arrangements can be achieved using e-beam

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lithography.[25] We and others have demonstrated that e-beam lithography patterning of reactive polymers is effective for fabricating patterns of proteins.[25,45–48] In this report we demonstrate that iterative e-beam lithography patterning, coupled with orthogonal Click reactions is effective to place two different proteins on surfaces (Scheme 1).

Experimental

Materials

8-Arm amine terminated graft PEG was purchased from NOF Corporation (Tokyo, Japan). 8-Arm aminooxy terminated PEG was synthesized as previously described.[25] α-Ketoamide myoglobin was synthesized as previously described.[41] Alexa Flour488 azide and all Alexa Flour secondary antibodies were purchased from Invitrogen (Carlsbad, CA). Goat anti-myoglobin antibody was purchased from Genetex (San Antonio, TX). Mouse anti-Ubiquitin antibody was purchased from Abcam (Cambridge, MA). All other reagents were purchased from Sigma Aldrich (St. Louis, MO) or Fisher Biotech (Pittsburgh, PA) and utilized as received unless otherwise indicated.

Instrumentation

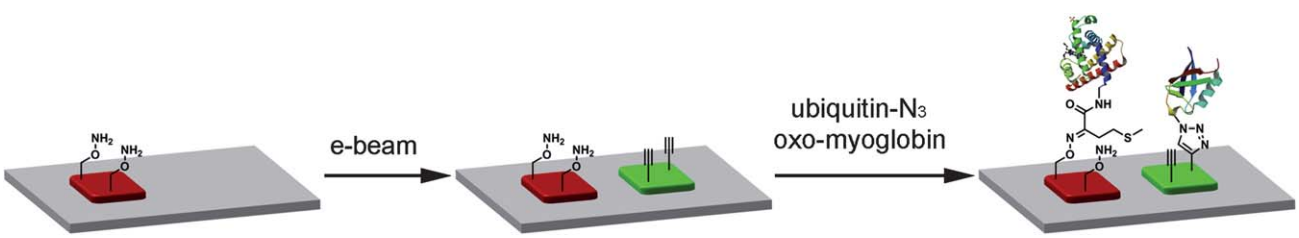
^{1}H NMR, ^{13}C NMR, and DEPT NMR were acquired on either a Bruker ARX 500 MHz or 400 MHz spectrometer. Infrared spectroscopy was recorded using a Perkin-Elmer SpectrumOne Fourier Transfer IR (FTIR). UV-Visible (Vis) spectroscopy analysis was performed with a BioMate 5 Spectrophotometer (Thermo Spectronic Instruments). MALDI-TOF Mass spectra were acquired using an Applied Biosystems Voyager-DE-STR MALDI-TOF instrument. AFM Images were obtained on a Veeco Dimension 5000 Scanning Probe Microscope in tapping mode equipped with a silicon AFM probe (BS-Tap300, BudgetSensors). For fluorescence visualization, a Zeiss Axiovert 200 fluorescent microscope equipped with an AxioCam MRm monochrome camera was used. Pictures were acquired and processed using AxioVision LE 4.1. Fluorescence intensity was measured using NIH Image and signal to noise ratio was determined as (signal – background)/standard deviation of background. Ellipsometry was conducted on a Gaertner LSE stokes ellipsometer. The spin coated polymer layer thicknesses were measured with a HeNe laser at a single wavelenght of 632.8nm and a 70 degree angle. The original Gaertner GEMP software was used to fit the layer in the following manner: First the silicon oxide on the piranah cleaned silicon wafer was measured and fitted using the refractive index of Palik (n1 =.54264, k1 = 0.00) and silicon as substrate (substrate thickness: 1000μm, n1 = 3.859, k1 = 0.016). The measurement was then repeated on the same wafer after spin coating the polymer. The polymer layer was fitted using values for the previously obtained silicon oxide thickness and an additional chauchy layer model (n1 = 1.45, k1 = 0.01). A minimum of 15 measurements were performed at 3 different locations and the values were then averaged.

