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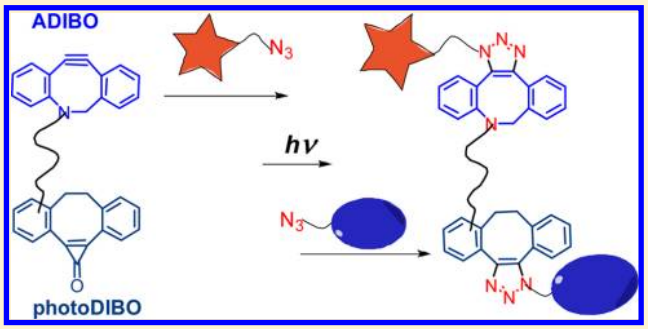
Sequential "Click" – "Photo-Click" Cross-Linker for Catalyst-Free Ligation of Azide-Tagged Substrates

Selvanathan Arumugam and Vladimir V. Popik*

Department of Chemistry, University of Georgia, Athens, Georgia 30602, United States

Supporting Information

ABSTRACT: Heterobifunctional linker allows for selective catalyst-free ligation of two different azide-tagged substrates via strained-promoted azide–alkyne cycloaddition (SPAAC). The linker contains an azadibenzocyclooctyne (ADIBO) moiety on one end and a cyclopropenone-masked dibenzocyclooctyne (photo-DIBO) group on the other. The first azide-derivatized substrate reacts only at the ADIBO end of the linker as the photo-DIBO moiety is azide-inert. After the completion of the first SPAAC step, photo-DIBO is activated by brief exposure to 350 nm light from a fluorescent UV lamp. The unmasked DIBO group then reacts with the second azide-tagged substrate. Both click reactions are fast (k = 0.4 and 0.07 M^{-1} s^{-1}, respectively) and produce quantitative yield of ligation in organic solvents or aqueous solutions. The utility of the new cross-linker has been demonstrated by conjugation of azide functionalized bovine serum albumin (azido-BSA) with azido-fluorescein and by the immobilization of the latter protein on azide-derivatized silica beads. The BSA–bead linker was designed to incorporate hydrolytically labile fragment, which permits release of protein under the action of dilute acid. UV activation of the second click reaction permits spatiotemporal control of the ligation process.



INTRODUCTION

Structural modifications of biomolecules and polymers, as well as derivatization of particles and surfaces, are commonly achieved using "click chemistry".[1] This term describes a set of bimolecular reactions that permits the efficient formation of a covalent link between two substrates (a.k.a. ligation in biochemistry) or between a substrate and a surface. The majority of "click" strategies are based on 1,3-dipolar or Diels–Alder cycloadditions, imine formation, and addition to carbon–carbon multiple bonds.[2−4] Since azide and alkyne moieties are very uncommon in natural products, i.e., "bioorthogonal", the copper-catalyzed (CuAAC) or strain-promoted azide–alkyne cycloadditions (SPAAC) have found many applications in labeling biomolecules,[5] protein modification,[6] synthesis of bioconjugates,[7] and developing biotech tools.[8] The chemical stability of the triazole linker made azide–alkyne click reactions a popular tool in material chemistry.[1b,e,9]

Chemoselective sequential click ligation brings the ability to introduce multiple functionalities to biological molecules,[10] drug delivery vehicles,[11] polymer,[12] or surfaces.[13] Cross-linking of biological molecules using sequential click chemistry allows for the preparation of complex macromolecules.[14] There are two different approaches to selective sequential click ligations. One method relies on two (or more) mutually orthogonal click reactions, e.g., CuAAC and Diels–Alder,[11a,12a−d] SPAAC and SPANC,[15,13a,b] SPAAC and hetero-Diels–Alder,[16] as well as other combinations.[11b,17] These strategies provide excellent selectivity but require derivatization of substrates with different functionalities. To avoid this complication, sequential click ligation may employ reactions of the same type, most commonly azide–alkyne cycloaddition. This approach relies on the differences in the reactivity of alkyne moieties[10c,13c,14c,18] or on the deprotection of terminal acetylenes.[10a,11c,19] The use of cytotoxic copper(I) catalyst and/or deprotecting reagents, however, somewhat reduces the utility of this method. In addition, terminal acetylene were found to inhibit cysteine proteases by forming thioether with the catalytically active thiol.[20]

