Carbohydrate and Protein Immobilization onto Solid Surfaces by Sequential Diels−Alder and Azide−Alkyne Cycloadditions

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We demonstrate the applicability of sequential Diels−Alder and azide−alkyne [3 + 2] cycloaddition reactions (click chemistry) for the immobilization of carbohydrates and proteins onto a solid surface. An α,ω-poly(ethylene glycol) (PEG) linker carrying alkyne and cyclodiene terminal groups was synthesized and immobilized onto an *N*-(ϵ-maleimidocaproyl) (EMC)-functionalized glass slide via an aqueous Diels−Alder reaction. In the process, an alkyne-terminated PEGylated surface was provided for the conjugation of azide-containing biomolecules via click chemistry, which proceeded to completion at low temperature and in aqueous solvent. As anticipated, alkyne, azide, cyclodiene, and EMC are independently stable and do not react with common organic reagents nor functional groups in biomolecules. Given an appropriate PEG linker, sequential Diels−Alder and azide−alkyne [3 + 2] cycloaddition reactions provide an effective strategy for the immobilization of a wide range of functionally complex substances onto solid surfaces.

INTRODUCTION

Robust immobilization techniques that preserve the activity of biomolecules have many potential applications. For example, microarrays, microbeads, and biosensor chips play increasingly important roles in characterizing the function and interaction of biomolecules, purification schemes, and point-of-care diagnostics (*1*). In addition, appropriate surface functionalization remains a critical variable for the optimal performance of a wide range of implantable medical devices (*2*). The design of solid-phase-based assays and bioactive implant surfaces often requires the immobilization of proteins and carbohydrates in their native state. Current immobilization techniques have relied on adsorption, direct covalent immobilization to chemically activated surfaces, or on the biosynthetic expression of a polypeptide 'tag' that mediates immobilization on an appropriately derivatized surface (*3*). Although appropriate for many applications, these schemes can, at times, be limited by the reduction in biomolecular activity due to protein denaturation, unintended reactions at or near the 'active' domain, or random orientation at the solid−liquid interface that reduces access to molecular recognition sites.

Diels−Alder cycloaddition has been recognized as an exceedingly useful bioconjugation procedure (*4*). The reaction takes place between an electronically matched pair of a double bond (dienophile) and a conjugated diene to form a six-membered unsaturated ring. Recently, this scheme has been applied to the immobilization of biomolecules onto a solid surface (*5*). Crucial to the selection of Diels−Alder cycloaddition as a preferential strategy for surface immobilization of biomolecules is (i) the recognition that water has an extraordinary rate-accelerating effect on the reaction process; (ii) the reaction occurs efficiently at room temperature; and (iii) cyclodiene and *N*-(ϵ-maleimidocaproyl) (EMC) are independently stable and easy to introduce.

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The Cu(I)-catalyzed 1,3-dipolar cycloaddition of azide and alkyne to form a triazole, termed 'click' chemistry, has been recently established as an important tool for chemically and biologically modifying biomolecules (*6*). The reactants, alkyne and azide, are convenient to introduce, independently stable, and do not react with common organic reagents or functional groups in biomolecules. Triazole formation is irreversible and usually quantitative. In addition, this reaction benefits from an extremely mild and regioselective copper(I) catalyst system that is surprisingly indifferent to solvent and pH. Thus, the potential for this reaction to modify a wide range of functionally complex substances is significant. The unique properties of this reaction appears transferable to surface-bound reactants and will likely provide access to a growing variety of novel functionalized surfaces (*7*). Indeed, azide-containing compounds have been successfully captured to alkynes via the Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction (*8*). Therefore, azide/alkyne-based click chemistry can be expected to provide a robust strategy for uniform, high-density biomolecular surface immobilization using a conjugation scheme that is both covalent and displays a high degree of selectivity.