2,5-Dioxopyrrolidin-1-yl pent-4-ynoate

To a flame dried round bottom flask containing 100 mL of freshly distilled methylene chloride (DCM) was added *N*-hydroxysuccinimide (NHS, 1.0 g, 8.7 mmol, 1 eq), pentynoic acid (0.94 g, 9.6 mmol, 1.1 eq), triethylamine (1.4 g, 14 mmol, 1.6 eq), and 4-dimethylaminopyridine (DMAP) (0.11 g, 0.87 mmol, 0.1 eq). The resulting solution was stirred for 30 min before addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 1.8 g, 9.6 mmol, 1.1 eq). The reaction was stirred for 4 h at 22 °C, and the solvent was removed *in vacuo* to give a crude product which was subjected to column chromatography (100% DCM, R_f 0.4). This product was further purified by recrystallization from 1 : 4 ethyl acetate : hexanes and sublimation at 0.2 Torr to obtain 2, 5-dioxopyrrolidin-1-yl pent-4-ynoate as a white solid (0.69g, 40% yield). ^{1}H NMR (500 MHz; $CDCl_3$) δ (ppm): 2.90–2.84 (bm, 5H, COC**H$_2$** + NHS), 2.62 (dt, 2H, J = 7.5, 2.6 Hz, $CH_2C\mathbf{H_2}C$), 2.05 (t, 1H, J = 2.6 Hz, CC**H**); ^{13}C (400 MHz; $CDCl_3$) δ (ppm): 169.1, 167.2, 81.0, 70.2, 30.4, 25.7, 14.2; DEPT-135 ^{13}C (400 MHz; $CDCl_3$) δ (ppm): 69.9(-), 30.18(+), 25.5(+), 14.0(+) ppm. FTIR ν_{max}/cm^{-1}: 3304, 2948, 2258, 1815, 1787, 1734, 1430, 1415, 1371, 1203, 1085, 1067, 993, 906.

Synthesis of alkyne PEG

8-Arm amine-terminated PEG was added (20 mg mL^{-1}) to a solution of 2,5-dioxopyrrolidin-1-yl pent-4-ynoate (5 mg mL^{-1}) in CH_2Cl_2 and allowed to react for 1 h at 23 °C. The solvent was removed *in vacuo*. The crude product was then re-dissolved in Milli-Q water and purified by centrifugal filtration (3,000 MW cutoff) followed by lyophilization to isolate the pure product. ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 6.48 (s, CON**H** end group 1H), 3.69–3.58 (m, PEG peaks), 3.39 (m, $CH_2C\mathbf{H_2}NH$ end group 2H), 2.52 (dt, J = 7.4 Hz, 2.6 Hz, $CH_2C\mathbf{H_2}C$ end group 2H), 2.38 (t, J = 7.4 $C\mathbf{H_2}CH_2C$ end group 2H), 2.00 (t, J = 2.6 Hz, CC**H** end group 1H). Conversion = 93% by comparison of the integrations of the alkyne end group with the $C\mathbf{H_2}NH_2$ peak of the unmodified PEG.



Scheme 1 Protein patterning by multiple Click reactions. Aminooxy and alkyne PEGs are patterned next to each other by e-beam lithography. α-Ketoamide-myoglobin followed by azide-modified ubiquitin are then conjugated to the surface.

Synthesis of imidazole-1-sulfonyl azide hydrochloride

Synthesis of imidazole-1-sulfonyl chloride proceeded in 62% yield following a literature procudure.[40] The product was isolated as colorless needles. ^{1}H NMR (500 MHz, D_2O) δ (ppm): 9.34 (1H), 7.99 (1H), 7.57 (1H). ^{13}C NMR (500 MHz, D_2O) δ (ppm): 137.6, 123.1, 120.0 FTIR ν_{max}/cm^{-1}: 3104, 3059, 2499, 2425, 2169,1913, 1582, 1508, 1425, 1298, 1229, 1189, 1159, 1137, 1103, 1068, 982, 967.