Here, we report the development of the SPAAC-based selective sequential click strategy, which permits cross-linking of two different azide-tagged substrates without the use of catalysts or activating reagents (Scheme 1). Dibenzocyclooctynes are apparently too large to fit into the active site cavity of a protease. Selection of the reactive moieties for the construction of the sequential SPAAC cross-linking agent was based on the orthogonality requirement. The first click reaction should occur only at one end of the linker, while the second ligation should not degrade connection to the first substrate. We have chosen azadibenzocyclooctyne (ADIBO, Scheme 1)[21] as the first SPAAC moiety, as this cyclooctyne combines high reactivity toward azides with excellent aqueous stability and long shelf life.[22] PhotoDIBO (Scheme 1), on the

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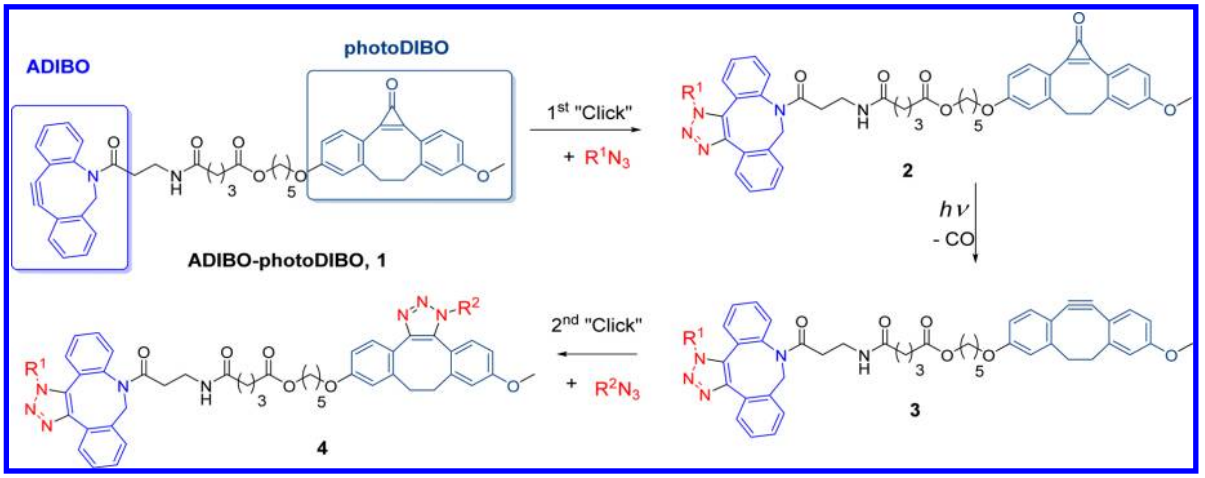
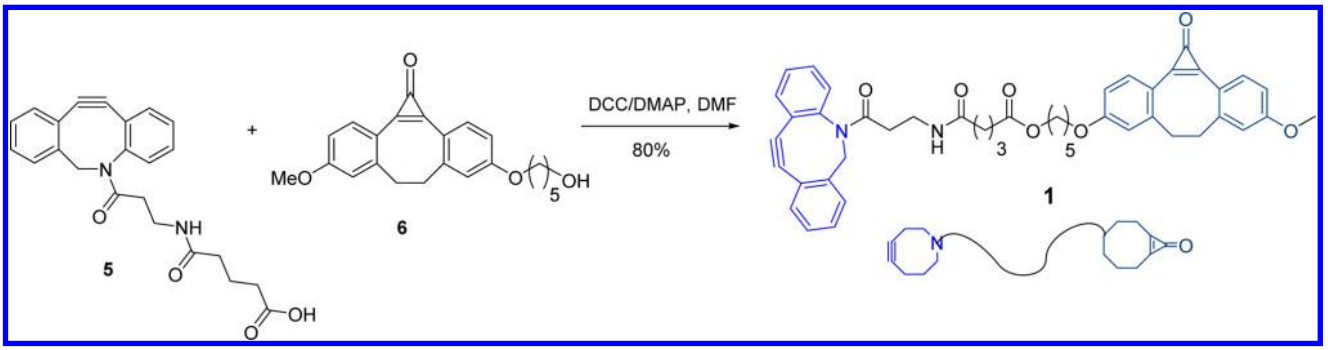
Scheme 1. Sequential Click−Photoclick Conjugation of Two Azides[a]



[a](a) R^1,R^2 = *n*-Bu; (b) R^1,R^2 = Bn; (c) R^1 = BSA, R^2 = Fluorescein; (d) R^1 = SiO_2-beads, R^2 = BSA.

