Surface Functionalization Using Catalyst-Free Azide−Alkyne Cycloaddition

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The utility of catalyst-free azide−alkyne [3 + 2] cycloaddition for the immobilization of a variety of molecules onto a solid surface and microbeads was demonstrated. In this process, the surfaces are derivatized with aza-dibenzocyclooctyne (ADIBO) for the immobilization of azide-tagged substrates via a copper-free click reaction. Alternatively, ADIBO-conjugated molecules are anchored to the azide-derivatized surface. Both immobilization techniques work well in aqueous solutions and show excellent kinetics under ambient conditions. We report an efficient synthesis of aza-dibenzocyclooctyne (ADIBO), thus far the most reactive cyclooctyne in cycloaddition to azides. We also describe convenient methods for the conjugation of ADIBO with a variety of molecules directly or via a PEG linker.

INTRODUCTION

Surface immobilization of biomolecules is a very important step in the manufacturing of biosensors, microbeads, biochips, probe arrays, medical implants, and other devices (*1*−*5*). The key requirements for this process are the preservation of biochemical properties of immobilized substrates and robustness of the linkage. Copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of azides to terminal acetylenes has emerged as one of the most convenient methods for the functionalization of various surfaces (*3, 5*−*10*). The triazol linker formed in the azide "click" reaction has excellent chemical stability due to the aromatic character of the heterocycle. Azide tags can be incorporated into biomolecules using a variety of different strategies, such as postsynthetic modification (*11*−*13*), in vitro enzymatic transfer (*14, 15*), the use of covalent inhibitors (*16*), and metabolic labeling by feeding cells a biosynthetic precursor modified with azido functionality (*17*). While conventional copper(I)-catalyzed click chemistry has become commonplace in surface derivatization, as well as in polymer and materials synthesis (*6*−*10, 18*−*20*), the use of metal catalyst often limits the utility of the method. Copper ions are cytotoxic (*21*), can cause degradation of DNA molecules (*22, 23*), and induce protein denaturation (*24*). In addition, the use of catalysts complicates kinetics of the immobilization process, requires polar solvents, and can alter surface properties (*25*).

Conventional copper-catalyzed azide click coupling methods employ terminal acetylenes, since internal alkynes react with azides only at elevated temperatures. Cyclooctynes, on the other hand, are known to form triazoles without a catalyst under ambient conditions, albeit at a rather slow rate (*26*). The triple bond incorporated into an eight-membered ring is apparently already bent into a geometry resembling the transition state of the cycloaddition reaction, thus reducing its activation barrier (*27*). Recently developed cyclooctyne derivatives are substantially more reactive toward azides and offer a convenient metal-free alternative to the copper-catalyzed click reaction (*28*−*33*). Metal-free click chemistry has been successfully employed for the modification of luminescent quantum dots (*25*) and protein labeling and purification (*34*−*37*), as well as for the introduction of fluorescent tags into live cells (*25, 38, 39*) and organisms (*40, 41*).

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Among reactive cyclooctyne derivatives, dibenzocyclooctynes are synthetically more accessible (*42*). The major limitation of these click reactants is the relatively low rate of azide cycloaddition (ca. 0.05 M^{-1} s^{-1}) (*34*−*37*). Substitution of one of the saturated carbons in the cyclooctyne ring for a nitrogen atom not only improves its reactivity, but also simplifies the synthesis (*28*−*33*). To extend the arsenal of bioorthogonal copper-free click reagents, we have developed an efficient synthesis of aza-dibenzocyclooctyne-containing compounds for azide-coupling reactions. In the present report, we describe a novel approach to efficient surface-functionalization using a catalyst-free azide click reaction. This method allows for the site-specific covalent anchoring of proteins and other substrates to various surfaces. The same metal-free click reaction is employed for the PEGylation of nonfunctionalized areas of the surface. Such treatment allows for a dramatic reduction or complete elimination of nonspecific binding. The copper-free click immobilization strategy discussed in the present report can be applied to the preparation of various types of arrays, as well as to the derivatization of microbeads and nanoparticles.

EXPERIMENTAL PROCEDURES

Materials and Methods. Purification of products by column chromatography was performed using 40−63 μm silica gel. All NMR spectra were recorded on a 400 MHz instrument in $CDCl_3$ and referenced to TMS unless otherwise noted. Images of fluorophore-patterned slides were recorded, and fluorescence intensity was quantified using a Typhoon 9400 variable mode imager (GE Healthcare) at an excitation/emission setting appropriate for fluorescein (488/520 nm) or Lissamine rhodamine B (532/580 nm) fluorophores in PBS solution; images of fluorescent microbeads were obtained using Zeiss Observer AX10 inverted microscope with an X-cite series 120 fluorescent light source and Chroma Technology filters. The relative fluorescent intensity (spot/background) was quantified using *ImageJ* program (NIH).

Tetrahydrofuran was distilled from a sodium/benzophenone ketyl; ether and hexanes were distilled from sodium; *N*,*N*-dimethylformamide (DMF) was dried by passing through an alumina column. Biotin-dPEG3+4-azide and Biotin-PEG4-acid were purchased from Quanta BioDesign; fluorescein SE, Oregon Green SE, Lissamine rhodamine B sulfonyl chloride, and FITC-Avidin were obtained from Invitrogen; (3-glycidyloxypropyl)-

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dimethoxymethylsilane was purchased from TCI America; 0.0067 M phosphate buffered saline (PBS) with pH 7.4 was obtained from Thermo Scientific. Bovine serum albumin (BSA) was obtained from Fisher BioReagents. Other reagents were purchased from Aldrich or VWR and used as received unless otherwise noted. Polished glass slides were obtained from VWR; NanoLink streptavidin magnetic microspheres (1% aqueous suspension) were purchased from SoluLink Biosciences. Aza-dibenzocyclooctyne **6** conjugates with fluorescein (ADIBO-fluor, **10**), Oregon green (ADIBO-OG, **11**), and Lissamine rhodamine B (ADIBO-Rhodamine, **12**) were prepared by treating **6** with equimolar amounts of fluorescein SE, Oregon Green SE, Lissamine rhodamine B sulfonyl chloride, respectively, in DMF in the presence of DIEA (ethyldiisopropylamine). Oregon green azide (**13**) and Lissamine rhodamine B azide (**14**) were prepared by reacting equimolar amounts of Oregon green SE or Lissamine rhodamine B sulfonyl chloride with 3-azidopropyl amine in DMF in the presence of DIEA. Conjugates **10**−**14** were purified by chromatography ($CHCl_3$/MeOH/AcOH 100:5:0.5) and their purity was confirmed by HPLC analysis. 1-Amino-11-azido-3,6,9-trioxaundecane (AminoPEG_4azide) (*43*) and 6-azidohexylamine (*44*) were prepared according to literature procedures.

*5,6-Dihydrodibenzo[b,f]azocine (**2**).* A solution of dibenzosuberenone (25 g, 121 mmol) and hydroxylamine hydrochloride (6.81 mL, 164 mmol) in pyridine (70 mL) was refluxed for 20 h. The reaction mixture was concentrated and poured into 5% aqueous hydrochloric acid (with crushed ice), stirred for 20 min, filtered, and dried in the air to provide 28.1 g of crude dibenzosuberenone oxime, as a white precipitate. Dibenzosuberenone oxime (16 g, 72.3 mmol) was added to 250 mL polyphosphoric acid at 125 °C; the reaction mixture was stirred for 60 min at this temperature, poured onto crushed ice (∼700 mL), stirred for another 30 min, and filtered. The filter cake was washed with water and dried under vacuum to provide crude dibenzo[*b,f*]azocin-6(5H)-one **1** (11.6 g, 52.4 mmol, 73%) as a gray powder.

