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Covalent protein-oligonucleotide conjugates by copper-free click reaction

Santoshkumar L. Khatwani^a, Daniel G. Mullen^a, Michael A. Hast^b, Lorena S. Beese^b, Mark D. Distefano^a, and T. Andrew Taton^{a,*}

^aDepartment of Chemistry, University of Minnesota, Minneapolis, MN 55455, USA

^bDepartment of Biochemistry, Duke University, Durham, NC 27710, USA

Abstract

Covalent protein-oligodeoxynucleotide (protein-ODN) conjugates are useful in a number of biological applications, but synthesizing discrete conjugates—where the connection between the two components is at a defined location in both the protein and the ODN—under mild conditions with significant yield can be a challenge. In this article, we demonstrate a strategy for synthesizing discrete protein-ODN conjugates using strain-promoted azide-alkyne [3+2] cycloaddition (SPAAC, a copper-free “click” reaction). Azide-functionalized proteins, prepared by enzymatic prenylation of C-terminal CVIA tags with synthetic azidoprenyl diphosphates, were “clicked” to ODNs that had been modified with a strained dibenzocyclooctyne (DIBO-ODN). The resulting protein-ODN conjugates were purified and characterized by size-exclusion chromatography and gel electrophoresis. We find that the yields and reaction times of the SPAAC bioconjugation reactions are comparable to those previously reported for copper-catalyzed azide-alkyne [3+2] cycloaddition (CuAAC) bioconjugation, but require no catalyst. The same SPAAC chemistry was used to immobilize azide-modified proteins onto surfaces, using surface-bound DIBO-ODN as a heterobifunctional linker. Cu-free click bioconjugation of proteins to ODNs is a simple and versatile alternative to Cu-catalyzed click methods.

Keywords

Click chemistry; Bioconjugation; Protein-oligodeoxynucleotide conjugates; Protein prenylation; Protein immobilization

1. Introduction

Protein-oligonucleotide conjugates have been used in a variety of biological and biotechnological applications.^{1,2} Oligodeoxynucleotide (ODN) tags have been attached to proteins in order to encode multiplexed protein libraries,³ to immobilize proteins to surfaces in the fabrication of protein arrays,^{4,5} and to identify targets in protein- and immuno-diagnostics via PCR⁶ or mass spectroscopy.⁷ Conjugating antisense ODNs to proteins has

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*Corresponding author. Tel.: +1-612-626-4681; fax: +1-612-626-7541; taton@umn.edu.

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Supplementary data

Supplementary data associated with this article can be found in the online version.

been shown to enhance cellular delivery of the nucleic acid component.⁸ In addition, ODNs attached to proteins have been used as architectural tools, as a means of spatially organizing multiple proteins into multimeric assemblies.^{9–13} Different strategies have been used to connect proteins to ODNs, using both noncovalent and covalent chemistries. Synthesizing discrete protein-ODN conjugates via noncovalent (e.g., biotin-streptavidin⁵ and nickel-histidine¹⁴) interactions is convenient, but the resulting conjugates can reversibly dissociate during purification and subsequent handling. Covalent protein-ODN conjugates have also been synthesized by connecting appropriately functionalized ODNs and proteins, using a wide variety of chemistries.¹ Some of these chemistries involve direct attachment of the ODN to an amino acid residue on the protein surface—usually a lysine amine¹⁵ or a cysteine thiol¹⁶—while other, more complex schemes require post-translational modification of the protein,^{10,17} or the involvement of a catalytic or reactive protein domain.^{18–21}

In all of these cases, the amounts of the biomolecule components used are typically small, and so conjugation is only efficient when the reaction rate is high, and when the reacting groups are indefinitely stable. Conjugation efficiency is improved even further if the reaction is bioorthogonal—if the reaction conditions do not also modify other functional groups found in the biomolecules. Copper-catalyzed azide-alkyne [3+2] cycloaddition²² (CuAAC, a “click” reaction²³) satisfies many of these criteria. Alkynes and azides are not typically found in biological molecules, and Cu-catalyzed click chemistry is fast and selective, making this method attractive for bioconjugate chemistry.^{24,25} We have previously used Cu-catalyzed azide-alkyne cycloaddition to attach post-translationally modified proteins to solid surfaces,²⁶ dye molecules¹⁰ and ODNs.¹⁰ In this past work, azides or alkynes were introduced into proteins via enzymatic prenylation of a C-terminal CVIA tag, using prenylazide diphosphates as alternative enzyme substrates. Subsequent reaction with alkyne- or azide-modified surfaces, small molecules or biomolecule targets yielded protein conjugates with high yield.

Nevertheless, applying CuAAC to bioconjugate chemistry has some important limitations. The Cu(II) that is used in many CuAAC protocols is known to oxidize amino acid sidechains²⁷ and contribute to DNA cleavage.^{28,29} The Cu-catalyzed reaction also requires optimization of metal, ligand, and auxiliary reductant concentrations. As a result, researchers have worked to apply alternative, Cu-free azide-alkyne cycloaddition reactions to biomolecules.³⁰ Strain-promoted [3+2] azide-alkyne cycloaddition (SPAAC),³⁰ for example, links azide- and cycloalkyne-modified biomolecules under catalyst-free reaction conditions and has found many applications in biotechnology^{31–44}. Researchers have developed a number of strained cycloalkynes for this reaction.^{34,45–49} Recently, we synthesized ODNs modified with one of these strained alkynes, dibenzocyclooctyne (DIBO), using solid-phase oligonucleotide synthesis (SPOS). We then showed that the resulting DIBO-modified ODNs reacted via SPAAC with organic azides with high yield and specificity.⁵⁰

In this article, we describe the synthesis of protein-ODN conjugates via SPAAC between DIBO-modified ODNs and prenylazide-functionalized proteins. Proteins engineered to contain a C-terminal CVIA tag were enzymatically prenylated with an artificial, azide-containing substrate to yield azidoprenylated proteins. Then, protein-ODN conjugates were synthesized by clicking the azidoprenylated proteins to DIBO-modified ODNs. We compare this Cu-free procedure to the Cu-catalyzed bioconjugation of the same proteins to ODNs bearing a simple, terminal alkyne. Finally, we demonstrate that the Cu-free SPAAC reaction can be used to click azidoprenylated proteins to surfaces, using DIBO-modified ODNs as attachment points. Overall, we find that Cu-free protocols for ODN-protein bioconjugates are comparable to Cu-catalyzed reactions in yield, speed and simplicity.