Poly(ethylene glycol) (PEG) is widely used as an inert and biocompatible linker in surface engineering and in the modification of proteins and peptides for drug delivery (*9*). Monofunctional PEG coupled to proteins is known to prolong circulation half-life and reduce immunogenicity (*10*). Moreover, PEG-modified liposomes have been used for cell targeting applications (*11*). While carboxyl or amine-terminated PEGs are widely available, they remain expensive and require further chemical modification to convert their corresponding groups. Further, these functional PEGs are available mainly as higher molecular weight compounds (>3400 Da), which under many conditions may limit grafting density onto solid surfaces due to steric repulsive effects. In this report, we describe the synthesis of a bifunctional short PEG linker with alkyne and cyclodiene groups at opposite chain termini to facilitate surface bioconjugation based upon sequential Diels−Alder and azide−alkyne cycloadditions. We demonstrate that this linker is suitable for the immobilization of biomolecules onto solid surfaces in a chemi-

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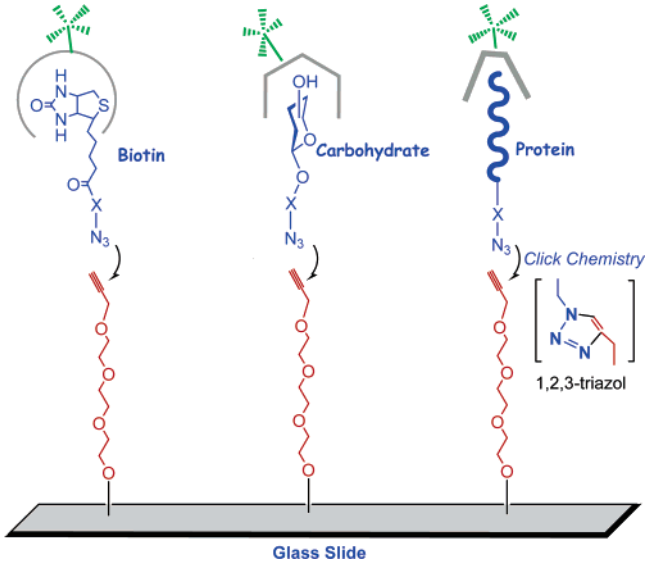


Figure 1. Schematic illustration of test substrates produced by sequential Diels−Alder and azide−alkyne cycloadditions of azide-derivatized biomolecules.

cally and biologically orthogonal fashion (Figure 1). Specifically, the alkyne and cyclodiene moieties are capable of conjugating under aqueous conditions and in a selective fashion to azide and EMC groups, respectively. As a covalent conjugation scheme that is orthogonal to diverse functionalities intrinsic to biological systems, this approach provides an attractive alternative to other currently available immobilization protocols.

EXPERIMENTAL PROCEDURES

General Methods. Thin-layer chromatography (TLC) was performed on Whatman silica gel aluminum backed plates of 250 μm thickness on which spots were visualized by UV light or by charring the plate after dipping in 10% H_2SO_4 in methanol. ^{1}H NMR spectra were recorded at 400 MHz at room temperature using a Varian INOVA 400 spectrometer. Fluorescence images were acquired using a Zeiss LSM 510 epifluorescence microscope under 10× magnification and illuminated using an argon ion laser at 485 nm. Images were acquired using eight co-added scans in line mode on air-dried glass slides stained with FITC-labeled proteins. Post-image processing was not performed.

Materials. All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. Amino PEG_4 azide **7** was prepared, as reported in the literature (*12*). 2-Azide-ethyl-*O*-lactoside **9** was synthesized, as previously reported (*18*). *N*-Hydroxsuccinimidobiotin and the lectin (*Arachis hypogaea*)-FITC were purchased from Sigma. The recombinant thrombomodulin construct (*r*TM-N_3) with an azide group and S-tag at the C- and N-termini, respectively, was prepared as previously reported (*21*). The FITC-conjugated S-protein and FITC-conjugated streptavidin, which specifically bind to S-Tag and biotin, respectively, were obtained from Novagen. EMC functionalized glass slides were purchased from XENOPORE Corp.