Preparation of azide-ubiquitin

Ubiquitin was modified with two azides by reaction with aqueous diazo transfer agent, imidazole-1-sulfonyl azide hydrochloride, following a literature procedure (CAUTION!).[39] To a solution of ubiquitin in Milli-Q water (200 μL, 2.5 mg mL^{-1}) was added imidazole-1-sulfonyl azide hydrochloride (85 μL, 2.0 mg mL^{-1}), K_2CO_3 (100 μL, 2.0 mg mL^{-1}), and Cu(II)SO_4•H_2O (25 μL, 1.0 mg mL^{-1}). The solution was allowed to react for 24 h at 4 °C. Subsequent purification by centrifugal filtration (3,000 MWCO) gave the azide-modified ubiquitin. The solution was diluted to 250 μL. Mass spectrum (MALDI-TOF) calcd for two transfers (based on 8635.51 observed for unreacted ubiquitin): 8687.51 *m*/*z*; observed: 8695.02 *m*/*z*.

Pattern formation

A 1% or 2% (w/v) solution was prepared in methanol for the aminooxy- and alkyne-functionalized polymers, respectively. Silicon wafer chips were prepared by cleaning in Piranha (3 : 1 H_2SO_4:H_2O_2, CAUTION!) for 5 to 10 min, followed by a 10 s rinse in Millipore H_2O, and blow drying with high pressure air. 15 μl of the polymer solution was then spincoated at 4000 rpm for 20 s. The resulting aminooxy-PEG films were approximately 48 nm thick, and the alkyne-PEG films 40 nm thick by ellipsometry measurements. Patterning was accomplished by electron beam exposure-induced crosslinking in a modified JEOL 5910 scanning electron microscope. Patterns were designed using a JC Nabity e-beam lithographic system (Nanometre Pattern Generation System, version 9.0). An acceleration voltage of 30 kV was used, with a beam current of approximately 5 pA. Aminooxy patterns were written with a 40 μC cm^{-2} area dose and alkyne patterns with a 20 μC cm^{-2} area dose. The writing time was 6 s for the aminooxy features 1 s for the alkyne features. Following pattern writing, samples were removed from the vacuum and immediately rinsed with methanol for 10 s followed by 10 s with Millipore H_2O.

Multiple patterns. For pattern alignment, gold alignment reference marks were first fabricated on the silicon wafer by conventional photolithography as described previously.[25] Following the procedure above, aminooxy polymer patterns were generated. After pattern development, the alkyne end-functionalized PEG was spincoated on top of the aminooxy features and patterned as described above in the appropriate position, according to the reference marks. AFM images were taken, and the samples were used directly for conjugation of proteins.

Protein conjugation and pattern visualization

Alkyne patterns. Patterns were incubated with azido-ubiquitin (35 μg mL^{-1}) and 5 mM CuBr dissolved in 1 : 39 EtOH:Milli-Q water for 3 h at 23 °C. After rinsing by gently squirting with PBS, the bound protein was stained with a mouse anti-ubiquitin antibody (50 μg mL^{-1} in PBS) for 1h and AlexaFluor® 488 anti-mouse secondary antibody (20 μg mL^{-1} in PBS) for 30 min. Samples were rinsed with PBS after each incubation step. Patterns were visualized with fluorescence microscopy. Signal to noise 37 : 1.

Controls. As the first control, propargyl amine was first Clicked to azido-ubiquitin and then the protein was incubated with the alkyne surface using identical conjugation conditions as above. As a second control, unmodified ubiquitin was subjected to Click conditions with the alkyne patterns. In both cases, no patterns were visualized.

Aminooxy patterns. The substrate was incubated with a solution of α-ketoamide myoglobin (10 μg mL^{-1} in PBS, 25 mM, pH 6.5) for 1 h. After rinsing by gently squirting with PBS, the bound protein was then stained with a goat anti-myoglobin antibody (10 μg mL^{-1} in PBS) for 1h and AlexaFluor® 568 anti-goat secondary antibody (20 μg mL^{-1} in PBS) for 30 min. Samples were rinsed with PBS after each incubation step. Patterns were visualized using fluorescence microscopy. Signal-to-noise 33 : 1.