A suspension of **1** (7.4 g, 33.4 mmol) and lithium aluminum hydride (2.494 mL, 66.9 mmol) in anhydrous ether (200 mL) was refluxed for 15 h. The reaction mixture was quenched by water and filtered, and the filter cake was washed with ether. The filter cake was dispersed in ether (100 mL), stirred for 10 min, and filtered. The combined organic layers were dried over $MgSO_4$, solvent was removed under vacuum, and the product was purified by chromatography (hexanes/ethyl acetate 2:1) to provide 4.04 g (19.49 mmol, 58%) of 5,6-dihydrodibenzo[*b,f*]-azocine (*2*). ^{1}H NMR: δ 7.27−7.23 (m, 1 H), 7.2−7.1 (m, 3 H), 6.96−6.9 (m, 1 H), 6.9−6.8 (m, 1 H), 6.65−6.55 (m, 1 H), 6.54−6.48 (m, 1 H), 6.40 (d, J = 8 Hz, 1 H), 6.38−6.29 (m, 1 H), 4.51 (d, J = 6.8 Hz, 2 H), 4.2 (br s, 1 H). ^{13}C NMR: δ 147.3, 139.3, 138.3, 134.9, 132.7, 130.3, 129.0, 128.1, 127.8, 127.6, 127.5, 121.8, 118.1, 117.9, 49.6. HRMS (ESI+) calcd for $C_{15}H_{14}N$ $[M+H]^+$ 208.1126, found 208.1120.

*N-(6-(Dibenzo[b,f]azocin-5(6H)-yl)-6-oxohexyl)trifluoroacetamide (**3**).* 6-(Trifluoroacetamido)hexanoyl chloride (*45*) (0.984 g, 4.52 mmol) was added to a solution of **2** (0.75 g, 3.62 mmol) and pyridine (0.859 g, 10.86 mmol) in CH_2Cl_2 (ca. 10 mL) at rt and stirred for 30 min. The reaction mixture was diluted with CH_2Cl_2 (ca. 20 mL), washed with water (2 × 30 mL), and dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by chromatography (hexanes/ethyl acetate 2:3) to provide 1.064 g (2.56 mmol, 71%) of **3** as yellowish oil. ^{1}H NMR: δ 7.32−7.22 (m, 4 H), 7.19−7.11 (m, 4 H), 6.77 (d, J = 13.2 Hz, 1 H), 6.58 (d, J = 13.2 Hz, 1 H), 5.46 (d, J = 14.8 Hz, 1 H), 4.20 (d, J = 14.8 Hz, 1 H), 3.29−3.15 (m, 2 H), 2.09−2.02 (m, 1 H), 1.93−1.85 (m, 1 H), 1.51−1.32 (m, 4 H), 1.25−1.04 (m, 2 H). ^{13}C NMR: δ 172.7, 157.5, 141.1, 136.1, 135.8, 134.8, 132.4, 131.8, 130.5, 128.5, 128.2, 128.1, 128.0, 127.3, 127.2, 117.5, 114.6, 54.8, 39.6, 34.3, 28.2, 25.9, 24.3. HRMS (ESI+) *m/z* calcd for $C_{23}H_{24}F_3N_2O_2$ $[M+H]^+$ 417.1790, found 417.1783.

*N-(6-Trifluoroacetamidohexanoyl)-5,6-dihydro-11,12-didehydrodibenzo[b,f]azocine (**5**).* Pyridine hydrobromide perbromide (0.948 g, 2.97 mmol) was added to a solution of **3** (1.05 g, 2.70 mmol) in CH_2Cl_2 (4 mL) at rt, and the reaction mixture was stirred overnight. The reaction mixture was diluted with CH_2Cl_2 (20 mL), washed with 5% aqueous hydrochloric acid (20 mL), dried over $MgSO_4$, and solvent removed under vacuum. The residue was passed through a short pad of silica gel (CH_2Cl_2) to give 1.2 g of crude *N*-(trifluoroacetamidohexanoyl)-5,6,11,12-tetrahydro-11,12-dibromodibenzo[*b,f*]azocine (*4*) as an oil. A solution of crude **4** (1.2 g, 2.08 mmol) in THF (5 mL) was added to a solution of potassium *t*-butoxide (0.584 g, 5.21 mmol) in THF (10 mL) at rt; the reaction mixture was stirred for 1 h, diluted with ethyl acetate (20 mL), washed with 5% aqueous hydrochloric acid and brine, then dried over $MgSO_4$, and the solvent was removed under reduced pressure. The crude product was purified by chromatography (hexanes/ethyl acetate, 2:1 to 1:1) to afford 0.76 g (1.83 mmol, 88%) of **5** as brown oil. ^{1}H NMR: δ 7.68 (d, J = 7.6 Hz, 1 H), 7.45−7.21 (m, 7 H), 6.79 (br s, 1 H), 5.16 (d, J = 14.4 Hz, 1 H), 3.67 (d, J = 13.6 Hz, 1 H), 3.22−3.14 (m, 1 H), 3.11−3.02 (m, 1 H), 2.24−2.16 (m, 1 H), 1.41−1.22 (m, 4 H), 1.11−0.9 (m, 2 H). MS: *m/z* 414 $[M^+]$. Calcd for $C_{23}H_{21}F_3N_2O_2$ 414.

*N-(6-Aminohexanoyl)-5,6-dihydro-11,12-didehydrodibenzo-[b,f]azocine (ADIBO-C6-amine, **6**).* A solution of K_2CO_3 (2 g, 14.47 mmol) in 15 mL of water was added to a solution of *N*-(6-trifluoroacetamido hexanoyl)-5,6-dihydro-11,12-didehydrodibenzo[*b,f*]azocine (**5**, 2.95 g, 7.12 mmol) in MeOH (30 mL) at rt and stirred overnight. Solvents were removed under reduced pressure, the residue was redissolved in CH_2Cl_2/ethyl acetate (1:4), then washed with brine and water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuum. The crude product was purified by chromatography (CH_2Cl_2/MeOH 10:1 to 10:4) to provide 1.31 g (4.11 mmol, 58%) of **6** as slightly yellow oil. ^{1}H NMR: δ 7.71 (d, J = 7.6 Hz, 1 H), 7.45−7.21 (m, 7 H), 5.18 (d, J = 14.4 Hz, 1 H), 3.63 (d, J = 13.6 Hz, 1 H), 3.32 (br, 2 H), 2.67 (t, J = 5.6 Hz, 1 H), 2.52 (t, J = 5.6 Hz, 1 H), 2.21−2.19 (m, 1 H), 1.95−1.90 (m, 1 H), 1.45−1.35 (m, 2 H) 1.28−1.20 (m, 2 H), 1.11−0.9 (m, 2 H). ^{13}C NMR: δ 173.3, 151.8, 147.8, 132.3, 132.1, 128.9, 128.2, 128.0, 127.8, 127.5, 126.5, 122.1, 123.0, 114.8, 107.9, 55.8, 41.4, 34.7, 31.9, 26.0, 24.8. HRMS (ESI+) *m/z* calcd for $C_{21}H_{23}N_2O$ $[M+H]^+$ 319.1810, found 319.1799.

*N-(3-Aminopropionyl)-5,6-dihydro-11,12-didehydrodibenzo[b,f]azocine (ADIBO-C3-amine, **7**).* **7** was prepared following the same protocol as the preparation of **6**. ^{1}H NMR (500 MHz): δ 7.68 (d, J = 7.5 Hz, 1 H), 7.45−7.33 (m, 5 H), 7.29 (t, J = 7.5 Hz, 1 H), 7.25 (t, J = 7 Hz, 1 H), 5.15 (d, J = 14 Hz, 1 H), 3.16 (d, J = 14 Hz, 1 H), 2.82−2.67 (m, 2H), 2.45−2.35 (m, 1 H), 2.01−1.92 (m, 1 H), 1.6−1.4 (br s, 2 H). ^{13}C NMR: δ 172.14, 151.48, 148.01, 132.12, 129.08, 128.29, 128.21, 127.99, 127.63, 127.01, 125.43, 122.85, 122.57, 114.97, 107.66, 55.25, 38.25, 38.15. HRMS (ESI+) calcd for $C_{18}H_{17}N_2O$ $[M+H]^+$ 277.1341, found 277.1339.