Scheme 2. Preparation of ADIBO−PhotoDIBO Cross-Linker (1)



other hand, does not react with azides in the dark and possesses excellent thermal stability.[23] Exposure of photo-DIBO moiety to a low intensity 350 nm light results in the efficient decarbonylation and the formation of azide-reactive dibenzocyclooctyne (DIBO).[23,24] ADIBO, DIBO, and corresponding triazole adducts have virtually no absorbance at 350 nm, and, therefore, are stable under photodecarbonylation conditions.[23]

RESULTS AND DISCUSSION

The heterobifunctional ADIBO-photoDIBO cross-linker (**1**) was prepared by the DCC/DMAP-assisted coupling of ADIBO-acid (**5**)[25] with of pehtamethylenehydroxy-derivatized photoDIBO (**6**, Scheme 2).

The efficiency and selectivity of cross-linker **1** were evaluated using its reaction with benzyl azide. The accurate rate measurements of the first and second click reaction were conducted by UV spectroscopy at 25 ± 0.1 °C in methanol under pseudo-first-order conditions. The UV spectrum of ADIBO-photoDIBO (**1**) contains characteristic features of both ADIBO (a band at 292 nm) and photoDIBO (intense bands at 331 and 347 nm) chromophores (Figure 1a, black trace). All stages of the conversion of **1** can be, therefore, conveniently monitored by UV spectroscopy. The addition of the excess of benzyl azide (0.1−20 mM) to the methanol solution of **1** leads to the rapid disappearance of the band at 292 nm, while photoDIBO bands remain unchanged (Figure 1a, red trace). The decay ADIBO band followed the single exponential equation well (Figure 1b). The dependence of the observed pseudo-first-order rate constants on azide concentration was linear and produced second-order rate constant k= 0.406 ± 0.001 M^{-1} s^{-1}. This value is consistent with literature data for ADIBO.[21,22] After the completion of the first click reaction, the cyclopropenone protection of the triple bond in **2** was removed by 2 min irradiation of the reaction mixture with 350 nm fluorescent lamps (Scheme 1). The conversion of photo-DIBO was followed by the disappearance of the characteristic cyclopropenone bands at 331 and 347 nm and the formation of the DIBO band at 319 nm (Figure 1a). The disappearance of the latter band due to the addition of the second molecule of benzyl azide to **3** also showed clean first-order kinetics (Figure 1b). The second-order rate of this reaction (k = 0.072 ± 0.004 M^{-1} s^{-1}) is similar to the values reported for DIBO.[22,23a]

HPLC analysis of the "click" − photoactivation − second "click" sequence indicates clean and quantitative conversion at every step (Figure 2). Thus, 50 μM methanol solution of **1** (Figure 2a) was treated with an equimolar amount of butyl acid and incubated for 48 h under ambient conditions to ensure complete conversion. The HPLC trace (Figure 2b) and ESI-HRMS of the resulting product (MH^+, Calc. for $C_{50}H_{54}N_5O_7$ 836.4018, found 836.4016) confirmed the quantitative formation of **2**a (Note: Apparently, head-to-tail and head-to-head isomers have very similar retention times on C-18 column). Photodecarbonylation step produces **3a** (MH^+, Calc. for $C_{49}H_{54}N_5O_6$ 808.4069, found 808.4067) with no detectable amounts of side products (Figure 2c). The addition of the second equivalent of butyl azide cleanly gives the final

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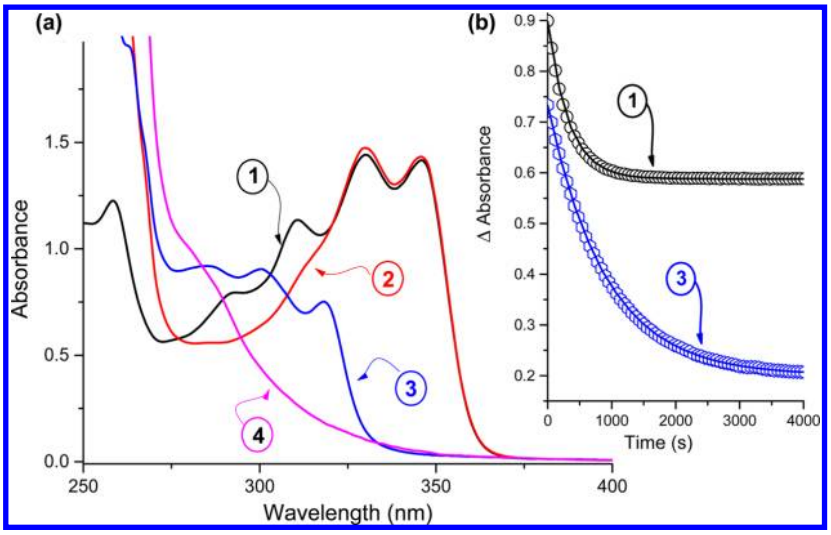