2. Materials and methods

2.1. Materials

Tris(2-carboxyethyl)phosphine (TCEP; Invitrogen, Carlsbad, CA), CuSO₄ (Mallinckrodt Baker, Phillipsburg, NJ), tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA; Aldrich, Milwaukee, WI), and *N*-hydroxysuccinimide-Sepharose 4 Fast Flow (Sigma, St. Louis, MO) were used as received. DIBO-phosphoramidite **1**,⁵⁰ 10-undecyn-1-yl-(2-cyanoethyl)-(N,N-diisopropyl)phosphoramidite **2**,¹⁰ 1-azido-6,7-dihydrogeranyl diphosphate **3(C₁₀)**,⁵¹ and 1-azido-10,11-dihydrofarnesyl diphosphate **3(C₁₅)**²⁶ were synthesized as described previously. Analytical and preparative size-exclusion HPLC was carried out using a Beckman model 125/168 instrument equipped with a UV-vis detector, ABI Analytical Spectroflow 980 fluorescence detector, and a Superdex 200 10/300 GL (GE Healthcare Life Sciences, Piscataway, NJ). Analytical reverse-phase HPLC was carried out using an Agilent 1100 instrument equipped with a UV-vis detector and a Microsorb 300 C₁₈ column (10 μm particle size; Varian, Palo Alto, CA). Samples were run using a two-solvent gradient between 0.03 M triethylammonium acetate (TEAA) buffered H₂O and 95:5 CH₃CN:0.03 M TEAA-buffered H₂O as the mobile phase. All buffers, including 0.1 M NaCl phosphate buffer (50 mM NaH₂PO₄, 0.1 M NaCl, pH 7.3) and 1.0 M NaCl phosphate buffer (50 mM NaH₂PO₄, 1.0 M NaCl, pH 7.3) were prepared in ultrapure deionized water (*R* > 18 MΩ).

2.2. Construction, overexpression and purification of ScFTase

To simplify the purification and improve the yield of recombinant *Saccharomyces cerevisiae* farnesyltransferase (ScFTase), a new expression plasmid for 6×His-tagged ScFTase was constructed using the pCDFDuet-1 vector (Novagen). The genes for the α and β subunits of ScFTase were amplified by standard PCR methods using Platinum Taq HiFi DNA Polymerase (Invitrogen) from a plasmid containing the cDNAs. The β subunit was amplified using the primers 5'-AATTCGGATTGCACATATGCGACAGAGAGTAGGAAG GTCCATAGC-3' (*Nde*I restriction site underlined) and 5'-TAGTAGGAACCTAGGTTAACTTGGAGAAGATAAATT GGATTTAAAGTAGTGG-3' (*Avr*II restriction site underlined), and the α subunit was amplified using the primers 5'-AGCGATTGCAGGATCCAATGGAGGAGTACGATTATT CAGACGTTAAACCTTTG-3' (*Bam*HI restriction site underlined) and 5'-GTAGTAGGAAGCGGCCGC TCAGTTCAG ATTATTTATTTGTGGTGCCACAAG-3' (*Not*I restriction site underlined). The α and β subunits were subcloned into pCDFDuet-1 multiple cloning sites I and II, respectively, using the restriction enzymes indicated above. In-frame cloning of the α subunit into multiple cloning site I of the plasmid resulted in the N-terminal, 6xHis-containing tag MGSSHHHHHSQDP preceding the wild-type ScFTase α subunit sequence. DNA sequencing (Duke University DNA Analysis Facility) confirmed correct construction of the vector.

The resulting plasmid was transformed into BL21[*DE3*] cells and grown on an LB-streptomycin (50 μg/mL) agar plate. A single colony was used to inoculate 100 mL of LB broth supplemented with streptomycin (50 μg/mL) and cells were allowed to grow overnight at 37 °C overnight under shaking at 250 rpm. The overnight cell culture was transferred to six 1 L flasks containing LB-streptomycin (50 μg/mL) and cells were allowed to grow until OD₆₀₀ reached 1.2. The overexpression of 6xHis-yPFTase was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) and ZnSO₄ (0.5 mM) for 4 h at 37 °C under shaking at 250 rpm. The cells were harvested by centrifuging at 5000 × *g* for 10 min at 4 °C. The cells were lysed, sonicated for 5 min using a 10-s “on” and 10-s “off” cycle. The lysis solution was centrifuged at 26,000 × *g* for 1 h followed by purification by affinity chromatography using Ni-NTA resin. The enzymatic activity of the ScFTase was

determined by a farnesylation reaction of dansyl-GCVIA (2.4 μ M, $\lambda_{\text{ex}} = 340$ nm, $\lambda_{\text{em}} = 505$ nm).²⁶