Synthesis of Bifunctional PEG Spacers. *O-Propargyl-tetra(ethylene glycol) (2).* To a solution of tetra(ethylene glycol) (**1**, 2 g, 5.30 mmol) in 20 mL of THF was added NaH (60% w/w in mineral oil, 272 mg, 11.34 mmol, 1.1 equiv.) at 0 °C with frequent venting. After stirring for 15 min, propargyl bromide (80% in toluene, 1.68 mL, 11.34 mmol, 1.1 equiv) was added slowly, and the mixture was stirred at 0 °C for 2 h and then 23 °C for an additional 2 h. The reaction mixture was passed through a silica gel column eluted with ethyl acetate to give the purified product **2** (1.552 g, 65%) as a clear oil. ^{1}H NMR ($CDCl_3$) δ: 4.16 (d, 2 H, J = 2.4 Hz, $OCH_2C{\equiv}CH$), 3.65−3.61 (m, 14 H, $-(OCH_2CH_2)O-$), 3.56 (t, 2 H, J = 4.4 Hz, OCH_2CH_2OH), 2.75 (br. s, 1 H, −OH), 2.40 (t, 1 H, J = 2.4 Hz, $OCH_2C{\equiv}CH$).

O-Propargyl-tetra(ethylene glycol) tosylate (3). *p*-Toluenesulfonyl chloride (1 g, 5.4 mmol) and DMAP (25 mg, 0.2 mmol) were added to a solution of **2** (1.044 g, 4.5 mmol) in 1:1 pyridine-dichloromethane (10 mL). After 5 h, the solution was poured into ice−water (20 mL), and the aqueous phase was extracted with dichloromethane (3 × 20 mL). The combined organic phases was washed with NH_4Cl (20 mL) and brine (20 mL), dried over anhydrous Na_2SO_4, and concentrated. The yellow oil was chromatographed on silica gel (EtOAc:hexane 3:1) to yield **3** (1.404 g, 81%) as a clear oil. ^{1}H NMR ($CDCl_3$) δ: 7.75 (d, 2 H, J = 8.4 Hz, Ph), 7.26 ((d, 2 H, J = 8.4 Hz, Ph), 4.19 (d, 2 H, J = 2.4 Hz, $OCH_2C{\equiv}CH$), 4.16 (t, 2 H, J = 7.8 Hz, OCH_2CH_2OTs), 3.70−3.55 (m, 14 H, $(OCH_2CH_2)O$), 2.54 (s, 3 H, Ph-CH_3), 2.52 (t, 1 H, J = 2.4 Hz, $OCH_2C{\equiv}CH$).

2-O-Propargyl-8-O-cyclopentadieneethyl tri(ethylene glycol) (4). Sodium cyclopentadienylide (0.75 mL, 2 M solution in THF) was added dropwise to a solution of **3** (390 mg, 1.01 mmol) in THF (10 mL) at −78 °C. The reaction mixture was stirred at −78 °C for 30 min and at room temperature for an additional 4 h. The solution was filtered through a pad of Celite, and the salts were washed thoroughly with THF. Evaporation of the filtrate and subsequent silica gel chromatography (EtOAc:hexane 1:1) afforded **4** (172 mg, 61%) as a clear oil. ^{1}H NMR ($CDCl_3$) δ: 6.44−6.05 (m, 3 H, cyclodiene), 4.18 (d, 2 H, J = 2.4 Hz, $OCH_2C{\equiv}CH$), 3.70−3.55 (m, 12 H, $(OCH_2CH_2)O$), 2.91 (dd, 2 H, J = 1.4, 9.8 Hz, OCH_2CH_2-cyclodiene), 2.66 (m, 2 H,-OCH_2CH_2-cyclodiene), 2.42 (t, 1 H, J = 2.4 Hz, $OCH_2C{\equiv}CH$).