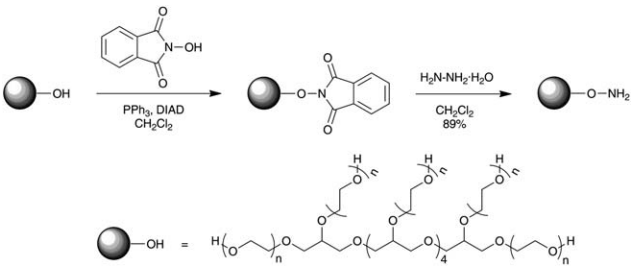
Control. The protein was pre-incubated with *O*-methoxyamine hydrochloride to consume the reactive carbonyl and then subjected to identical staining procedures. Fluorescent patterns were not observed.

Alkyne and aminooxy patterns. Patterns were first stained using α-oxoamide modified myoglobin as described above, followed by staining with azido-ubiquitin using the procedure described above.

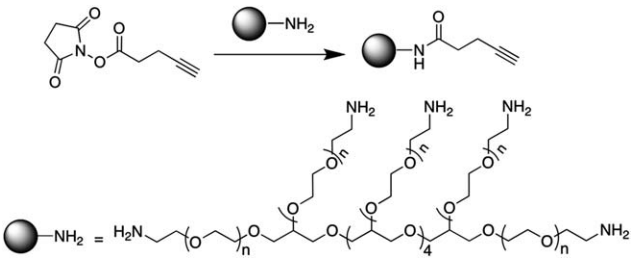
Results and discussion

Poly(ethylene glycol) PEGs have been shown to cross-link to the surfaces of either the native oxide of silicon[49] or a PEG film when exposed to focused electron beams.[45,48] This procedure results in the formation of surface-bound hydrogels in the desired patterns. The crosslinking process is thought to occur *via* a radical mechanism.[49] End-functionalized polymers form features that bear that functionality. In the work described herein, this process was employed to produce aminooxy-functionalized and alkyne-functionalized micropatterns. PEGs with chain ends bearing aminooxy groups or alkynes were designed. The end groups would result in features with the desired Click partners, and eight arm stars were employed rather than linear polymers in order to achieve a higher density of surface functionality. PEG was used so that the polymer could be cross-linked to the surface and so that nonspecific binding would be minimized.

The aminooxy polymer was synthesized by a Mitunobu reaction of 8-arm PEG with hydroxyphthalimide and subsequent cleavage using hydrazine (Scheme 2). The 8-arm end functionalized alkyne PEG was prepared by first synthesizing 2,5-dioxopyrrolidin-1-yl pent-4-ynoate (NHS-alkyne). This was achieved by EDC coupling of NHS to pentynoic acid to afford



Scheme 2 Synthesis of aminooxy-8-arm PEG.



Scheme 3 Synthesis of 8-arm alkyne PEG.

the activated ester alkyne in 40% yield. Subsequent reaction with amine-terminated 8-arm PEG (Scheme 3) resulted in 93% conversion of the end groups as determined by ^{1}H NMR.

The polymers were then subjected to e-beam patterning to produce aminooxy-functionalized PEG triangle shapes and alkyne-functionalized PEG squares. A 1% or 2% (w/v) solution in methanol of the aminooxy- or alkyne-functionalized polymer, respectively, was spincoated onto a silicon wafer with a native oxide. Pattern formation was optimized for each polymer individually and found to occur at doses of 20 μC cm^{-2} for alkyne-functionalized PEG and 40 μC cm^{-2} for aminooxy-functionalized PEG. At lower doses the polymer was insufficiently crosslinked for hydrogel formation, whereas at significantly higher doses irradiation-induced side-reactions could lead to chain scission and degradation of the patterned hydrogels. The uncrosslinked polymer was rinsed away by washing with methanol, followed by water.

Proteins were then modified for Click immobilization. In this case, the myoglobin was subjected to pyridoxal 5′-phosphate to install an N-terminal α-ketoamide.[41] This PLP transamination was introduced by Francis and coworkers.[50] This same transformation can also be undertaken using the Dixon and Fields synthesis.[20,41,51] Ubiquitin was transformed using a recently reported diazo transfer reaction.[39] This method employs imidazole-1-sulfonyl azide hydrochloride to introduce azides at the amine side chains of lysine residues and at the N-terminus of proteins. To this end, imidazole-1-sulfonyl azide hydrochloride was synthesized in 62% yield and incubated with ubiquitin. Ubiquitin was modified with an average of two azides according to MALDI-TOF analysis.