2.3. Synthesis of alkyne-functionalized ODNs

DNA synthesis reagents were purchased from Glen Research (Sterling, VA). ODNs **4a** and **4b** were made by standard solidphase oligonucleotide synthesis on a Perseptive Biosystems Expedite 8909 from controlled-pore glass (CPG, 500 Å, 1 μ mol scale). 3'-Amine-modified ODNs were synthesized from amino-modifier C7 CPG (Glen Research). DIBO-modified ODNs (Scheme 1; **DIBO-4a**, **DIBO-4b**, and **DIBO-4a-NH₂**) and terminal-alkyne-modified ODNs (**HC $\equiv$ C-4a** and **HC $\equiv$ C-4b**) were synthesized by manually coupling either alkyne phosphoramidite **1** or **2**, respectively, to the 5' end of the synthesized ODN on solid phase, followed by deprotection, cleavage from support, and reverse-phase (C₁₈) HPLC purification.^{10,50} Purified fractions corresponding to alkyne-modified ODNs were collected, lyophilized to dryness, and redissolved in water. The concentration of each ODN was determined from its optical absorbance at 260 nm ($\epsilon_{4a/b,260} = 2.4 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$).⁵² All DNA samples (15 μ L) were mixed with 5 μ L of 6X loading buffer (0.25% bromophenol blue and 40% sucrose in deionized water) and analyzed using a 1.5% agarose gel in 1X TBE buffer at 80 V for 1 h. Gels were imaged for green fluorescence using a Molecular Dynamics Storm 840 phosphorimager (Amersham Biosciences, Piscataway, NJ), or for red fluorescence using a Molecular Imager FX (BioRad, Hercules, CA), or were stained with ethidium bromide (EtBr) and imaged for blue fluorescence using the Molecular Imager FX.

2.4. Synthesis of C-terminal CVIA-tagged proteins

The overexpression and purification of **eGFP-CVIA** and **mCherry-CVIA** were performed as reported previously.^{26,53} Purified proteins were concentrated using Centricon centrifugal filters (MWCO 10,000 Da) and exchanged in 0.1 M NaCl phosphate buffer. The concentration of protein was determined from its absorbance at 488 nm ($\epsilon_{\text{eGFP},488} = 55,000 \text{ M}^{-1} \text{ cm}^{-1}$) for eGFP⁵⁴ and 587 nm ($\epsilon_{\text{mCherry},587} = 72,000 \text{ M}^{-1} \text{ cm}^{-1}$) for mCherry.⁵⁵ CVIA-tagged glucose-dependent insulinotropic polypeptide (**GIP-CVIA**) was generated by chemical synthesis on solid phase as previously reported.⁵⁶

2.5. Synthesis of azide-functionalized proteins

eGFP-CVIA, **mCherry-CVIA** and **GIP-CVIA** were enzymatically prenylated with **3(C₁₀)** or **3(C₁₅)** as described previously.²⁶ To attach an azidogeranyl group, a 10 mL solution of either **eGFP-CVIA** or **mCherry-CVIA** (2.4 μ M), DTT (5 mM), MgCl₂ (10 mM), ZnCl₂ (10 μ M), Tris-HCl (50 mM, pH 7.5), **3(C₁₀)** (40 μ M) and ScFTase (150 nM) was incubated at 30 °C for 2 h. To attach an azidofarnesyl group, **3(C₁₅)** was used in place of **3(C₁₀)**, the amount of ScFTase was reduced (10 nM), and the solution was incubated at room temperature for 4 h. After completion of the reaction, the mixture was concentrated to ~500 μ L using Centricon centrifugal filters (MWCO 10,000 Da). The excess azide was removed by gel filtration chromatography through a NAP-5 column (GE Healthcare), and eluted with 0.1 M NaCl phosphate buffer to yield prenylated proteins **eGFP-(C₁₀)N₃**, **eGFP-(C₁₅)N₃**, **mCherry-(C₁₀)N₃**, **mCherry-(C₁₅)N₃**, and **GIP-(C₁₅)N₃**. The concentrations of azide-functionalized eGFP and mCherry were determined from sample absorbance, using the extinction coefficients for the unmodified proteins listed above.

Azide-modified HIV nucleocapsid protein (**NC-N₃**) was synthesized on solid phase by a modification of a published procedure.⁵⁷ To resin-bound, side-chain protected NC(11–55)⁵⁷ was added a solution of Fmoc-mini-PEG (0.04 mmol, 15.4 mg), Bop (0.04 mmol, 17.7 mg) and DIEA (0.08 mmol, 13.9 μ L) dissolved in DMF (1.5 mL). After 5 h reaction, the resin was washed with DMF and the Fmoc group removed with 20% piperidine in DMF. After

washing with DMF, this coupling/deprotection sequence was repeated. Then 4-azidomethylbenzoic acid (0.04 mmol, 7.1 mg), Bop (0.04 mmol, 17.7 mg), and DIEA (0.08 mmol, 13.9 μ L) dissolved in DMF (1.5 mL) were added to the resin. The resin was allowed to tumble 24 h, after which it was washed with DMF, CH_2Cl_2 , and dried *in vacuo*. The resin was cleaved with freshly prepared reagent K^{58} for 2.5 h. The resulting, 4-azidomethylbenzoate-(mini-PEG)₂-NC(11–55) peptide was precipitated with ether and centrifuged to form a pellet, which was washed twice with ether. The pellet was dissolved in 0.1% aqueous TFA, filtered, and purified by preparative HPLC. Yield: 11.4 mg, T_r = 29.0 min, purity by RP-HPLC: 98%, deconvoluted ESI-MS: calculated 5684.7, found 5684.6.

2.6. Synthesis of dibenzocyclooctyltriazole (DBTrz)-linked protein-ODN conjugates by Cu-free SPAAC

Azide-functionalized protein (Scheme 2; 14 μ M) was combined with a DIBO-modified ODN (**DIBO-4a** or **DIBO-4b**, respectively; 4.6 μ M) in H_2O or 0.1 M NaCl phosphate buffer unless otherwise indicated, and incubated at room temperature overnight with gentle shaking in the dark. The crude reaction mixture was analyzed by agarose gel electrophoresis (1.5% agarose). Gels were directly imaged for red or green fluorescence in the case of **eGFP-DBTrz-4a** and **mCherry-DBTrz-4b**. Gels were stained with ethidium bromide as described above for DNA, in order to image the DNA component of the conjugate.