Figure 1. (a) UV spectra of 50 μM methanol solutions of ADIBO-photoDIBO (**1**, black line); product of the first click reaction **2b** (red line); product of photoactivation **3b** (blue line); product of the second click reaction **4b** (purple line). (b) Kinetics traces of the first (black circles) and the second (blue hexagons) click reactions in the presence of 10 mM of benzyl azide.

adduct **4a** after 48 h incubation (Figure 2d; MH^+, Calc. for $C_{53}H_{63}N_8O_6$ 907.4865, found 907.4867).

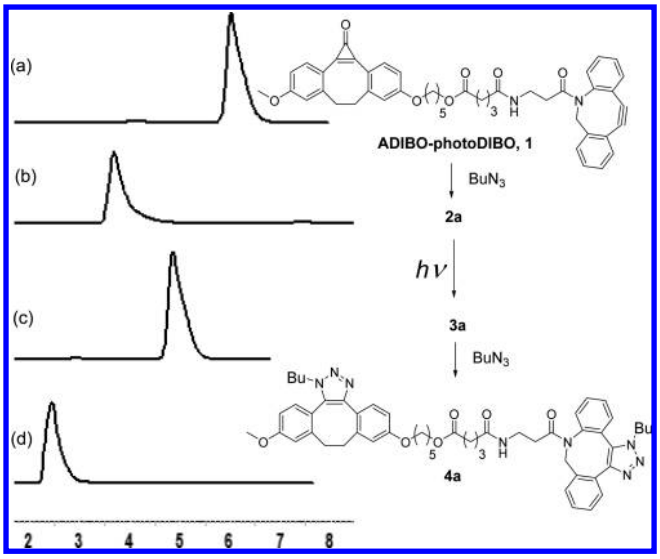
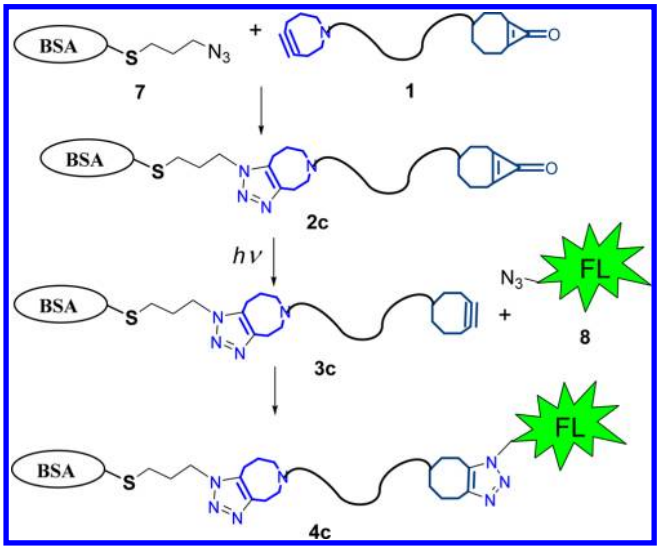


Figure 2. HPLC traces of the sequential click ligation (Scheme 1): (a) starting linker **1**; (b) reaction mixture after the reaction of **1** with equimolar amount of butyl azide; (c) product of photodecarbonylation step (**3a**); (d) product (**4a**) of the second click reaction with equimolar amount of azide.

As an illustrative example, we have employed ADIBO-photoDIBO cross-linker (**1**) for the selective conjugation of azido-derivatized BSA (**7**) with (3-azidopropylcarbamoyl)-fluorescein (azido-Fl, **8**, mixture of 5- and 6-isomers, Scheme 3). After each click reaction the functionalized protein was isolated and characterized by MALDI. The azido-derivatized BSA (**7**) was prepared by treating the native BSA with 1-azido-3-iodopropane. Protein **7** was incubated with an excess of the cross-linker **1** in aqueous solution overnight and resulting BSA derivative **2c** (Scheme 3) was isolated by gel filtration. An aqueous solution of **2c** was irradiated for 2 min using 350 nm fluorescent lamps and treated with 50-fold excess of azidoFL **8** overnight and purified by gel filtration

Scheme 3. Conjugation of Azido-Derivatized Bovine Serum Albumin (BSA) with Azido-Fluorescein (FL) Using ADIBO-PhotoDIBO Cross-Linker



(Scheme 3). The resulting Fluorescein-derivatized BSA (**4c**) clearly shows characteristic absorbance and emission of the Fluorescein chromophore (Figure 3).