*Aza-dibenzocyclooctyne−Biotin Conjugate (ADIBO-biotin, **8**).* HBTU (1.916 g, 5.05 mmol) was added to a solution of biotin-PEG4-acid (1.9 g, 3.87 mmol) and DIEA (0.647 g, 5.61 mmol) in CH_2Cl_2 (15 mL) at rt and stirred for 15 min. A solution of **7** (1.238 g, 4.48 mmol) in CH_2Cl_2 (2 mL) was added dropwise, the reaction mixture was stirred for 3 h and concentrated under reduced pressure. The product was purified by chromatography (CH_2Cl_2 to CH_2Cl_2/MeOH 20:1 to 100:15) to provide 2.44 g (3.26 mmol, 87%) of **8** as colorless semisolid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.66 (d, J = 7 Hz, 1 H),

7.42−7.3 (m, 7 H), 7.28−7.25 (m, 1 H), 7.05−6.99 (m, 1 H), 6.79−6.75 (m, 1 H), 6.61 (br s, 1 H), 5.13 (d, J = 14 Hz, 1 H), 4.5−4.45 (m, 1 H), 4.32−4.25 (m, 1 H), 3.68 (d, J = 14 Hz, 1 H), 3.65−3.45 (m, 17 H), 3.44−3.36 (m, 2 H), 3.32−3.22 (m, 2 H), 3.15−3.07 (m, 2 H), 2.9−2.82 (m, 1 H), 2.75−2.65 (m, 1 H), 2.55−2.46 (m, 1 H), 2.33 (q, J = 6 Hz, 2 H), 2.2 (t, J = 7.5 Hz, 2 H), 2.0−1.92 (m, 1 H), 1.75−1.6 (m, 4 H), 1.45−1.35 (m, 5 H). ^{13}C NMR: δ 173.51, 171.99, 171.13, 164.01, 151.1, 148.09, 132.13, 129.14, 128.68, 128.21, 127.83, 127.19, 125.58, 123.07, 122.45, 114.72, 107.86, 70.45, 70.43, 70.39, 70.3, 70.17, 70.03, 69.98, 97.17, 61.83, 60.26, 55.69, 55.52, 53.67, 42.0, 40.52, 39.14, 36.79, 35.89, 35.26, 34.71, 28.29, 28.11, 25.63, 18.59, 17.44, 11.88. HRMS (ESI+) *m/z* calcd for $C_{39}H_{53}N_5O_8S$ $[M+H]^+$ 750.3537, found 750.3542.

*Aza-dibenzocyclooctyne-PEG$_4$-Amine (ADIBO-PEG$_4$-amine, **9**).* EDC (0.75 g, 4.68 mmol) was added to a solution of Boc-NH-$(CH_2CH_2O)_4$-$CH_2CH_2CO_2H$ (*46*) (1.57 g, 4.32 mmol) in CH_2Cl_2 (15 mL) and DIEA (0.7 g, 5.4 mmol) at rt and stirred for 15 min. A solution of ADIBO-amine **7** (1 g, 3.6 mmol) in CH_2Cl_2 (1 mL) was added to the reaction mixture and stirred for 4 h, at which time the solvent was removed under reduced pressure and the crude product purified by chromatography (ethyl acetate/hexanes 1:1 to 9:1) to provide 1.8 g (2.8 mmol, 80%) of crude *N*-Boc-protected ADIBO-PEG$_4$-amine (9-Boc) as yellow oil.

A solution of TFA (0.48 g, 4.2 mmol) in THF (15 mL) was added to a solution of **9-Boc** (1.8 g, 2.8 mmol) in THF (30 mL) at rt. The reaction mixture was stirred overnight, and the solvent was removed under reduced pressure. The residue was purified by chromatography (CH_2Cl_2/MeOH 10:1 to 10:4) to provide 1.15 g (2.2 mmol, 79%) of ADIBO-PEG$_4$-amine (**9**) as slightly yellow oil. ^{1}H NMR (500 MHz): δ 7.67 (d, J = 7.5 Hz, 1 H), 7.43−7.34 (m, 5 H), 7.31 (t, J = 7.5 Hz, 1 H), 7.29−7.24 (m, 1 H), 6.95−6.88 (m, 1 H), 5.13 (d, J = 14 Hz, 1 H), 4.45−4.2 (br s, 2 H), 3.7−3.5 (m, 22 H), 3.37−3.2 (m, 3 H), 2.68 (t, J = 5 Hz, 2 H), 2.57−2.42 (m, 1 H), 2.4−2.32 (m, 2 H), 2.02−1.92 (1 H). ^{13}C NMR (100 MHz, $CDCl_3$): 172.13, 171.21, 151.20, 148.16, 129.3, 129.2, 128.78, 128.44, 128.31, 127.9, 127.29, 127.25, 125.68, 123.16, 122.55, 114.81, 107.92, 70.46, 70.39, 70.35, 70.32, 70.30, 70.26, 70.1, 55.62. 48.86, 36.69, 35.41, 34.67. HRMS (ESI+) *m/z* calcd for $C_{29}H_{38}N_3O_6$ [M+H] 524.2761, found 524.2756. (Note: Integration of ^{1}H spectra of compounds containing PEG commonly overestimates the number of protons due to the presence of tightly bound water and/or hydroxylic solvents.)

Cleaning and Activation of Glass Slide Surfaces. All glass slides were sonicated for 30 min in methanol, rinsed with acetone, and dried in an oven at 145 °C for 1 h. Piranha solution (90 mL) was prepared by the addition of H_2O_2 (25 mL of 35% v/v) in one portion to 65 mL of conc H_2SO_4 in a 100 mL Pyrex beaker, which was kept in a water bath (**Caution:** Piranha solution reacts violently with organic compounds and should be handled with extreme care. Wear thick plastic gloves, lab coat, and safety glasses. Any piranha solutions should be handled in a fume hood at all times). The solution was carefully stirred with a glass rod, followed by inserting glass slides into the solution. After 1 h, slides were removed from the solution and rinsed with copious amounts of distilled water, then acetone, and dried in an oven for 20 min at 145 °C. Glass slides prepared in this fashion were submitted to derivatization procedures immediately.

Preparation of Epoxy-Derivatized Glass Slides. Freshly activated glass slides were immersed in a solution of freshly distilled (3-glycidyloxypropyl)dimethoxy-methylsilane (1% v/v) and DIEA (1% v/v) in dry toluene (100 mL) at 25 °C for 16 h. Slides were sonicated two times in methanol for 15 min, thoroughly rinsed with acetone, and dried under a stream of nitrogen.

Preparation of Aza-dibenzocyclooctyne (ADIBO, 6)-Coated Glass Slides. Freshly prepared epoxy-coated glass slides were placed in a solution of aza-dibenzocyclooctyne amine **6** (75 mg) and DIEA (1 mL) in DMF (100 mL) and incubated overnight at rt. Slides were then rinsed with acetone, sonicated for 15 min in methanol, rinsed with acetone, and dried under a stream of nitrogen.

Preparation of Azide-Coated Glass Slides. Freshly prepared epoxy-coated glass slides were immersed in a solution of 6-azidohexylamine (1 mL) and DIEA (1 mL) in DMF (100 mL) overnight at rt. The slides were then sonicated for 15 min in methanol, rinsed with acetone, and dried under a stream of nitrogen.