The crude conjugate was concentrated to ~ 100 μ L, and each protein-DNA conjugate was purified from unreacted starting materials via size exclusion HPLC (0.5 mL/min 50 mM NaH_2PO_4 , pH 7.3, 1 M NaCl). The multi-wavelength HPLC diode array detector was set to monitor the elution of molecules containing DNA ($\lambda_{\text{abs}} = 260$ nm) and, in the case of eGFP and mCherry, protein ($\lambda_{\text{abs}} = 488$ nm for eGFP, $\lambda_{\text{abs}} = 587$ nm for mCherry) components. The fluorescence detector was set to monitor fluorescent protein ($\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 510$ nm for eGFP; $\lambda_{\text{ex}} = 588$ nm, $\lambda_{\text{em}} = 610$ nm for mCherry). Fractions containing both DNA and protein signals, corresponding to protein-ODN conjugates, were collected, concentrated using Centricon ultracentrifugal filters (MWCO 3000 Da), and finally exchanged with 0.1 M NaCl phosphate buffer. The concentration of each protein-DNA conjugate was determined from its absorbance at $\lambda = 488$ nm (for **eGFP-DBTrz-4a**) or $\lambda = 587$ nm (for **mCherry-DBTrz-4b**).

2.7. Synthesis of triazole (Trz)-linked protein-ODN conjugates by CuAAC

Cu-catalyzed click chemistry was used to synthesize eGFP- and mCherry-ODN conjugates using a modification of a previously published protocol.¹⁰ In short, **eGFP-(C₁₀)N₃** or **mCherry-(C₁₀)N₃** (14 μ M) was reacted with **HC $\equiv$ C-4a** or **HC $\equiv$ C-4b** (17 μ M) in the presence of TCEP (1 mM), TBTA ligand (0.2 mM) and CuSO_4 (1 mM) for 1 h at room temperature in the dark. A reaction containing all the constituents except CuSO_4 was also run as a negative control. In order to purify the protein-ODN conjugate from unreacted ODN and proteins, the crude mixture was diluted to ~ 500 μ L, passed through a NAP-5 column, and eluted with 0.1 M NaCl phosphate buffer. The sample was concentrated to ~ 100 μ L using Centricon ultracentrifugal filters (MWCO 3000 Da). The concentrated mixture was analyzed on an agarose gel (1.5%), and the protein-ODN conjugate (**eGFP-Trz-4a** or **mCherry-Trz-4b**) was purified from unreacted protein and DNA by size exclusion chromatography as described above for DBTrz-linked conjugates.

2.8. Formation of protein-protein heterodimers by hybridization of protein-ODN conjugates

Protein-protein heterodimers containing both eGFP and mCherry were assembled by combining either DBTrz-linked or Trz-linked protein-ODN conjugates under DNA-hybridizing conditions. The annealing mixture (10 μ L) contained 1 μ M each of protein-ODN conjugate (combining **eGFP-DBTrz-4a** and **mCherry-DBTrz-4b**, or **eGFP-Trz-4a** and

mCherry-Trz-4b) in 0.1 M NaCl phosphate buffer containing 5 mM MgCl₂. The reaction mixtures were either incubated at room temperature, or annealed by heating at 55 °C for 3 min followed by cooling at 4 °C for 2 min using a PCR Sprint Thermal Cycler (Thermo Electron, Millford, MA). The reaction mixture was analyzed on a 1.5% agarose gel and imaged for red and green fluorescence, or stained in EtBr and imaged for blue fluorescence as described above.

2.9. Immobilization of proteins on Sepharose beads by Cu-free SPAAC

In a microcentrifuge tube, 50 µL of a suspension of NHS-activated Sepharose (Sigma, St. Louis, MO) was washed by diluting the suspension into 500 µL water, centrifuging at $14,000 \times g$ for 20 min at room temperature, removing supernatant, resuspending the beads in 0.1 M NaCl phosphate buffer, and allowing the beads to equilibrate in the buffer for 10 min. The beads were subjected to this wash cycle two more times. Then, 50 µL of either 10 µM **DIBO-4a-NH₂** or 10 µM **4a** (as a negative control) was added to the washed beads, and the mixture was incubated at room temperature under gentle shaking overnight. The beads were centrifuged, washed with 0.1 M NaCl phosphate buffer, and incubated with 50 mM Tris-HCl (pH 8.3) for 2 h to cap any unreacted NHS-groups on the beads.⁵⁹ The beads were centrifuged, washed with 0.1 M NaCl phosphate buffer, and incubated with 50 µL of 40 µM **eGFP-(C₁₅)N₃**, **mCherry-(C₁₅)N₃**, or **eGFP-CVIA** (as a negative control) at room temperature under gentle shaking overnight in dark. The beads were then washed with 500 µL of 1.0 M NaCl phosphate buffer three times as described above. For bead imaging, 2 µL of the bead suspension was diluted to 100 µL in deionized water, placed in a clear-bottomed microtiter plate and imaged under a PixCell II LCM inverted microscope (Arcturus Engineering, Sunnyvale, CA). Both brightfield and fluorescent images (green fluorescence for eGFP or red fluorescence for mCherry) were obtained by choosing appropriate filter settings in the microscope.

3. Results and Discussion

Previously, we showed that azide-functionalized proteins could be conjugated to 5'-alkyne-modified ODNs using Cu-catalyzed click chemistry.^{10,26} This method is simple to perform, and bioconjugation yields are typically high, but the optimal reaction conditions are not always the same from protein to protein, and some optimization is necessary. A primary goal of this work was to develop a Cu-free alternative to this method that required only the two reacting partners—protein and oligonucleotide—and thus required no optimization. To accomplish this, we combined model azidoprenylated proteins with 5'-DIBO-ODNs, which we previously showed would react with azides via SPAAC.⁵⁰ We chose fluorescent eGFP⁵⁴ and mCherry⁵⁵ as model proteins for this study, because of the ease with which they can be analyzed and imaged by fluorescence techniques. We previously engineered each of these proteins to display a C-terminal CVIA prenylation tag,^{26,53} which is recognized and prenylated by ScPFTase, a substrate-promiscuous prenyltransferase. When combined with ScPFTase and azide-functionalized prenyl diphosphate **3(C₁₀)** or **3(C₁₅)** as a substrates, CVIA-tagged eGFP and mCherry were both converted to azide-modified protein products.