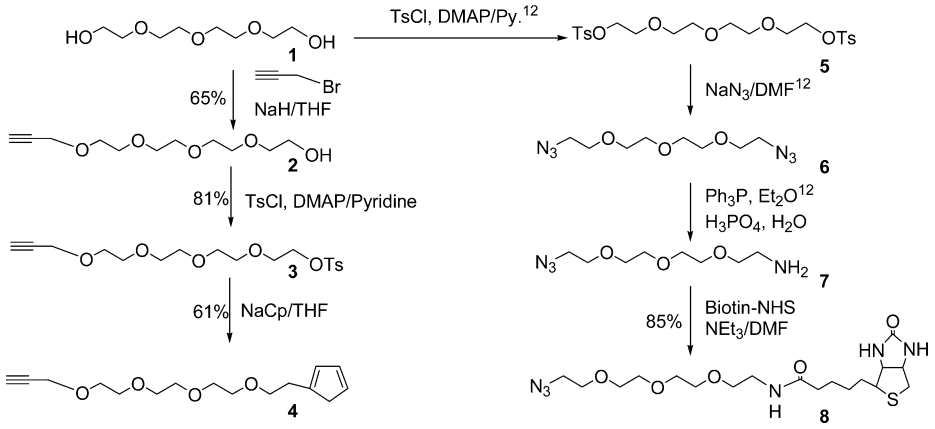
Azidoethyl-di(ethylene glycol) ethylamine (7) was synthesized from tetra(ethylene glycol) **1** as reported in the literature (*12*).

Azidoethyl-di(ethylene glycol) ethylamino biotin (8). Triethylamine (0.1 mL, 0.71 mmol) was added to a solution of **7** (200 mg, 0.917 mmol) in DMF (5 mL). After the solution was stirred for 30 min, a solution of *N*-hydroxsuccinimidobiotin (375 mg, 1.10 mmol) was added. The reaction mixture was stirred for 12 h at room temperature and then concentrated in a vacuum to give a residue, which was purified by silica gel column chromatography using acetone:hexane (4:1) as eluent to afford **8** (345 mg, 85%). ^{1}H NMR (CD_3OD) δ: 4.51 (m, 1 H, C*H*-1-Biotin), 4.32 (m, 1 H, C*H*-4-Biotin), 3.70−3.63 (m, 8 H, $O(CH_2CH_2O)$-PEG), 3.39 (m 4 H, CH_2NH and CH_2N_3-PEG), 3.20 (m 1 H, C*H*-3-Biotin), 2.93 (dd, 1 H, J = 4.8, 12.8 Hz, CH-2a-Biotin), 2.71 (m 1 H, CH-2b-Biotin), 2.23 (t, 1 H, J = 7.6 Hz, CH_2CO-Biotin), 1.76−1.43 (m, 6 H, $(CH_2)_3$-Biotin).

Aqueous Diels−Alder Alkyne PEGylation of EMC Solid Surface. An EMC functionalized glass slide (0.5 cm^2) was placed in a glass vial and incubated overnight on a shaker at room temperature in a solution of alkyne PEG cyclodiene **4** (0.5 mg) in 500 μL of H_2O-*tert*-BuOH (1:1). The PEG solution was removed, and the slide was washed three times with 1 mL of H_2O-*tert*-BuOH (1:1). The unreacted EMC groups were blocked by incubation in a 500 μL solution of cysteine (10 mg/L in PBS pH 7.4) for 1 h at room temperature, followed by three washes with dd water.

Click Biotinylation of an Alkyne PEGylated Glass Slide. A total of 75 μL of biotin PEG azide **8** (0.2 mg) in PBS was added to a glass vial containing an alkyne PEGylated glass slide (0.5 cm^2). This was followed by the sequential addition of 5 μL of *tert*-BuOH, 10 μL of 2 mM ligand (tris(triazolyl)amine) solution in PBS, 10 μL of 1 mM $CuSO_4$ solution, and 2 mM TCEP in PBS. The vial was shaken at 4 °C for 12 h. The reaction solution was removed and the slide washed three times with 1 mL of dd water.

Scheme 1. Synthesis of Bifunctional PEG Linkers



FITC-conjugated streptavidin (Novagen) was diluted 1:50 in PBST. The biotin-PEG immobilized slide was incubated in 500 μL of the diluted streptavidin solution (0.02 mg/mL) for 2 h at 4 °C and then washed three times with 1 mL of dd water.

Click Immobilization of Azide Lactose onto a Glass Slide. A total of 75 μL of lactose azide **9** (*18*) (0.2 mg) in PBS was added to a glass vial containing an alkyne PEGylated glass slide (0.5 cm^2). This was followed by the sequential addition of 5 μL of *tert*-BuOH and then 10 μL of 2 mM tris(triazolyl)amine solution in PBS, and 10 μL of 1 mM $CuSO_4$ and 2 mM TCEP solution in PBS. The vial was shaken at 4 °C for 14 h. The reaction solution was removed and the slide washed three times with 1 mL of dd water.