Patterned Derivatization of Glass Slides with Aza-dibenzocyclooctyne 6. One microliter drops of a 10 mM PBS solution of dibenzocyclooctyne **6** were spotted using a micropipet on a freshly prepared epoxy-coated slide, followed by incubation in a humidity chamber containing PBS buffer for 12 h at rt. The slide was washed with copious amounts of acetone, then water, and sonicated in DMF for 30 min.

Patterned Immobilization of Fluorescent Dyes on ADIBO-Coated Slides. Solutions of Oregon green azide (**13**, 0.1 mM and 1 mM in PBS) or Lissamine rhodamine B azide (**14**, 0.1 mM) were spotted using a pipet (2 μL) on a freshly prepared dibenzocyclooctyne plate and incubated in a humidity chamber for various periods of time (vide infra). Slides were rinsed with acetone, then sonicated for 15 min in methanol, rinsed with acetone, and dried under a stream of nitrogen.

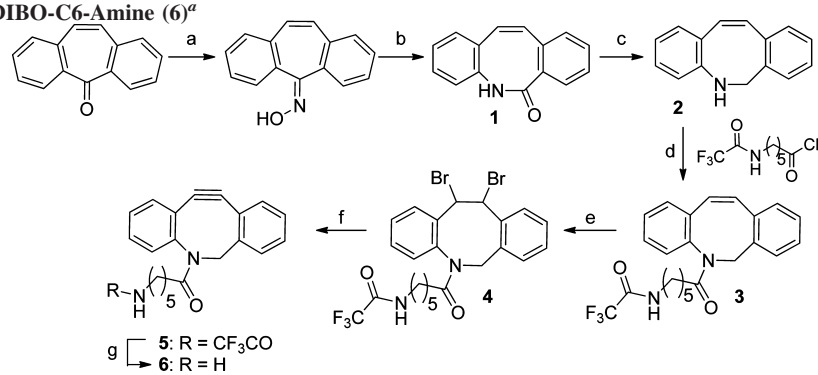
Patterned Immobilization of Avidin on ADIBO-Coated Slides. One microliter drops of biotin-dPEG3+4-azide PBS solutions of different concentrations (10 mM, 1 mM, and 0.1 mM) were spotted on a ADIBO-coated glass slide. Slides were incubated in a humidity chamber for 1 h at rt, rinsed with copious amounts of acetone, then water, and sonicated in DMF for 30 min. Slides were then immersed in a blocking solution containing 1% aminoPEG$_4$azide in DMF and incubated overnight at rt. The slides were then rinsed with acetone, sonicated in DMF for 30 min, and rinsed with distilled water, followed by immersion into a solution of avidin-FITC (50 μL of 2 mg/mL in 10 mL of PBS) at 2 °C for 15 min. The slides were sonicated in PBS containing 0.1% Tween 20 for 30 min, washed with distilled water, incubated in PBS containing 0.1% of BSA for 12 h at 2 °C, sonicated again in PBS (1% of Tween 20) for 30 min, and rinsed with distilled water.

Patterned Immobilization of Fluorescent Probes on Azide-Coated Slides. One microliter drops of 1 mM of ADIBO-fluor (**10**) solution in PBS were spotted on an azide plate, followed by incubation in a humidity chamber for 12 h at rt. Slides were rinsed with acetone, then sonicated for 15 min in methanol, rinsed with acetone, and dried under a stream of nitrogen.

For two-color derivatization, azide-coated slides patterned with ADIBO-fluor (**10**) spots as described above were immersed in a solution of ADIBO-Rhodamine (**12**, 1 mM in PBS) and incubated for 3 h at rt. Slides were rinsed with acetone, then sonicated for 15 min in methanol, rinsed with acetone, and dried under a stream of nitrogen.

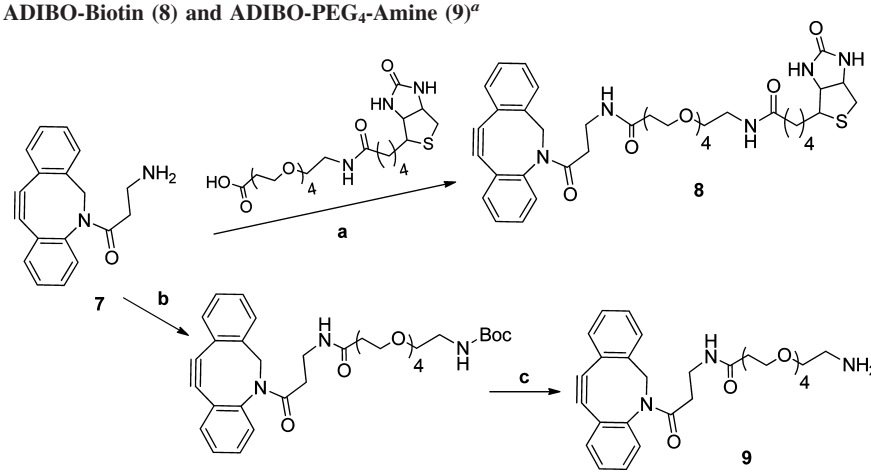
Patterned Immobilization of Avidin on Azide-Coated Slides. One microliter drops of ADIBO-biotin (**8**) solutions of different concentrations (10 mM, 1 mM, 0.1 mM, and 0.01 mM in PBS) were spotted on an azide-coated glass slide. Slides were incubated in a humidity chamber for 1 h at rt, rinsed with copious amounts of acetone, then water, and sonicated in DMF for 30 min. Slides were then immersed in a blocking solution containing 0.1% ADIBO-PEG4-amine (**9**) in DMF and incu-

Scheme 1. Synthesis of ADIBO-C6-Amine (6)[a]



[a] Reagents and conditions: (a) $NH_2OH.HCl$, pyridine, 60%; (b) PPA, 125 °C, 73%; (c) $LiAlH_4$, ether, 58%; (d) pyridine, CH_2Cl_2, 71%; (e) pyridinium tribromide, 78%; (f) *t*-BuOK, THF, 88%; (g) K_2CO_3, aq MeOH, 58%.

Scheme 2. Synthesis of ADIBO-Biotin (8) and ADIBO-PEG$_4$-Amine (9)[a]



[a] Reagents and conditions: (a) HBTU, DIEA, CH_2Cl_2, 89%; (b) Boc-NH-$(CH_2CH_2O)_4$-$CH_2CH_2CO_2H$, EDC, DIEA, CH_2Cl_2, 80%; (c) TFA, THF, 79%.

bated overnight at rt. The slides were rinsed with acetone, sonicated in DMF for 30 min, and rinsed with distilled water, followed by immersion into a solution of avidin-FITC (50 μL of 2 mg/mL in 10 mL PBS) at 2 °C for 15 min. The slides were sonicated in a PBS solution containing 0.1% Tween 20 for 30 min, washed with distilled water, incubated in PBS containing 0.1% BSA for 12 h at 2 °C, sonicated again in PBS (1% Tween 20) for 30 min, and rinsed with distilled water.

Preparation of ADIBO-Coated Magnetic Beads. A solution of ADIBO−biotin conjugate (**8**) (25 μL, 10 mM in PBS) was added to a suspension of streptavidin-coated magnetic beads (25 μL of 10 mg/mL) in 450 μL PBS. The resulting mixture was stirred by shaking for 2 h at rt. The reaction mixture was centrifuged at 11 000 rpm for 2 min, the supernatant liquid was decanted, and the pellet resuspended in PBS (450 μL). The washing step was repeated two times.

Preparation of Azide-Coated Magnetic Beads. A solution of Biotin-dPEG3+4-azide (25 μL, 10 mM in PBS) was added to a suspension of streptavidin-coated magnetic beads (25 μL of 10 mg/mL) in 450 μL PBS. The resulting mixture was stirred by shaking for 2 h at rt. The reaction mixture was centrifuged at 11 000 rpm for 2 min, the supernatant liquid was decanted, and the pellet resuspended in PBS (450 μL, pH 7.4). The washing step was repeated twice.