As an initial test of Cu-free SPAAC reactions with these proteins, azide-modified GFP (**eGFP-(C₁₀)N₃**) was incubated with DIBO-modified oligonucleotide (**DIBO-4a**) at room temperature overnight. The reaction mixture was analyzed by non-denaturing agarose gel electrophoresis, and the gel was imaged for protein fluorescence (Fig. 1A) and for DNA (Fig. 1B). The reaction mixture (lane 3) contained, in addition to unreacted starting material, a product that had both protein and DNA components and that migrated faster than the protein alone, but slower than the ODN alone. This electrophoretic behavior is characteristic of protein-DNA conjugates⁶⁰ and complexes,⁶¹ and indicated the successful formation of

the click reaction product (**eGFP-DBTrz-4a**) between the azide-modified protein and DIBO-modified ODN. Conjugation of ODN **DIBO-4a** to **eGFP-(C₁₀)N₃** required both the DIBO and azidoprenyl groups, as confirmed by control reactions between **DIBO-4a** and non-prenylated **eGFP-CVIA**, and between unmodified ODN **4a** and **eGFP-(C₁₀)N₃**. Neither of these control experiments generated new product bands in agarose gel analysis (data not shown). This indicates that the observed band was indeed the result of a click reaction, and not due to non-specific binding of **DIBO-4a** to the protein.

To show that the change in electrophoretic behavior was due to an attached, single-stranded DNA (ssDNA) molecule, we incubated **eGFP-DBTrz-4a** with an unmodified oligonucleotide (**4b**) that was complementary to the attached sequence **4a**, under DNA hybridization conditions. The resulting double-stranded complex was likewise analyzed by gel electrophoresis (Fig. 1A–B, lane 4). The double-stranded DNA (dsDNA) conjugate migrated faster than the ssDNA conjugate, and absorbed the dsDNA-binding EtBr dye more strongly. These results are consistent with hybridization of **4b** to eGFP-bound **4a**, and confirmed the success of the click conjugation reaction.

In principle, the Cu-free click bioconjugation reaction described here should be applicable to any protein, as long as the protein bears a C-terminal CVIA tag and can be efficiently prenylated by PFTase. To test the versatility of SPAAC bioconjugation, we expressed and purified another model protein, **mCherry-CVIA**, and subjected to the same enzymatic azidoprenylation and click conjugation as **eGFP-CVIA**. The resulting azide-modified protein, **mCherry-(C₁₀)N₃**, was reacted with ODN **DIBO-4b**, and the reaction mixture was analyzed by agarose gel electrophoresis (Fig. 2, lane 5). Again, a new band was observed that migrated faster than **mCherry-(C₁₀)N₃**, but slower than **DIBO-4b**, corresponding to the clicked bioconjugate **mCherry-DBTrz-4b**. A control reaction containing clickable oligonucleotide **DIBO-4b** and unmodified **mCherry-CVIA** failed to show a similar band (Fig. 2, lane 4). We found that the click reaction succeeded for a variety of proteins containing prenyl (C₁₀), geranylgeranyl (C₁₅), and non-lipid azides. To test this, we conducted copper-free click conjugation reactions between **DIBO-4a** and five other model, azide-modified proteins: **eGFP-(C₁₅)N₃**, **mCherry-(C₁₅)N₃**, **NC-N₃**, **GIP-(C₁₅)N₃**, and a hexahistidine-tagged **His₆-eGFP-(C₁₅)N₃**. We analyzed all of these reactions by gel electrophoresis (Fig. 3). In each case, we observed new, slower-migrating bands corresponding to clicked protein-ODN conjugates. Reactions performed at low pH (**NC-N₃** + **DIBO-4a**, 0.1 M PBS pH 5) and high pH (**GIP-(C₁₅)N₃** + **DIBO-4a**, 0.1 M PBS pH 11) yielded identical conjugate product bands by gel electrophoresis (data not shown), indicating that the reaction can accommodate proteins with different isoelectric points at different pH values.

The success of any protein bioconjugate reaction depends in part on how quickly the reaction can be completed, and whether proteins are stable to the conditions and duration of the reaction. In all of the click reactions shown above, product bands were observed by gel electrophoresis within 1 h of the start of the reaction (data not shown). This was reasonable given the reagent concentrations used in these experiments; assuming the same bimolecular rate constant previously measured for the reaction between DIBO and azides ($0.9\text{--}2.3\text{ M}^{-1}\text{ s}^{-1}$),^{48,50} we calculated that the test reaction would be ~6% complete after 1 h. In order to further evaluate the conditions and kinetics of SPAAC for protein modification, we monitored the reaction between **eGFP-(C₁₅)N₃** and **DIBO-4a** by reverse-phase HPLC, and tracked the amount of unreacted **eGFP-(C₁₅)N₃** over time (Fig. 4). Proteins frequently need to be handled below room temperature, and so we conducted kinetic experiments both at room temperature and at 4 °C. Both reactions could be fit to second-order reaction kinetics, and gave second-order rate constants that were similar to those measured previously for SPAAC reactions. The lower-temperature kinetic data makes it clear that SPAAC

bioconjugation reactions at 4 °C would require days to complete, but would still succeed. A variety of strategies for more rapid, low-temperature conjugation of proteins to DNA have been described, including photoconjugation methods.⁶³ Nevertheless, given the simplicity of SPAAC, we argue that this approach could still be useful at temperatures commonly used for proteins.