The protein content of the solution of **4c** was determined using Coomassie brilliant blue dye assay, while the emission intensity of **4c** and the absorbance in the fluorescein region were compared to the fluorescence and absorbance of an aqueous solution of an authentic fluorescein sample of the same concentration (Figure 3). These experiments confirm high cross-linking efficiency (84–92%) of the ADIBO-photoDIBO (**1**). The high yield of azido-BSA (**7**) labeling was somewhat surprising result since it is commonly accepted that commercial BSA samples contain 0.6–0.8 equiv of free thiols. The rest of Cys34 moieties are believed to form a disulfide with cysteine or glutathione.[26] We believe that free thiol content of the BSA is actually higher than reported previously, since Ellman's test, which uniformly used in these measurements, often underestimates SH contents in pro-

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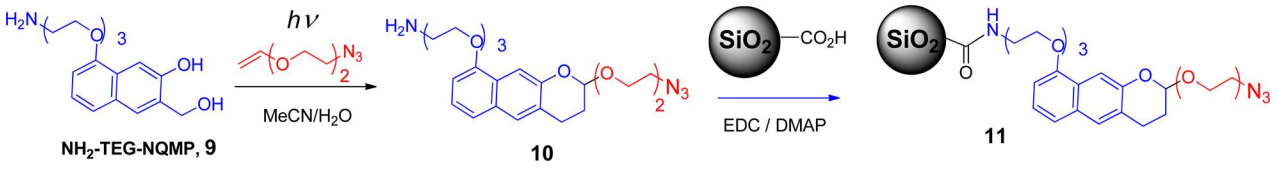
Scheme 4. Photo-Diels-Alder Click Derivatization of Silica Microbeads with Azide Moiety



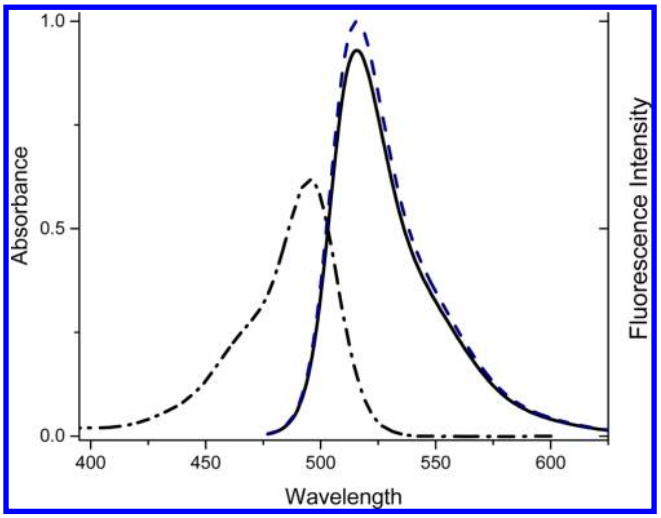


Figure 3. Absorption (dash–dotted line) and emission spectra (solid line) of 11 μM PBS solution of BSA–fluorescein conjugate (**10**) and emission spectrum of a 11 μM PBS solution of fluorescein (dotted line).

teins,[27] especially in proteins containing acidic Cys residues.[28,29]

To test the suitability of ADIBO-photoDIBO (**1**) for protein immobilization, we have employed this cross-linking agent for the attachment of azido-BSA (**7**) to azide-functionalized silica microbeads. First, a photo-Diels–Alder click reaction[30] between 8-(2-(2-(2-aminoethoxy)ethoxy)-ethoxy)-3-(hydroxymethyl)naphthalen-2-ol (**9**, NH_2-TEG-NQMP)[17d] and (2-(2-azidoethoxy)ethoxy)ethylene produced heterobifunctional linker **10** (Scheme 4). It is important to note that 2-alkoxybenzochroman **10** is stable under neutral conditions, but becomes hydrolytically labile at pH < 3.[30] Linker **10** was then EDC-coupled to commercial carboxylate-functionalized silica microbeads to yield target azide-derivatized microbeads bearing acid-labile linker (**11**, Scheme 4).