Fluorescent Labeling of ADIBO-Coated Magnetic Beads. A solution of Oregon Green-azide (**13**) (10 mM in PBS) was added to a suspension of ADIBO-coated magnetic beads (25 μL of 10 mg/mL) and incubated for 3 h at rt. Beads were centrifuged at 11 000 rpm for 1 min, the supernatant liquid was decanted, and the pellet was resuspended in 450 μL PBS containing 0.1% Tween 20, centrifuged, washed with PBS, centrifuged, and resuspended in 450 μL PBS for fluorescent microscopy imaging.

Fluorescent Labeling of Azide-Coated Magnetic Beads. A solution of ADIBO-fluor (**10**, 25 μL of 10 mM in PBS) was added to a suspension of azide-coated magnetic beads (25 μL of 10 mg/mL) in 450 μL PBS and incubated for 3 h at rt. Beads were centrifuged at 11 000 rpm for 1 min, the supernatant liquid was decanted, and the pellet was resuspended in 450 μL PBS containing 0.1% of Tween 20, centrifuged, washed with PBS, centrifuged, and resuspended in 450 μL PBS for fluorescent microscopy imaging.

RESULTS AND DISCUSSION

Synthesis of Aza-dibenzocyclooctyne−Amine Conjugate (ADIBO-amine, 6). The efficient preparation of ADIBO-amine developed in our lab is outlined in Scheme 1. It starts with the polyphosphoric acid-catalyzed Beckman rearrangement of dibenzosuberenone oxime, which is readily prepared by treating commercially available dibenzosuberenone with hydroxylamine. The lactam **1** produced in this reaction is then reduced with lithium aluminum hydride to give dihydrodibenzo[*b*,*f*]azocine (**2**). The secondary amino group in **2** is converted to amide **3** by reacting with 1.25 equiv of 6-(trifluoroacetamido)hexanoyl chloride in the presence of pyridine. The olefin in **3** is smoothly converted into an acetylene moiety via a bromination−dehydrobromination procedure to give aza-dibenzocyclooctyne **5** in excellent yield (88%). Saponification of the trifluoroacetamide moiety with potassium carbonate in aqueous methanol gives

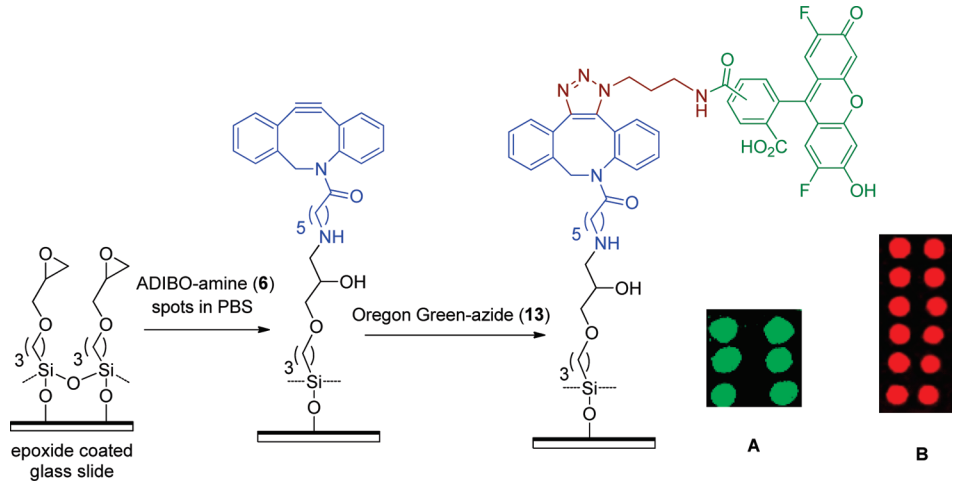


Figure 1. Schematic representation of ADIBO derivatization of an epoxy-coated slide followed by copper-free click immobilization of Oregon green dye. Inserts show fluorescent images ADIBO slides patterned with (A) Oregon green azide (**13**) and (B) Lissamine rhodamine B azide (*14*).

target ADIBO-C6-amine (**6**). ADIBO-amine with a shorter aminoakyl side chain, ADIBO-C3-amine (**7**), was prepared following the same synthetic sequence by replacing 6-(trifluoroacetamido)hexanoyl chloride in step "d" with 3-(trifluoroacetamido)propionyl chloride.

Aza-dibenzocyclooctyne−Biotin Conjugate (ADIBO-biotin, 8). 8 was prepared by HBTU (1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium-3-oxide hexafluorophosphate)-promoted coupling of ADIBO-C3-amine (*7*) with biotin-PEG4-acid (Scheme 2). EDC-induced coupling of compound **7** with *N*-Boc-15-amino-4,7,10,13-tetraoxapentadecanoic acid, followed by trifluoroacetic acid-catalyzed removal of *N*-Boc protection gave Aza-dibenzocyclooctyne-PEG$_4$-amine (ADIBO-PEG$_4$-amine, **9**, Scheme 2).

ADIBO and Azide Functionalization of Glass Slides. Derivatization experiments were performed on glass surfaces due to the ready availability, low cost, high mechanical stability, low intrinsic fluorescence, and easy surface modification techniques of the glass substrate. Freshly prepared epoxide-coated slides were incubated in a DMF solution of ADIBO-amine **6** or 6-azidohexylamine in the presence of Hünig's base (*N*-ethyl-*N,N*-diisopropylamine) overnight and washed with acetone, then methanol (Figures 1 and 5).

Oregon Green azide (**13**) was used as a model compound to assess the efficiency and kinetics of the metal-free azide click immobilization on ADIBO-coated slides. To ensure that a fluorescent dye is immobilized on the slides only by the click reaction and not due to physical absorption or other chemical reactions, we spotted the epoxy-coated plate with ADIBO-amine (**6**). After overnight incubation and washing, these plates were immersed in a PBS solution of Oregon green azide (**13**, 0.01 mM) for 100 min and washed with acetone, then sonicated for 15 min in methanol, rinsed with acetone, and dried under a stream of nitrogen. The fluorescence image of the resulting slide demonstrates that dye **13** specifically binds to the ADIBO-derivatized surface and not to the rest of the slide (Figure 1, insert A). While the value of relative fluorescence intensity (spot versus background) depends on the starting epoxy plate (we have also tested VWR Microarray Epoxy 2 Slides and Corning Epoxide Slides), Oregon green spots always showed bright fluorescence with 2000−6000 contrast ratio. In the following fluorescent dye patterning experiments, 2 μL drops of 0.1 mM or 0.01 mM PBS solutions of azide−dye conjugate were applied onto ADIBO-derivatized slides. Thus, 0.1 mM solution of Lissamine rhodamine B azide (**14**) was spotted on the ADIBO-

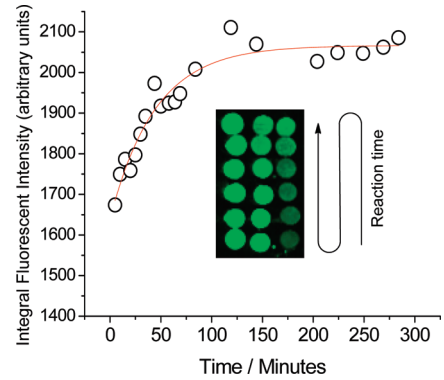


Figure 2. Integral fluorescent intensity of Oregon green azide (**13**) spots on ADIBO-derivatized slide versus reaction time.