Protein-ODN conjugates could be analyzed and purified from reaction mixtures by size exclusion chromatography (Fig. 5A–B). In the case of **eGFP-DBTrz-4a** and **mCherry-DBTrz-4b**, each eluted biomolecule was identified by simultaneous UV-vis absorbance and fluorescence detection. The extinction coefficients of the ODNs at $\lambda = 260$ nm were comparable to the extinction coefficients of the protein chromophores at visible wavelengths, such that both components could be monitored by the SEC absorbance detector. Eluting eGFP and mCherry proteins were also detected by their characteristic fluorescence signatures. In the SEC of each conjugation reaction, the protein-ODN conjugate—identified by detector signals corresponding to both protein and ODN components—eluted from the SEC column first. Lower-molecular-weight, unreacted protein and ODN molecules, with their independent detector signatures, eluted later. The ratio of fluorescence signal to visible-wavelength absorbance was the same for the conjugate and unreacted protein, indicating that neither the click reaction, nor the attachment of DNA, quenched the fluorescence of eGFP. The overlapping peaks in the SEC absorbance traces were deconvoluted by fitting the spectrum to Gaussian functions; based on the integrals of the peak fits, we estimate that 32% of the starting **eGFP-(C₁₀)N₃**, and 9% of the **mCherry-(C₁₀)N₃**, was conjugated to DIBO-functionalized DNA after 24 h. Both reactions were conducted with excess protein, and so these conversions corresponded to the click reactions proceeding to 95% (for eGFP) and 27% (for mCherry) completion. It is not clear why the click reaction was less efficient for **mCherry-(C₁₀)N₃** than for **eGFP-(C₁₀)N₃**, but both SPAAC reactions successfully generated the desired protein-ODN conjugate. Fractions collected from an SEC purification run on **eGFP-DBTrz-4a** were further analyzed by agarose gel electrophoresis (Fig. 5C). A fraction collected at $t = 31$ min, corresponding to the earliest SEC peak, showed a major, fast-migrating band in the agarose gel corresponding to the protein-DNA conjugate (Fig. 5C, lane 1). A fraction collected at $t = 35$ min, on the other hand, gave primarily a slow-migrating band in the agarose gel, corresponding to unreacted **eGFP-(C₁₀)N₃** (Fig. 5C, lane 2). Together with the SEC traces, these gel electrophoresis experiments confirmed the identity of the eGFP-ODN conjugate.

Cu-catalyzed click reactions are now extensively used in bioconjugate chemistry, and so to assess the relative utility of SPAAC bioconjugation, we compared the synthesis of protein-ODN conjugates via SPAAC with that performed by Cu-catalyzed azide-alkyne cycloaddition (CuAAC). To synthesize eGFP/mCherry-ODN conjugates via CuAAC, we followed a previously reported procedure using alkyne phosphoramidite **2**.¹⁰ Alkyne-modified oligos (**HC≡C-4a** and **HC≡C-4b**) were reacted with protein azides (**eGFP-(C₁₀)N₃** and **mCherry-(C₁₀)N₃**, respectively) to obtain triazole-linked eGFP- and mCherry-ODN conjugates **eGFP-Trz-4a** and **mCherry-Trz-4b**. Agarose gel electrophoresis analysis of each unpurified CuAAC reaction mixture showed similar results as the SPAAC reactions described above, with a faster-moving band corresponding to the protein-DNA product (Fig. 6A, lanes 4 and 6) and a slower-moving band for unreacted protein (Fig. 6A, lane 2). Control reactions between alkyne-modified ODNs and modified proteins in the absence of CuSO₄ failed to show a similar product band in the agarose gel analysis (Fig. 6, lanes 3 and 5), consistent with our past work on CuAAC-generated protein-ODN conjugates.¹⁰ Triazole-linked protein-DNA conjugates were further separated by size exclusion chromatography, following the same procedure described above for DIBO-linked conjugates (Fig. 6B–C). Retention times for CuAAC-generated conjugates were similar to those prepared by SPAAC. From the SEC chromatograms, we estimate that CuAAC reaction of **eGFP-**

(C₁₀)N₃ went 90% to completion after 24 h, and reaction of **mCherry-(C₁₀)N₃** went 81% to completion. These results suggest that both SPAAC and CuAAC can be utilized to synthesize protein-DNA conjugates, with comparable success.

Attaching ODNs to proteins allows the resulting conjugates to be organized into supramolecular assemblies and attached to surfaces via hybridization of the DNA.⁶⁴ For example, we have previously demonstrated the applicability of DNA-mediated assembly of proteins by using protein-ODN conjugates synthesized by CuAAC.¹⁰ In order to confirm that SPAAC-generated protein-ODN conjugates could likewise be used for this application, we attempted to assemble the simplest supramolecular structure: a protein heterodimer, with the protein units connected by hybridized, complementary DNA sequences. The complementary SPAAC bioconjugates **eGFP-DBTrz-4a** and **mCherry-DBTrz-4b** were combined at room temperature, or annealed at 55 °C for 3 min followed by cooling at 4 °C for 2 min, in 0.1 M NaCl—both protocols that are commonly used to hybridize complementary DNA sequences. Non-denaturing, agarose gel electrophoresis analysis of the mixtures showed that the SPAAC-generated conjugates did indeed hybridize to form a slower-moving, product band in the gel, but only under the hightemperature (55 °C) annealing conditions (Fig. 7A–C). By contrast, mixtures of CuAAC-generated conjugates showed the same heterodimer band when mixed not just at high temperature, but also at room temperature (Fig. 7D–E). In both cases, the new product bands were observed in both the red and green fluorimager channels, indicating the presence of both mCherry and eGFP in each heterodimer. SDS-PAGE analysis of the hybridization mixtures, which would be expected to denature the proteins but not the DNA, likewise showed the presence of a product band at a molecular weight approximately equal to sum of the masses of the individual protein-DNA conjugates (data not shown). It is not clear why conjugates prepared by these two different click chemistries exhibited slightly different hybridization behavior. The DBTrz group that links the protein and ODN in the SPAAC bioconjugate is more hydrophobic than the Trz linker in the CuAAC product, and it may be that this DBTrz group impedes DNA hybridization at low temperatures. Nevertheless, these results demonstrate the viability of both Cu-mediated and Cu-free click reaction strategies to assemble DNA-templated, multimeric protein structures without altering the proteins' properties.