The lactose-PEG immobilized slide was incubated in 500 μL of a FITC-conjugated galactose binding lectin (*Arachis hypogaea*, Sigma) solution (0.5 mg/mL) in PBST for 5 h at 4 °C and then washed three times with 1 mL of dd water.

Click Immobilization of *r*TM-N_3 onto a Glass Slide. A total of 75 μL of *r*TM-N_3 (0.1 mg) in PBS was added to a glass vial containing an alkyne PEGylated glass slide (0.5 cm^2). This was followed by the sequential addition of 5 μL of *tert*-BuOH, 10 μL of 2 mM tris(triazolyl)amine solution in PBS, and 10 μL of 1 mM $CuSO_4$ solution in PBS. A small piece of Cu wire was placed in contact with the above mixture. The vial was incubated at 4 °C for 72 h without shaking. The reaction solution and the wire, which has become black due to oxidation, were removed and the slide washed three times with 1 mL of dd water.

The *r*TM-N_3 immobilized slide was incubated overnight at 4 °C in 500 μL of the diluted FITC-conjugated S-protein solution (1: 500 diluted commercial sample from Novagen as manufacture protocol in PBST) and then washed three times with 1 mL of dd water.

RESULTS AND DISCUSSION

1. Synthesis of Bifunctional PEG Spacers. The alkyne PEG_4 cyclodiene (**4**) was synthesized from tetra(ethylene glycol) (**1**, PEG_4) as shown in Scheme 1. First, PEG_4 **1** was converted to *mono*-alkyne-PEG_4 **2** by reacting with 1.1 equivalent of propargyl bromide in the presence of sodium hydride. Treatment of **2** with *p*-toluenesulfonyl chloride followed by reaction with sodium cyclopentadienylide afforded alkyne-PEG_4-cyclodiene **4** in good yield. For further functionalization of an alkyne-derivatized surface, biotin PEG_4 azide **8** was synthesized by biotinylation of amino PEG_4 azide **7** (*12*) with commercial available *N*-hydroxsuccinimidobiotin in good yield.

2. Alkyne PEG Surface Functionalization via an Aqueous Diels−Alder Reaction. An EMC-functionalized glass slide (XENOPORE Corp.) was incubated with alkyne-PEG_4-cyclodiene **4** in H_2O-*tert*-BuOH (1:1) at room temperature for 12 h, followed by three washes in H_2O-*tert*-BuOH (1:1) to provide an alkyne-PEG functionalized surface (Figure 2B). The unreacted EMC groups on the slide were blocked with cysteine by incubation in PBS solution at room temperature, followed by three washes with dd water.

A biotin PEG azide was used as a model compound to assess both the extent of Diels−Alder driven surface PEGylation and the capacity of this surface to be further functionalized via click chemistry. Biotin exhibits high affinity binding to strept(avidin) and has been widely used in bioconjugation (*13*) and surface immobilization applications (*14*). As shown in Figure 2, alkyne-PEG functionalized glass slides were treated with biotin-PEG-azide **8** in the presence of $CuSO_4$, tris(carboxethyl)phosphine (TCEP), and tris(triazolyl)amine in PBS (pH 7.0)-*tert*-BuOH (1:1) at 4 °C for 12 h followed by PBS and water washes. In this reaction, Cu(I) is the direct catalyst and is prepared in situ by reduction of inexpensive and air-stable Cu(II) salt $CuSO_4$ with TCEP, and tris(triazolyl)amine is used as ligand to enhance the reaction rate greatly and inhibit oxidative alkyne coupling. Notably, the low temperature condition was investigated for later protein immobilization since many proteins are thermally sensitive. The success of alkyne PEGylation and the capacity of this surface to participate in click immobilization chemistry are evident by confocal fluorescence images that demonstrate FITC-labeled streptavidin specifically binds to a biotinylated surface (C), with no binding to either untreated EMC-glass slides (A) or slides functionalized with alkyne-PEG alone (B) (Figure 2). In addition, binding was not observed upon incubation with either a biotin-saturated FITC-labeled streptavidin to biotinylated surfaces or to EMC slides deactivated with cysteine prior to PEG immobilization (data not show).