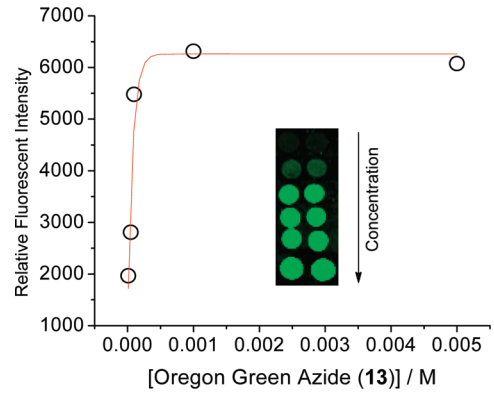


Figure 3. Integral fluorescent intensity of Oregon green azide (**13**) spots on ADIBO-derivatized slide versus concentration (10 μM, 50 μM, 0.1 mM, 1 mM, and 5 mM).

slide, incubated for 1 h, and thoroughly washed. The fluorescence image also has a good contrast ratio (Figure 1, insert B).

Kinetics of Metal-Free Click Immobilization. To evaluate the kinetics of the ADIBO−azide reaction on the surface, we spotted 2 μL drops of a 0.1 mM Oregon green azide (**13**) PBS solution onto ADIBO-derivatized slides. The first spot was allowed to react for 284 min; subsequent drops were applied at different times, with the last drop applied just 5 min before washing. The slides were stored in a humidity chamber during this procedure. The immobilization reaction shows excellent kinetics. Within 5 min, the relative fluorescent intensity reaches

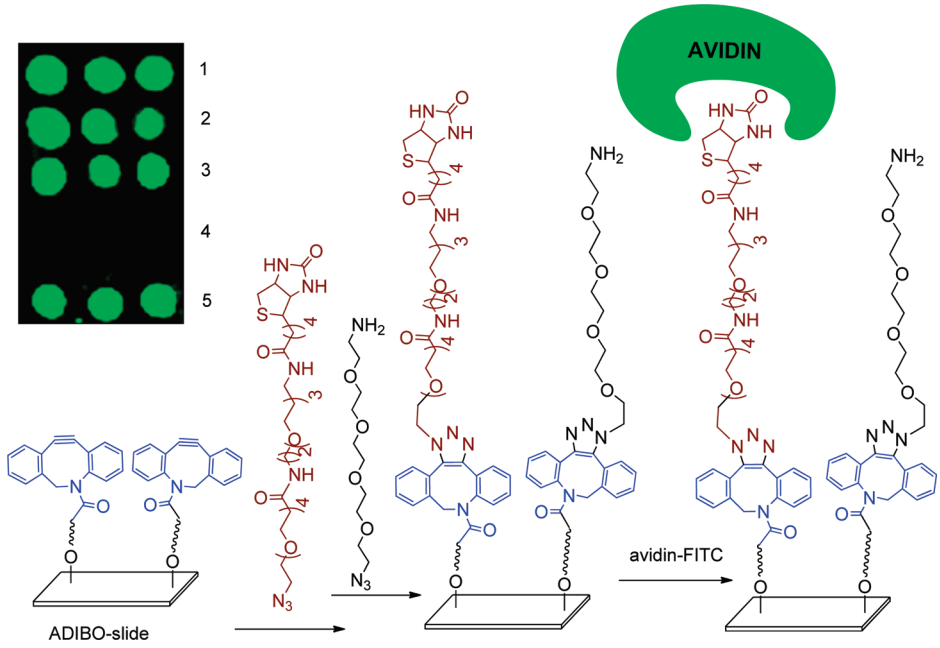


Figure 4. Schematic representation of patterned biotinylation of ADIBO-coated slide followed by selective immobilization of avidin-FITC. The insert shows the fluorescent image of ADIBO-slide spotted with 1 μL of the following solutions: lanes 1−3, 10 mM, 1 mM, and 0.1 mM PBS solutions of Biotin-dPEG3 + 4-azide; lane 4, 1 mM biotin-PEG$_4$-C≡CH; lane 5, 0.1 mM of Oregon green azide (**13**). The slide was then treated with aminoPEG$_4$azide and developed with avidin−FITC solution.

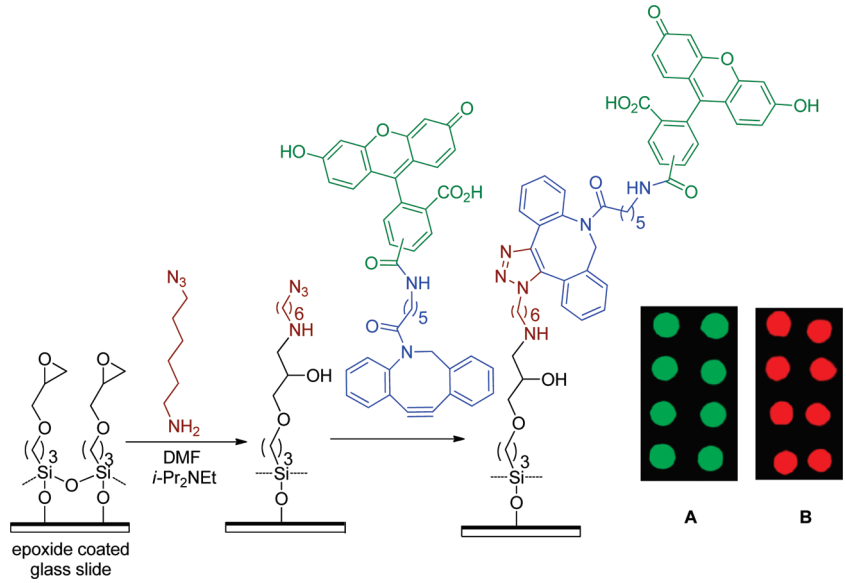


Figure 5. Schematic representation of azide derivatization of an epoxy-coated slide followed by copper-free click immobilization of ADIBO-fluor (*10*). Inserts show fluorescent images of azide slides patterned with (A) ADIBO-fluor (*10*) and (B) ADIBO-Rhodamine (*12*).

44−80% of the maximum value, and saturation of fluorescence is achieved at ca. 100 min at 0.1 mM of azide (Figure 2). As a result, incubation with the coupling reagent for 100 min was selected as a standard procedure for subsequent experiments.

To optimize the concentration of the substrate for the metal-free click immobilization, we spotted an ADIBO slide with PBS solutions of various concentration of Oregon green azide (**13**): 10 μM, 50 μM, 0.1 mM, 1 mM, and 5 mM. After incubation for 100 min and washing, the image of the slide was recorded and the integral fluorescent intensity analyzed (Figure 3). The saturation of Oregon green fluorescence is achieved at approximately 0.1 mM concentration of the azide **13**.

Biotinylation of ADIBO-Slides and Immobilization of Avidin. The exceptional selectivity and high binding constant between avidin and biotin (dissociation constant = 10^{-15} M) (*47*) is widely used in bioconjugation and surface immobilization applications (*1*−*5, 10, 48*−*53*). Therefore, we decided to test the efficiency of surface biotinylation using a copper-free azide click reaction. As shown in Figure 4, 1 μL drops of three different concentrations (10 mM, 1 mM, and 0.1 mM) of biotin-dPEG3+4-azide solutions in PBS were applied onto an ADIBO-functionalized glass slide. For a comparison, 1 μL drops of a 0.1 mM PBS solution of Oregon green azide (**13**) were also spotted on the slide. To test for the possibility of nonspecific absorption of biotin conjugates on the ADIBO slides, a 1 mM PBS solution of biotin-PEG$_4$-C≡CH was also spotted on the slide. After an hour-long incubation and washing, slides were immersed in a 1% DMF solution of aminoPEG$_4$azide and incubated overnight. Our initial experiment showed that dibenzocyclooctynes have significant affinity toward proteins. Ex-

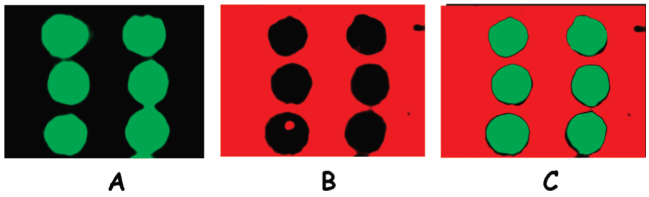


Figure 6. Two-color derivatization of azide slides. The slide was spotted with ADIBO-fluor (**10**), washed, and immersed in a solution of ADIBO-Rhodamine (*12*). (A) Fluorescent image recorded using 495/520 nm filter; (B) image recorded using 532/580 nm filter; (C) images A and B merged.

posure to the aminoPEG$_4$azide solution converts unreacted aza-dibenzocyclooctyne fragments into triazole−PEG conjugates. Patterned biotinylated slides were developed with an avidin-FITC PBS solution for 15 min at 2 °C and washed thoroughly. The fluorescent image of this slide shows selective immobilization of the protein in the biotinylated areas, while nonspecific binding of avidin was not observed (Figure 4). Incubation of the avidin-FITC-patterned slides in a PBS solution containing BSA does not reduce the fluorescence, confirming the immobilization of avidin via specific biotin−avidin interactions.