In principle, catalyst-free click chemistry can improve bioconjugate chemistry not just in solution, but also at surfaces, where achieving reactive concentrations of participating species can be challenging.^{42,44,65} In the case of attaching proteins to surfaces, forcing the surface reaction to completion by increasing the concentration of the protein reactant often leads to nonspecific protein adsorption and protein inactivation. One way to address this problem is to passivate the surface with macromolecules that inhibit non-specific adsorption, but still possess reactive groups that permit specific bioconjugate chemistry. This is often accomplished with synthetic polymers,⁶⁶ but ODNs have also been used.^{59,67} Motivated by this past work, and also our own demonstration of CuAAC-mediated surface immobilization of azidoprenylated proteins,^{10,26} we attempted to use SPAAC to specifically attach proteins to ODN-functionalized surfaces. First, ODN **DIBO-4b-NH₂** was synthesized using 3'-amino-modifier CPG and DIBO phosphoramidite. This ODN was then incubated with Sepharose beads bearing *N*-hydroxysuccinimidyl ester groups, in order to tether the ODNs to the bead surfaces at their 3'-ends. Finally, these 5'-DIBO-ODN-functionalized beads were incubated with either **eGFP-(C₁₅)N₃** or **mCherry-(C₁₅)N₃** to click the proteins to the Sepharose surface. Fluorescence microscope images of the beads demonstrated that the fluorescent proteins were bound to the bead surfaces (Fig. 8B/G, C/H). Control experiments, either using a non-clickable oligonucleotide (lacking DIBO) or a non-clickable eGFP (lacking azide) showed no discernable adsorption of the protein onto the beads (Fig. 8D/I, E/J). The reaction conditions of protein immobilization by SPAAC on agarose beads were comparable to those used in CuAAC reactions,^{10,26} but no lower concentrations of

azidoprenylated protein were required for SPAAC.²⁶ In principle, SPAAC immobilization of proteins on surfaces could be a useful alternative to CuAAC when a limited amount of protein is available.

4. Conclusions

In summary, we have demonstrated the utility of SPAAC reaction for site-specific attachment of DNA to two model proteins. The efficiency of the SPAAC reaction was found to be comparable to that of the Cu-catalyzed click reaction. In addition, the SPAAC bioconjugation method described herein proceeds without Cu catalyst and does not affect the activity of the protein and DNA involved. Moreover, in comparison to other fusion protein methodologies, the method described here requires a shorter recognition tag (C-terminal CVIA). We envision that our method can be an excellent alternate route to Cu-catalyzed click reaction for synthesizing myriads of protein-DNA conjugates with structural and functional importance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References and notes

1. Niemeyer CM. Angew. Chem. Int. Ed. 2010; 49:1200.
2. Niemeyer CM. Trends Biotechnol. 2002; 20:395. [PubMed: 12175771]
3. Bailey RC, Kwong GA, Radu CG, Witte ON, Heath JR. J. Am. Chem. Soc. 2007; 129:1959. [PubMed: 17260987]
4. Niemeyer CM, Boldt L, Ceyhan B, Blohm D. Anal. Biochem. 1999; 268:54. [PubMed: 10036162]
5. Niemeyer CM, Sano T, Smith CL, Cantor CR. Nucleic Acids Res. 1994; 22:5530. [PubMed: 7530841]
6. Sano T, Smith C, Cantor C. Science. 1992; 258:120. [PubMed: 1439758]
7. Zhang L, Kasif S, Cantor RC. Proc. Natl. Acad. Sci. U. S. A. 2007; 104:3061. [PubMed: 17360609]
8. Rajur SB, Roth CM, Morgan JR, Yarmush ML. Bioconjugate Chem. 1997; 8:935.
9. Diezmann F, Seitz O. Chem. Soc. Rev. 2011; 40:5789. [PubMed: 21589953]
10. Duckworth BP, Zhang Z, Hosokawa A, Distefano MD. ChemBioChem. 2007; 8:98. [PubMed: 17133644]
11. Park SH, Yin P, Liu Y, Reif JH, LaBean TH, Yan H. Nano Lett. 2005; 5:729. [PubMed: 15826117]
12. Yan H, Park SH, Finkelstein G, Reif JH, LaBean TH. Science. 2003; 301:1882. [PubMed: 14512621]
13. Chhabra R, Sharma J, Ke Y, Liu Y, Rinker S, Lindsay S, Yan H. J. Am. Chem. Soc. 2007; 129:10304. [PubMed: 17676841]
14. Goodman RP, Erben CM, Malo J, Ho WM, McKee ML, Kapanidis AN, Turberfield AJ. ChemBioChem. 2009; 10:1551. [PubMed: 19449345]
15. Jablonski E, Moomaw EW, Tullis RH, Ruth JL. Nucleic Acids Res. 1986; 14:6115. [PubMed: 3748805]
16. Corey D, Schultz P. Science. 1987; 238:1401. [PubMed: 3685986]