3. Immobilization of an Azide-Containing Sugar. Cells universally carry a sugar coating formed by glycoproteins, proteoglycans, and glycolipids, which are involved in highly specific recognition events between cells and proteins, hormones, antibodies, and toxins (*15*). Understanding the mechanism of these processes may lead to the development of new antimicrobial, anticancer, and antiinflammatory therapies. Therefore, simple and readily accessible methods for high-throughput analysis, such as carbohydrate microarrays or glycoarrays, are an area of active investigation (*16*). We investigated the click immobilization of carbohydrates onto alkyne-PEG-functionalized glass slides using lactose-N_3 as a model ligand (*17*).

As depicted in Figure 3, an alkyne-PEG functionalized glass slide was treated with an azide-derivate lactose **9** (*18*) in the presence of $CuSO_4$, TCEP, and tris(triazolyl)amine in PBS (pH 7.0)-*tert*-BuOH (1:1) at 4 °C for 14 h. Binding of a fluorescently labeled lectin confirmed the effectiveness of sugar immobilization. Specifically, uniform fluorescence was noted upon incubation of glycosylated test surfaces with FITC-lectin from *Arachis hypogaea* that binds to terminal β-galactose, but was not

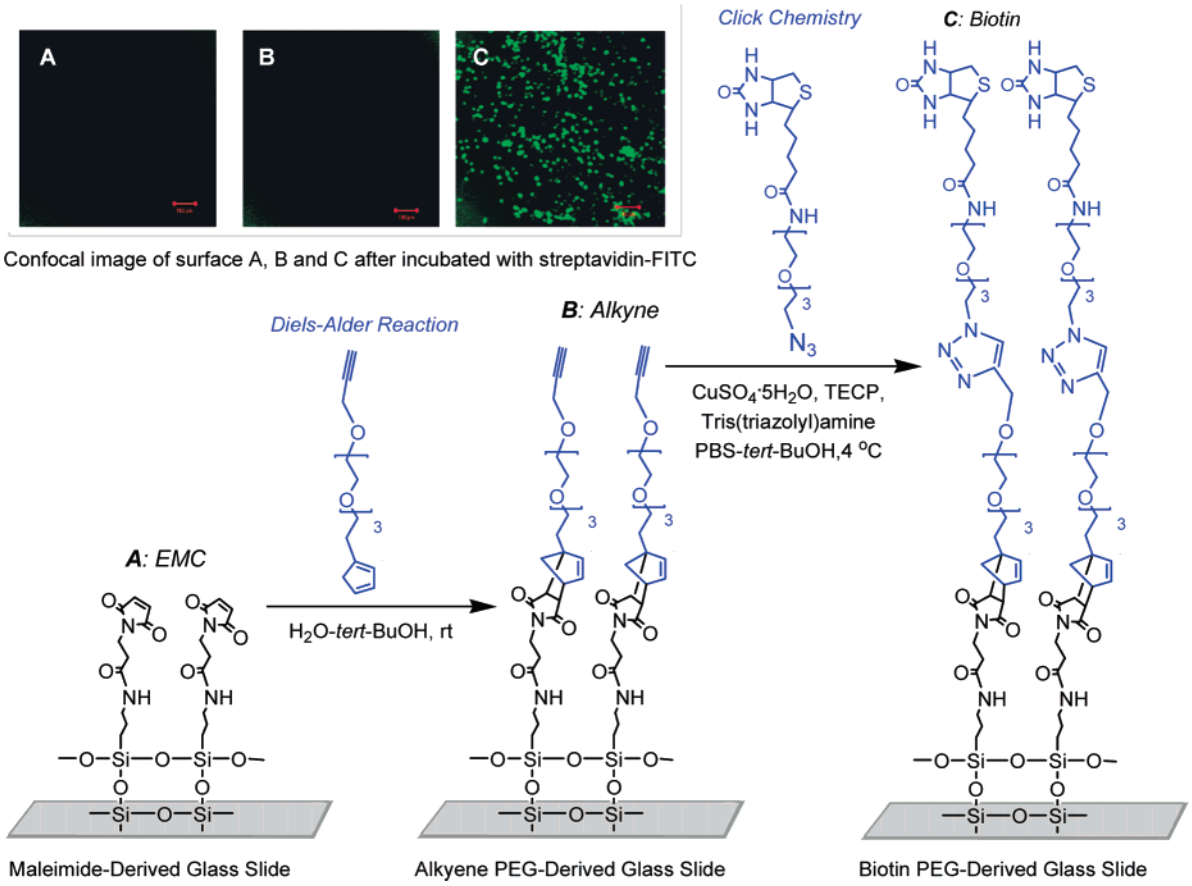