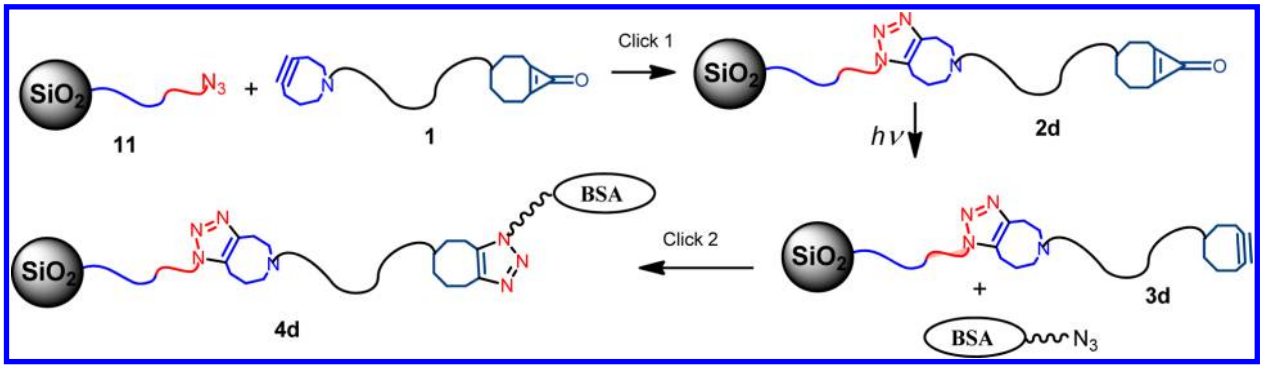
Silica beads bearing azido functionality (**11**) were first derivatized with linker **1** (click 1) by overnight incubation with ADIBO-photoDIBO (**1**) in DMF (Scheme 5). The resulting microbeads were added to 10 μM PBS solution of azido-BSA (**7**) and irradiated with 350 nm light for 2 min (Scheme 5). The reaction mixture was incubated under ambient conditions for 16 h and the beads were separated and washed. The Bradford assay of the combined supernatants from the reaction mixture after click reaction **2** showed that there was virtually no protein present in the solution. This observation illustrates the high efficiency of azido-BSA (**7**) immobilization using linker **1**.

As BSA was immobilized to silica beads via an acid-sensitive 2-alkoxybenzochroman fragment,[30] the protein can be cleaved from the solid support at low pH (Scheme 6). To validate the release of the protein, silica microbeads bearing immobilized BSA were incubated in 0.1 M perchloric acid overnight. The protein released from the beads was isolated from the supernatant via spin filtration and reconstituted to the original volume in PBS. Total protein concentration in the resulting solution was determined to be 9.12 μM by Bradford assay, which corresponds to the ca. 91% recovery. The BSA protein is stable in an aqueous 0.1 M perchloric acid solution, at least for the time frame (16 h) used for the release of protein from silica microbeads.

■ CONCLUSIONS

The heterobifunctional ADIBO-photoDIBO linker (**1**) allows for the efficient ligation of azide-tagged substrates, or for the immobilization of azide-functionalized molecules on an azide-coated surfaces. Since the covalent conjugation is achieved via strained-promoted azide–alkyne cycloaddition (SPAAC) and photochemical activation, this method does not require any catalysts or other reagents. This sequential click strategy is applicable to both homogeneous and heterogeneous cross-linking. The use of phototrigger in the sequential click system enables the spatiotemporal control to the cross-linking chemistry. Additionally, the incorporation of a hydrolytically

Scheme 5. Immobilization of Azido-BSA on Azide-Functionalized Silica Microbeads



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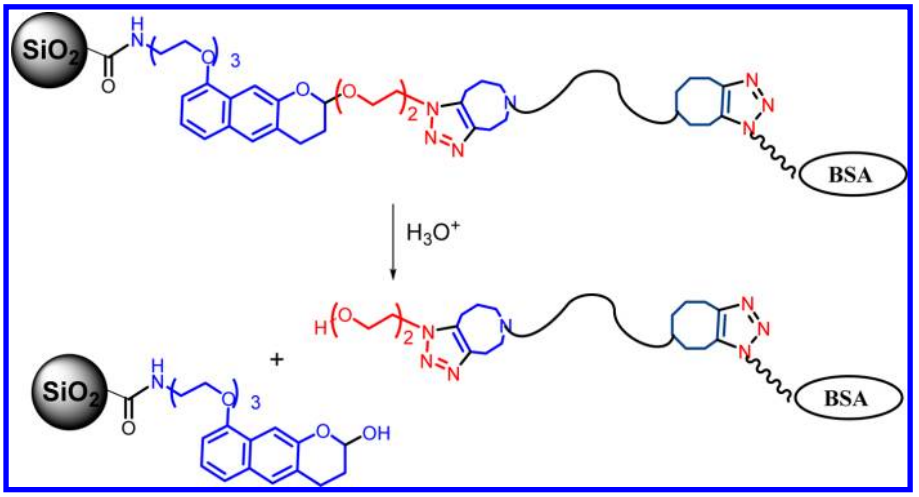
Scheme 6. Release of BSA from Silica Microbeads