Subsequent reaction of the oxo-myoglobin with aminooxy patterns in slightly acidic pH, and azido-ubiquitin (azido-Ub) with the alkyne patterns in the presence of Cu(I) were undertaken (Scheme 1) The proteins were visualized by fluorescence after staining with primary and secondary antibodies (signal-to-noise ratios of 33 : 1 for myoglobin and 37 : 1 for ubiquitin). In both cases, fluorescence was clearly visualized on the patterns (Fig. 1). Myoglobin was stained red and ubiquitin green, and red triangles and green squares were observed by fluorescence microscopy (Fig. 1).

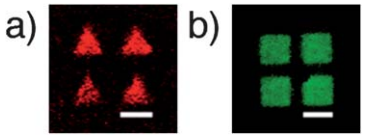


Fig. 1 Features prepared by electron beam were modified with proteins. (a) Myoglobin with a N-terminal α-oxoamide attached to aminooxy-PEG triangle patterns. (b) Azido-ubiquitin immobilized to square alkyne patterns. Proteins were visualized by primary and secondary antibody staining. Scale bar = 5 μm.

Patterns were subjected to controls in which the proteins were first incubated with a small molecule that would saturate the reactive group on the protein. In the case of the α-ketoamide-myoglobin, the protein was reacted with *O*-methoxyamine hydrochloride, and in the case of azido-Ub the protein was Clicked with propargyl amine. Another control was carried out in which unmodified ubiquitin was incubated with patterns of 8-arm alkyne-functionalized PEGs under identical Click conditions. In all cases, patterns were not observed, suggesting that the proteins reacted with the surfaces *via* Click chemistries as designed.

We next exploited e-beam lithography to pattern both types of polymers side-by-side (Scheme 1). Two different types of patterns were produced. First 1 μm × 1 μm features (with 1 μm spacing) were designed in which alternating squares of alkyne and aminooxy PEGs were precisely aligned and used to immobilize ubiquitin and myoglobin, respectively. Green and red alternating squares were observed by fluorescence (Fig. 2a) indicating that the proteins were patterned as expected. In the second pattern, an alkyne representation was drawn from the alkyne PEG and the letter "A" was fabricated from the aminooxy PEG. AFM showed that the desired shapes had been

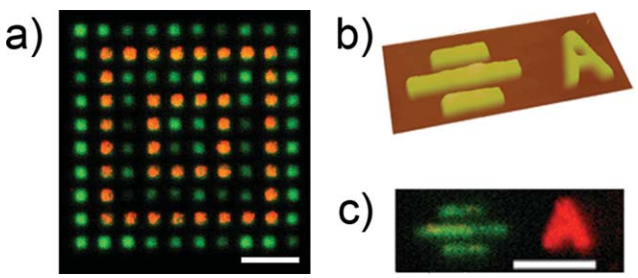


Fig. 2 Dual patterns of aminooxy-PEG and alkyne-PEG cross-linked next to each other by e-beam lithography. Myoglobin was conjugated to the aminooxy patterns and Ubiquitin was conjugated to the alkyne patterns. Proteins (myoglobin, red and ubiquitin, green) were visualized by primary and secondary antibody staining. (a) Micron patterns of 1 × 1 μm dots (1 μm spaces) of myoglobin on aminooxy PEG and ubiquitin on alkyne PEG patterns arranged into alternating squares. (b) Alkyne and "A" shaped polymer patterns were observed in the AFM height image taken in tapping mode. (c) Patterns are visualized by fluorescence imaging. Scale bars 5 μm.

prepared (Fig. 2b). Staining of these features revealed green fluorescent ubiquitin and red fluorescent myoglobin on the respective features as expected (Fig. 2c). Quantification of adsorbed protein based on signal-to-noise ratios showed low levels of cross-reactivity compared to the single protein experiments (3 : 1 for myoglobin staining on alkyne features and 8 : 1 for ubiquitin staining on aminooxy features).