Figure 2. Diels−Alder cycloaddition was used to modify an EMC-derivatized substrate with an alkyne-terminated PEG linker, which was followed by surface biotinylation via click chemistry. Representative confocal image of initial EMC surface incubated with streptavidin-FITC (A); alkyne-derivatized surface incubated with streptavidin-FITC (B); and a biotinylated surface produced by sequential Diels−Alder and azide−alkyne cycloadditions incubated with streptavidin-FITC (C). Bar size: 100 μm.

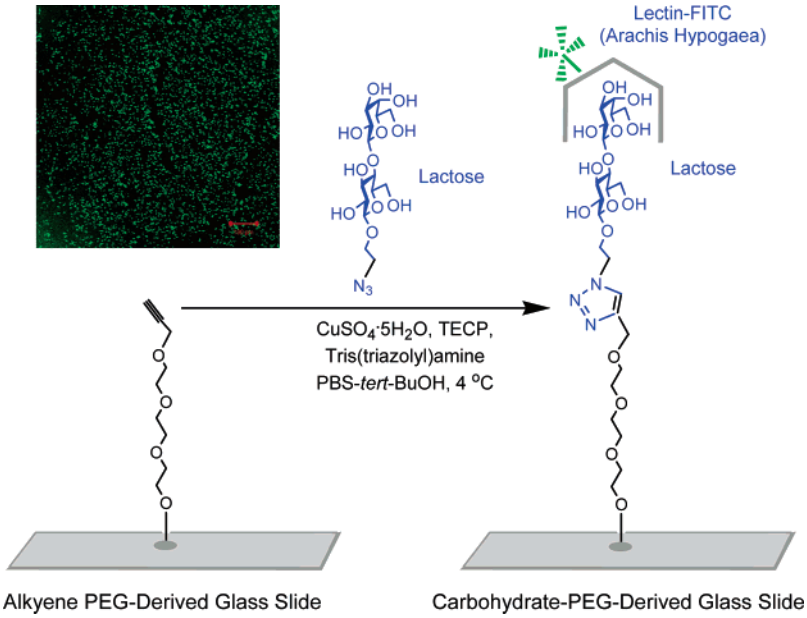


Figure 3. Immobilization of lactose-N_3 onto an alkyne-PEG-functionalized glass substrate via azide−alkyne cycloaddition. Representative confocal image of a lactose-derivatized surface after incubation with FITC-labeled *Arachis hypogaea*. Bar: 100 μm.

observed when untreated EMC or alkyne-derivatized glass substrates were used. Furthermore, binding was not observed when a glucose/mannose specific lectin, concavalian A was used (data not show).

4. Immobilization of a Recombinant Protein with a C-Terminal Azide Group. The immobilization of a protein onto a solid surface in the correct orientation is imperative for optimal interaction with other species in which it may form a functional complex. However, most current surface conjugation schemes offer somewhat limited control over the three-dimensional presentation of bound proteins due to their reliance on functional groups that are often abundantly present within the protein of interest. Falsey et al. has recently reported chemoselective N-terminal attachment of peptides onto glyoxylic