Patterning of Fluorescent Dyes on Azide-Coated Slides. To assess the efficiency of a reverse copper-free click surface derivatization, we prepared azide-coated glass slides (vide supra). Such a surface provides a convenient platform for immobilization using both conventional (i.e., copper-catalyzed) and catalyst-free azide click reactions. One microliter drops of a 1 mM PBS solutions of aza-dibenzocyclooctyne **6** conjugates with fluorescein (ADIBO-fluor, **10**) or with Lissamine rhodamine B (ADIBO-Rhodamine, **12**) were allowed to react for 12 h, then washed thoroughly. Fluorescent images of the resulting slides with fluorescein (Figure 5, insert A) and Lissamine rhodamine B (Figure 5, insert B) illustrate the efficiency of substrate immobilization on the azide-derivatized surface using metal-free click chemistry.

After immobilization, slides are often subjected to vigorous washing procedures, including the use of detergents and sonication in aqueous solutions and organic solvents. To test the stability of the azide surface to the washing procedures and to explore the feasibility of multisubstrate surface derivatization using copper-free click chemistry, we studied the sequential immobilization of two fluorescent dyes. The azide-coated glass slide was initially spotted with 1 μL drops of a 1 mM PBS solution of ADIBO-fluor (**10**) and incubated in a humidity chamber for 12 h at rt. The slide was rinsed with acetone, sonicated in DMF for 30 min, and rinsed with distilled water, then immersed in a 0.1 mM solution of ADIBO-Rhodamine (**12**) for another 12 h, then thoroughly washed. The fluorescent image recorded with a green (495−520 nm) filter clearly shows a pattern of fluorescein-immobilized spots (Figure 6A). The bright background of Lissamine rhodamine B fluorescence and dark spots, where azide groups were consumed in the first step, are visible on the image recorded with a red (532−580 nm) filter (Figure 6B). Figure 6C shows merged images A and B, which demonstrates that reactivity of an azide-derivatized surface is not significantly affected by washing procedures.

Patterned Biotinylation of Azide-Slides and Selective Immobilization of Avidin. To further demonstrate the versatility of the copper-free click reaction for protein immobilization, we have conducted patterned biotinylation of azide-derivatized slides, followed by the immobilization of avidin. Azide-functionalized glass slides were spotted with 1 μL drops of four different concentrations (10 mM, 1 mM, 0.1 mM, and 0.01 mM) of a PBS solution of ADIBO−biotin (*8*). Slides were incubated in a humidity chamber for 30 min, 2.5 h, and 3.5 h and washed with copious amounts of acetone, then water, and sonicated in DMF. To reduce nonspecific binding of the protein, slides were immersed in a blocking solution containing 0.1% ADIBO-PEG$_4$-amine (**9**) in DMF and kept overnight. The slides were then washed, incubated

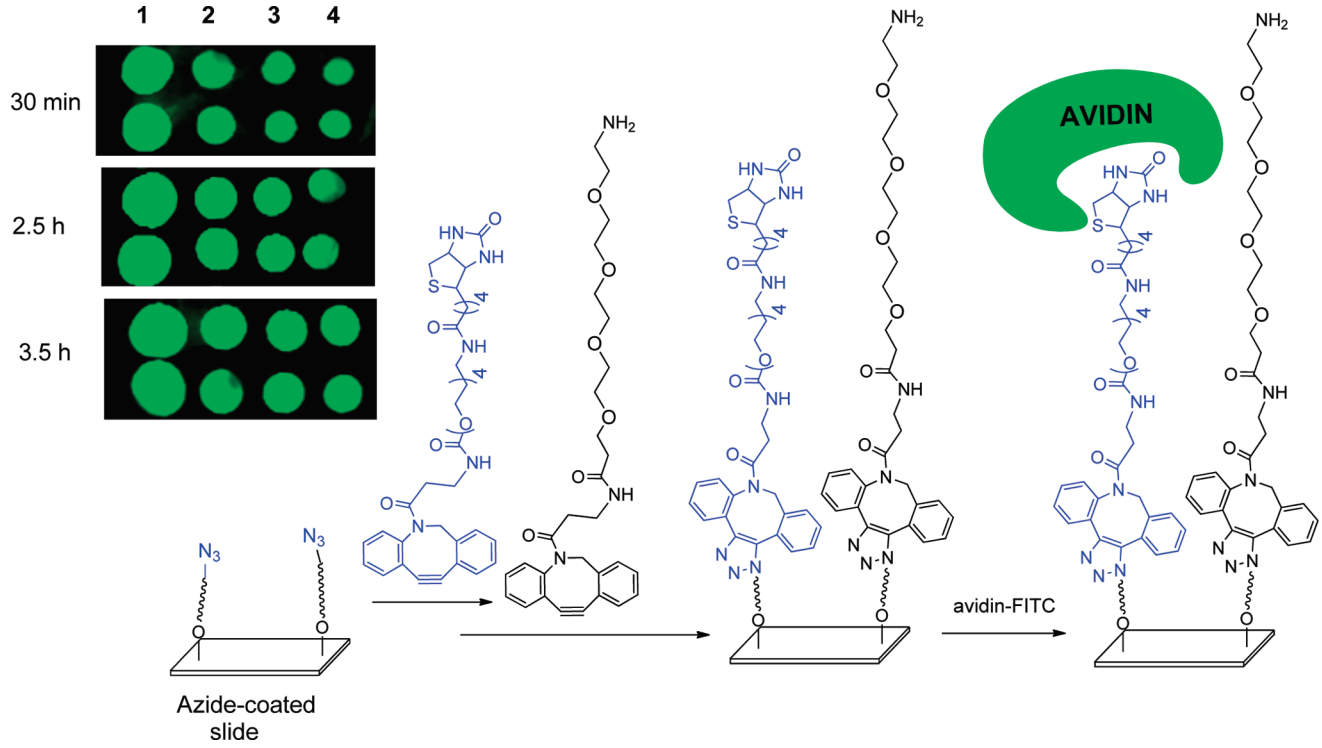


Figure 7. Schematic representation of patterned biotinylation of azide-coated slides followed by selective immobilization of avidin-FITC. The insert shows the fluorescent image of azide-derivatized slide spotted with 1 μL of ADIBO-biotin (**8**) solutions of the following concentrations: (1) 10 mM; (2) 1 mM; (3) 0.1 mM; (4) 0.01 mM. The first slide was incubated for 30 min, the second for 2.5 h, and the third for 3.5 h. Slides were then immersed in a solution of ADIBO-PEG$_4$-amine and developed with a solution of avidin-FITC.