labile fragment in protein−surface linker, allows for the release of a protein under the action of dilute acid.

EXPERIMENTAL SECTION

General Information. All organic solvents were dried and freshly distilled before use. Flash chromatography was performed using 40−63 μm silica gel. All NMR spectra were recorded on 400 MHz instruments in $CDCl_3$ and referenced to TMS unless otherwise noted. Solutions for HPLC and UV−vis analysis were prepared using HPLC grade solvents. HPLC analysis was conducted using analytical C-18 column and a diode array detector. Photochemical decarbonylation of photoDIBO moiety was conducted by the irradiation of the reaction mixture for 1−2 min in a photochemical reactor equipped with four fluorescent UV lamps (4 W, 350 nm).

Materials. Bovine serum albumin (BSA) was purchased from Boehringer Mannheim. ADIBO-carboxylic acid (**5**),[25] cyclopropenone-masked DIBO-OH (**6**),[23a] 8-(2-(2-(2-aminoethoxy)ethoxy)-ethoxy)-3-(hydroxymethyl)naphthalen-2-ol (**9**),[17d] (2-azidoethoxy)-ethyl vinyl ether,[17d] 5- and 6-(3-azidopropylcarbamoyl)fluorescein (azido-Fl, **8**, mixture of isomers)[23b] were prepared following previously reported procedures. Carboxy-functionalized silica microspheres (0.01 mmol of acid/g, 1 μm) was purchased from Polysciences Inc.

ADIBO-PhotoDIBO Cross-Linker (1). *N,N′*-Dicyclohexylcarbodiimide (62 mg, 0.3 mmol) and a catalytic amount of DMAP were added to a solution of ADIBO-carboxylic acid (**5**) (150 mg, 0.38 mmol) in 8 mL of dry DMF, followed by a dropwise addition of a solution of alcohol **6** (151 mg, 0.42 mmol) in 2 mL of DMF. The mixture was stirred for 12 h at r.t., the solvent was removed in a vacuum, the residue was dissolved in DCM, washed with $NaHCO_3$ solution, brine, dried over anhydrous magnesium sulfate, and concentrated under reduced pressure. The residue was purified by column chromatography (10% MeOH in DCM) to yield 225 mg (80%) of ADIBO-photoDIBO (**1**). ^{1}H NMR: 7.93 (dd, J = 8.9, 6.6 Hz, 2H), 7.66 (dd, J = 7.4, 1.5 Hz, 1H), 7.43−7.21 (m, 7H), 6.83−6.93 (m, 4H), 6.06 (t, J = 6.6 Hz, 1H), 5.12 (d, J = 13.9 Hz, 1H), 4.07 (t, J = 6.4 Hz, 2H), 4.02 (t, J = 6.4 Hz, 2H), 3.87 (s, 3H), 3.59 (d, J = 13.9 Hz, 1H), 3.29−3.39 (m, 3H), 3.16−3.27 (m, 1H), 2.55−2.65 (m, 2H), 2.30−2.50 (m, 1H), 2.28 (t, J = 7.5 Hz, 2H), 2.04 (t, J = 7.5 13 Hz, 2H), 1.76−1.99 (m, 3H), 1.72−1.38 (m, 6H). ^{13}C NMR: 173.6, 172.7, 172.3, 162.9, 162.5, 154.2, 151.5, 148.5, 148.3, 148.3, 142.8, 142.6, 136.3, 136.3, 132.6, 129.5, 129.1, 128.9, 128.8, 128.3, 127.7, 126.1, 123.4, 123.0, 116.8, 116.7, 116.3, 115.3, 112.8, 112.3, 108.2, 68.6, 64.8, 63.3, 55.99, 55.98, 35.9, 35.7, 35.2, 34.0, 29.5, 29.1, 26.2, 21.3. FW calc $[(C_{46}H_{44}N_2O_7)H^+]$: 737.3221; ESI-HRMS: 737.3217.