Since e-beam lithography has high alignment capabilities, we next demonstrated that mutilayer patterns could be prepared. These were achieved by precisely aligning the chip in order to pattern four 1 × 1 μm or sixteen 500 × 500 nm features of alkyne-PEG on top of 5 × 5 μm aminooxy-PEG boxes (Fig. 3a). After protein immobilization and visualization (Fig. 3c and d), the fluorescence overlay clearly showed the co-localization of the fluorescence in the desired features (Fig. 3b), thus demonstrating the ability to prepare multilayer constructs.

Taken together the data demonstrate that dual Click reactions coupled with e-beam lithography is effective for producing multicomponent protein arrays. The results also show that side-by-side and multilayer constructs can be prepared. Several of the positive features of e-beam lithography were exploited, namely the ability to pattern different features and shapes, as well as high alignment capability to produce three dimensional constructs. While several shapes and configurations were studied here, a myriad of designs are possible utilizing this technique. This should be useful for applications in which topography and chemistry of the system must be precisely spatially controlled. In addition, since with e-beam lithography smaller features are easily accessed, nanoscale patterns should be possible.

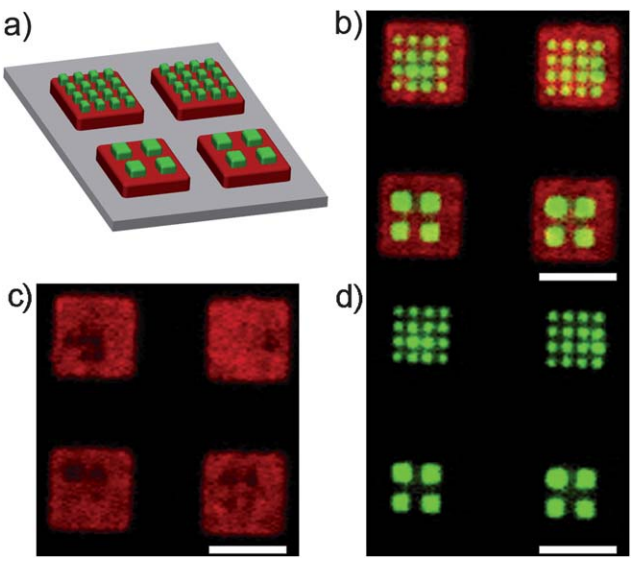


Fig. 3 Dual multilayer Click protein patterns. Aminooxy PEG was first cross-linked to the native oxide of silicon. Alkyne PEG was then patterned on top of the aminooxy patterns. (a) Schematic of multilayer patterning where four 1 × 1 μm or sixteen 500 × 500 nm alkyne PEG was patterned on top of 5 × 5 μm aminooxy PEG and subsequently ubiquitin and myoglobin were immobilized, respectively. (b) Overlay of myoglobin (green) and ubiquitin (red) fluorescence images. (c) Ubiquitin reacts selectively with alkyne patterns and (d) myoglobin reacts selectively with aminooxy patterns. The patterns were visualized with primary and secondary antibodies. Scale bar = 5 μm.

Click chemistry is an effective means to conjugate proteins to surfaces and multiple, orthogonal Click reactions provide an efficient route to surfaces where more than one type of biomolecule is desired. We employed myoglobin and ubiquitin as model systems. Yet the diazo transfer agent utilized herein, as well as the transamination reaction employed, provide facile means to modify proteins with Click partners; thus the number of possible combinations of proteins that could be envisioned is large. In addition, although two Click reactions were utilized, this approach should be amenable to other Click reactions such as thiol-ene, potentially increasing the number of different proteins that could be simultaneously immobilized on a surface.

Conclusions

Herein, we described use of e-beam lithography to create micropatterns of a protein reactive scaffold of 8-arm aminooxy PEG and 8-arm alkyne PEG. A facile azide modification of ubiquitin utilizing an aqueous diazo transfer agent allowed immobilization of the azo-protein to alkyne PEG patterns *via* a Huisgen 1,3-dipolar cycloaddition. Myoglobin that had been subjected to a PLP transamination reaction was immobilized on aminooxy-PEG features. Iterative conjugation of the proteins to surfaces either side-by-side or in multilayer constructs produced multicomponent features. The proteins were subsequently visualized by fluorescent antibody staining.

Acknowledgements

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