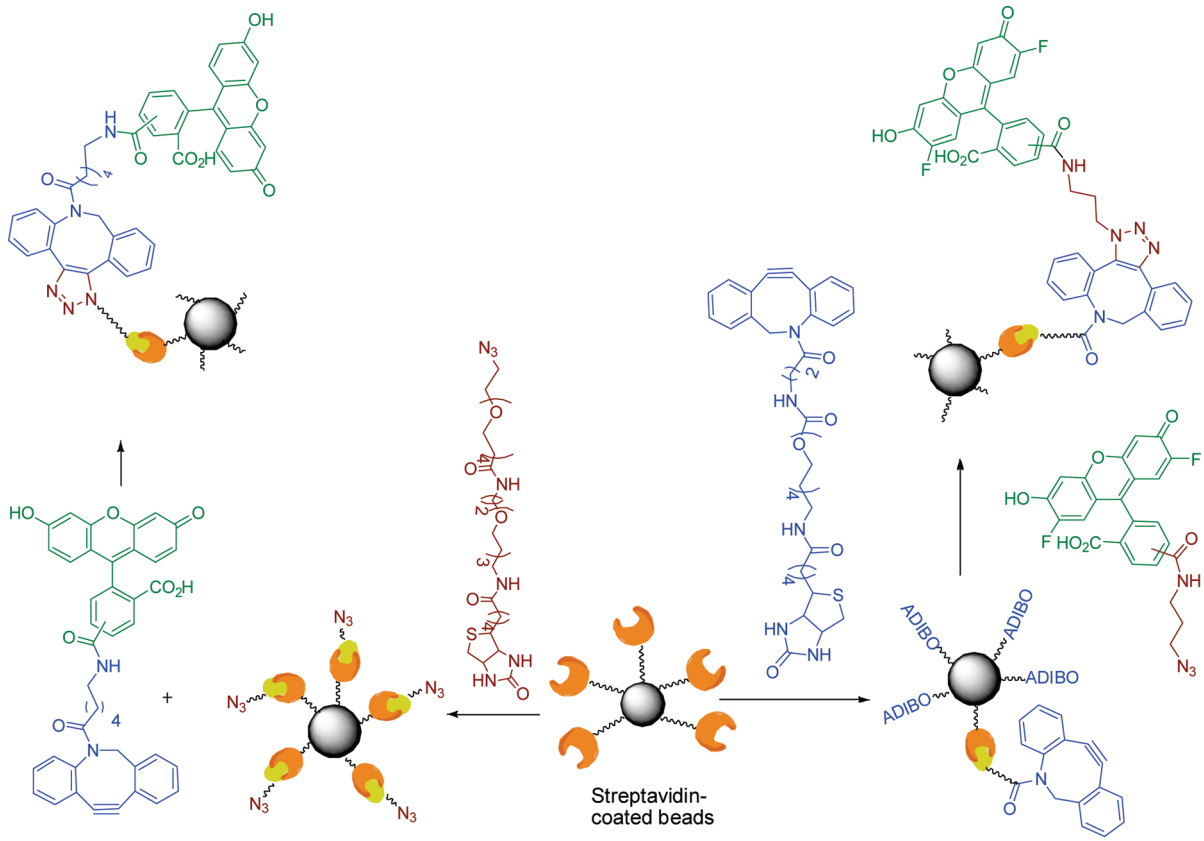


Figure 8. Schematic representation of derivatization of streptavidin-coated magnetic beads with azide and aza-dibenzocyclooctyne moieties, followed by the metal-free azide click coupling to fluorescent dyes.

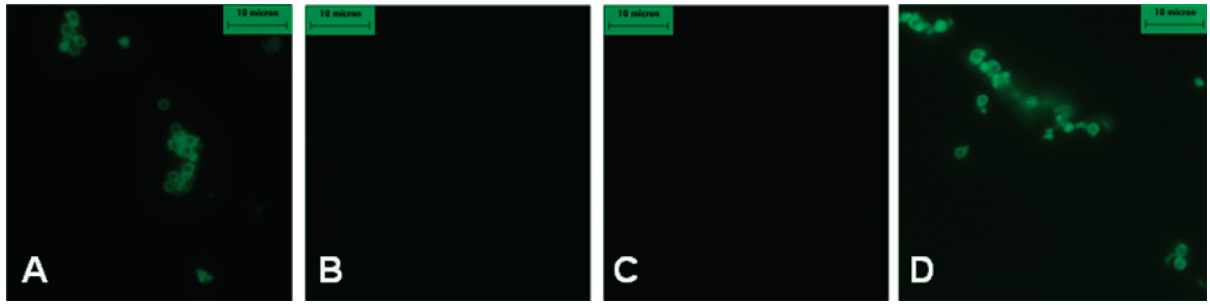


Figure 9. Fluorescent confocal microscope images of PBS suspensions of streptavidin-coated magnetic beads: (A) treated with ADIBO−biotin (**8**) followed by reaction with Oregon green azide (**13**); (B) treated with biotin-PEG_4-C≡CH and then with Oregon green azide (*13*); (C) incubated with ADIBO-fluor only (**10**); (D) treated with biotin-dPEG3 + 4-azide and then with incubated ADIBO-fluor (*10*).

in a solution of avidin-FITC at 2 °C for 15 min, and washed again. As in the case of patterned biotinylation of ADIBO slides, fluorescent images show selective immobilization of the fluorescently labeled protein in biotinylated areas. No significant nonspecific binding of avidin to the slides was observed (Figure 7). Fluorescent intensity of the spots produced at various concentrations of ADIBO-biotin and incubation time illustrate the efficiency of the reaction. Even at 10 μM concentration of ADIBO-biotin (**8**), a 30 min incubation produces a brightly fluorescent spot after avidin-FTIC development.

Catalyst-Free Derivatization of Microbeads. To demonstrate the utility of the copper-free azide click reaction for the modification of microparticle surfaces, we have explored the application of this reaction to the fluorescent labeling of streptavidin-coated magnetic beads. The surface of the beads was derivatized with azide groups by treating a suspension of the beads with biotin-dPEG3+4-azide (Figure 8). The aza-dibenzocyclooctyne functionalization of streptavidin beads was achieved by reacting the former with ADIBO-biotin (**8**, Figure 8).

The ADIBO-functionalized streptavidin beads were suspended in a PBS solution of Oregon green azide (**13**) and incubated for 3 h at rt. Beads were washed and resuspended in PBS for imaging via fluorescent confocal microscopy. Figure 9A shows the bright fluorescence of these Oregon green-labeled beads. The starting (unmodified) streptavidin magnetic beads treated with **13** under the same conditions show no detectable emission. As an additional control experiment, we have derivatized the beads with terminal acetylene groups by treating streptavidin beads with biotin-PEG_4-C≡CH. Incubation of the resulting particles in an Oregon green azide (**13**) solution, followed by thorough washing, did not induce detectable fluorescence in the beads (Figure 9B). Azide-derivatized microbeads were labeled with fluorescein by reacting them with an ADIBO−fluorescein

conjugate (**10**, Figure 9C). Magnetic streptavidin beads directly treated with ADIBO-fluor (**10**) showed no fluorescence (Figure 9D).

CONCLUSIONS

We have described an efficient synthesis of aza-dibenzocyclooctynes (ADIBO) starting from inexpensive precursors. This method allows for preparation of ADIBO derivatives on a large scale. The utility and excellent kinetics of catalyst-free ADIBO-azide cycloaddition for the patterned derivatization of glass slides and streptavidin beads has been demonstrated. The same metal-free click reaction was employed for the PEGylation of unfunctionalized areas of the surface. Such treatment allowed for dramatic reduction or complete elimination of nonspecific binding of proteins to the surface. The strategy described in this report provides a convenient tool for the site-specific covalent immobilization of various biomolecules. These procedures can be especially useful in cases where the presence of copper ions has to be avoided.

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Supporting Information Available: NMR spectra of newly synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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