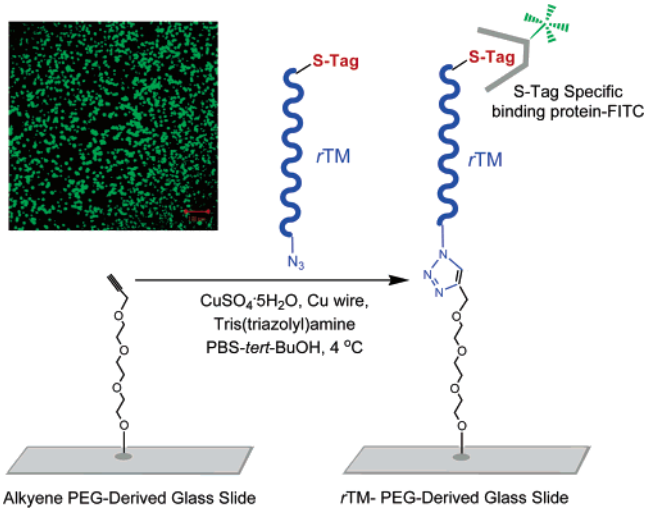


Figure 4. Immobilization of recombinant thrombomodulin (*r*TM-N_3) containing an N-terminal S-tag peptide seuqence onto an alkyne-PEG-functionalized glass surface via azide−alkyne cycloaddition. Representative confocal image of a thrombomodulin-derivatized surface after incubation with an S-tag binding FITC-labeled S-protein. Bar: 100 μm.

acid functionalized slides via oxime-bond or thiazolidine-ring ligation reaction (*19*). Although elegantly designed for site-specific coupling of peptides, the oxime bond is relatively unstable and its formation less selective. Zhu et al. has reported a method for site-specific immobilization of His-tag proteins on slides functionalized with Ni−NTA (*20*). Although an appropriate strategy for protein purification in which elution of the bound protein is required, the binding between His-tag proteins and the Ni−NTA complex is not very strong and is incompatible with many common chemicals such as DTT, SDS, and EDTA. Specifically, decoupling occurs outside pH 4−10 or when the buffer contains high concentrations of common salts. In the current report, the use of a recombinant protein containing a nonnatural azide-containing amino acid at the C-terminus provides an example of site-specific and regiospecific protein immobilization to an alkyne-derivatized surface.

We have recently reported a strategy for site-specific PEGylation of a truncated thrombomodulin (*r*TM-N_3) mutant through an azide group located at the C-terminus. The thrombomodulin (TM) construct was expressed in *E. coli* and contains epidermal growth factor (EGF)-like domains 4−6 and an azido-functionalized alanine analogue as a C-terminal linker (*21*). As shown in Figure 4, alkyne-PEG functionalized glass slides were treated with *r*TM-N_3 in the presence of $CuSO_4$, Cu wire, and tris-(triazolyl)amine in PBS (pH 7.0)-*tert*-BuOH (95:5) at 4 °C for 72 h. Cu wire was used for reduction of Cu(II) to Cu(I) and to avoid the risk of cleavage of the dithiosulfide bonds in the protein (6c and 6d). The recombinant TM also contains an N-terminal peptide sequence, referred to as an S-Tag that was used to qualitatively assess the extent of surface immbolization. As shown in Figure 4, the TM-functionalized surface was incubated with a FITC-labeled S-protein that specifically binds the S-tag (*22*) and examined by confocal microscopy. S-Tag binding was consistent with the production of a surface grafted at a high density with *r*TM-N_3. S-Protein binding was not observed for either untreated EMC-glass slides or for those functionalized with alkyne-PEG alone (data not show). On inspection of Figures 2−4, differences in surface domain size and morphology are noted, which likely reflects varying steric effects attributed to the use of ligands and binding proteins of varying size.

In conclusion, sequential Diels−Alder cycloaddition and azide−alkyne [3 + 2] cycloaddition reactions were successfully applied to the functionalization of EMC-derivatized glass slides with a diverse series of ligands, including biotin, lactose, and a recombinant thrombomodulin protein. This approach was enabled by the synthesis of a bifunctional poly(ethylene glycol) (PEG) linker carrying alkyne and cyclodiene groups at opposite chain termini. Significantly, azide, alkyne, cyclodiene, and EMC groups were stable under bioconjugation conditions and did not appear predisposed to unintended side reactions. In principle, the potential to immobilize a wide range of functionally complex substances onto solid surfaces can be achieved, which will likely extend design schemes directed at the fabrication of microarrays and medical devices whose performance is dictated by precisely defined surface molecular engineering.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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