Sequential Labeling of BSA with Azido-Fl. 3-Azidoprop-1-yl iodide (2.1 mg, 10 μmol) was added to a solution of BSA (66 mg, ca. 1 μmol) in 3 mL of 0.1 N phosphate buffer (pH = 8) containing 0.5 mL of acetonitrile and gently shaken for 12 h at r.t. The aqueous layer was washed with ethyl acetate and was freeze-dried to produce azido-derivatized BSA (80 mg, contains some phosphate salt). Ellman's test performed after completion of the reaction gave negative results. Azido-derivatized BSA (**7**) was further purified by spin filtration. MALDI TOF: 66546.

0.3 mg of linker **1** (400 μmol) was added to the solution of azido-BSA **7** (13.2 mg, ca. 0.2 μmol) in 2 mL of PBS and the mixture was incubated for 16 h at r.t. on a mechanical shaker. The excess of **1** was removed by filtration through a Dextran desalting column. The product **2c** was characterized by MALDI-TOF: 67283.

A solution of BSA **2c** (13.2 mg, ca. 0.2 μmol) in 2 mL of PBS was irradiated for 2 min and a solution of azido-Fl **8** (0.5 mg ca 1.1 μM) in 2 μL of DMF was added. The reaction mixture was incubated 16 h at r.t. on a mechanical shaker. The unreacted azido-fluorescein was removed by filtration through a Dextran desalting column. Fluorescein−BSA conjugate **4c** was characterized by MALDI-TOF: 67713. Total protein content in the solution of **4c** was determined by Bradford assay using Brilliant Blue G-250 dye. The Bradford assay standard curve was obtained from a series of standard BSA solution in the concentration range 1−15 μM and the unknown protein concentration was determined from the quadratic fit of the standard data. The concentration of protein in fluorescein−BSA conjugate **4c** was found to be 11.13 μM. The emission (519 nm) and absorbance (494 nm) of this solution were compared to corresponding spectral features of 11 μM solution of fluorescein, confirming 84−92% of BSA derivatization.

Preparation of Azide-Derivatized Silica Beads. (2-Azidoethoxy)-ethyl vinyl ether (96 mg, 0.6 mmol) was added to a solution of 8-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-3-(hydroxymethyl) naphthalen-2-ol (**9**, NH_2-TEG-NQMP, 70 mg, 0.22 mmol) in 20 mL of aqueous acetonitrile (1:1). The reaction mixture was irradiated for 1 h. The solvent and excess of (2-azidoethoxy)ethyl vinyl ether was removed under reduced pressure. The crude amine **11** was taken into the next step without further purification. EDC.HCl (66 mg, 0.3 mmol) and a catalytic amount of DMAP were added to a dispersion of carboxy-functionalized silica microspheres (2 g) in 20 mL of dry DMF, followed by addition of amine **11**. The mixture was stirred for 48 h at r.t. and the solvent was removed by centrifugation. The azido derivatized silica beads (**12**) were thoroughly washed with DMF trice and dried under nitrogen.

Immobilization of Azido-BSA 7 on Azide-Coated Silica Microbeads (12). A solution of ADIBO-photoDIBO **1** (1.5 mg, 2 μM) in 10 μL of methanol was added to a suspension of the azide-derivatized silica beads **11** (100 mg) in DMF (10 mL) and incubated overnight. The beads were filtered, washed, and allowed to dry. Excess of silica beads (50 mg, 0.5 μmol in ADIBO-photo DIBO) were added to 3.5 mL of 10 μM PBS solution azido-BSA **7** (2.33 mg, 0.035 μmol) and

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irradiated under vigorous stirring with 350 nm light for 2 min (Scheme 5). The reaction mixture was incubated under ambient conditions for 16 h and the beads were separated by centrifugation and washed.

Release of BSA from Solid Support. 50 mg of silica microbeads with immobilized BSA were incubated in 1 mL of 0.1 M perchloric acid overnight under mild shaking. The beads were separated and washed with 1 mL PBS buffer 5 times. The protein was isolated from combined supernatants (6 mL) by spin-filtration and reconstituted in 3.5 mL of PBS. The resulting solution was analyzed by the Bradford Assay.

■ ASSOCIATED CONTENT

Supporting Information

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

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: vpopik@uga.edu.

Notes

The authors declare no competing financial interest.

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