17. Ou W, Uno T, Chiu H, Grünewald J, Cellitti SE, Crossgrove T, Hao X, Fan Q, Quinn LL, Patterson P, Okach L, Jones DH, Lesley SA, Brock A, Geierstanger BH. *Proc. Natl. Acad. Sci. U. S. A.* 2011; 108:10437. [PubMed: 21670250]
18. Lovrinovic M, Seidel R, Wacker R, Schroeder H, Seitz O, Engelhard M, Goody RS, Niemeyer CM. *Chem. Comm.* 2003:822. [PubMed: 12739629]
19. Takeda S, Tsukiji S, Nagamune T. *Bioorg. Med. Chem. Lett.* 2004; 14:2407. [PubMed: 15109622]
20. Jongsma MA, Litjens RH. *Proteomics.* 2006; 6:2650. [PubMed: 16596705]
21. Tominaga J, Kemori Y, Tanaka Y, Maruyama T, Kamiya N, Goto M. *Chem. Commun.* 2007:401.
22. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. *Angew. Chem. Int. Ed.* 2002; 41:2596.
23. Kolb HC, Finn MG, Sharpless KB. *Angew. Chem. Int. Ed.* 2001; 40:2004.
24. Baskin JM, Bertozzi CR. *QSAR Comb. Sci.* 2007; 26:1211.
25. Best MD. *Biochemistry.* 2009; 48:6571. [PubMed: 19485420]
26. Duckworth BP, Xu J, Taton TA, Guo A, Distefano MD. *Bioconjugate Chem.* 2006; 17:967.
27. Requena JR, Chao C, Levine RL, Stadtman ER. *Proc. Natl. Acad. Sci. U. S. A.* 2001; 98:69. [PubMed: 11120890]
28. Burrows CJ, Muller JG. *Chem. Rev.* 1998; 98:1109. [PubMed: 11848927]
29. Sigman DS, Mazumder A, Perrin DM. *Chem. Rev.* 1993; 93:2295.
30. Becer CR, Hoogenboom R, Schubert US. *Angew. Chem. Int. Ed.* 2009; 48:4900.
31. Kele P, Mezö G, Achatz D, Wolfbeis OS. *Angew. Chem. Int. Ed.* 2009; 48:344.
32. Beatty KE, Fisk JD, Smart BP, Lu YY, Szychowski J, Hangauer MJ, Baskin JM, Bertozzi CR, Tirrell DA. *ChemBioChem.* 2010; 11:2092. [PubMed: 20836119]
33. Jewett JC, Bertozzi CR. *Chem. Soc. Rev.* 2010; 39:1272. [PubMed: 20349533]
34. Baskin JM, Prescher JA, Laughlin ST, Agard NJ, Chang PV, Miller IA, Lo A, Codelli JA, Bertozzi CR. *Proc. Natl. Acad. Sci. U. S. A.* 2007; 104:16793. [PubMed: 17942682]
35. Dommerholt J, Schmidt S, Temming R, Hendriks LJA, Rutjes FPJT, van Hest JCM, Lefebvre DJ, Friedl P, van Delft FL. *Angew. Chem. Int. Ed.* 2010; 49:9422.
36. Chang PV, Prescher JA, Sletten EM, Baskin JM, Miller IA, Agard NJ, Lo A, Bertozzi CR. *Proc. Natl. Acad. Sci. U. S. A.* 2010; 107:1821. [PubMed: 20080615]
37. Neef AB, Schultz C. *Angew. Chem. Int. Ed.* 2009; 48:1498.
38. Laughlin ST, Baskin JM, Amacher SL, Bertozzi CR. *Science.* 2008; 320:664. [PubMed: 18451302]
39. Dieterich DC, Hodas JLL, Gouzer G, Shadrin IY, Ngo JT, Triller A, Tirrell DA, Schuman EM. *Nat. Neurosci.* 2010; 13:897. [PubMed: 20543841]
40. DeForest CA, Sims EA, Anseth KS. *Chem. Mater.* 2010; 22:4783. [PubMed: 20842213]
41. Ornelas C, Broichhagen J, Weck M. *J. Am. Chem. Soc.* 2010; 132:3923. [PubMed: 20184364]
42. Bernardin A, Cazet AL, Guyon L, Delannoy P, Vinet F, Bonnaffé D, Texier I. *Bioconjugate Chem.* 2010; 21:583.
43. Canalle LA, van Berkel SS, de Haan LT, van Hest JCM. *Adv. Funct. Mater.* 2009; 19:3464.
44. Kuzmin A, Poloukhine A, Wolfert MA, Popik VV. *Bioconjugate Chem.* 2010; 21:2076.
45. Agard NJ, Prescher JA, Bertozzi CR. *J. Am. Chem. Soc.* 2004; 126:15046. [PubMed: 15547999]
46. Codelli JA, Baskin JM, Agard NJ, Bertozzi CR. *J. Am. Chem. Soc.* 2008; 130:11486. [PubMed: 18680289]
47. Jewett JC, Sletten EM, Bertozzi CR. *J. Am. Chem. Soc.* 2010; 132:3688. [PubMed: 20187640]
48. Ning X, Guo J, Wolfert MA, Boons G-J. *Angew. Chem. Int. Ed.* 2008; 47:2253.
49. van Delft P, Meeuwenoord NJ, Hoogendoorn S, Dinkelaar J, Overkleef HS, van der Marel GA, Filippov DV. *Org. Lett.* 2010; 12:5486. [PubMed: 21049910]
50. Marks IS, Kang JS, Jones BT, Landmark KJ, Cleland AJ, Taton TA. *Bioconjugate Chem.* 2011; 22:1259.
51. Xu J, DeGraw AJ, Duckworth BP, Lenevich S, Tann C-M, Jenson EC, Gruber SJ, Barany G, Distefano MD. *Chem. Biol. Drug Des.* 2006; 68:85. [PubMed: 16999773]
52. Tataurov AV, You Y, Owczarzy R. *Biophys. Chem.* 2008; 133:66. [PubMed: 18201813]

53. Chung JA, Wollack JW, Hovlid ML, Okesli A, Chen Y, Mueller JD, Distefano MD, Taton TA. *Anal. Biochem.* 2009; 386:1. [PubMed: 18834849]
54. Heim R, Cubitt AB, Tsien RY. *Nature.* 1995; 373:663. [PubMed: 7854443]
55. Shaner NC, Campbell RE, Steinbach PA, Giepmans BNG, Palmer AE, Tsien RY. *Nat. Biotech.* 2004; 22:1567.
56. Rashidian M, Song JM, Pricer RE, Distefano MD. *J. Am. Chem. Soc.* 2012
57. Liu H-W, Cosa G, Landes CF, Zeng Y, Kovaleski BJ, Mullen DG, Barany G, Musier-Forsyth K, Barbara PF. *Biophys. J.* 2005; 89:3470. [PubMed: 16100256]
58. King DS, Fields CG, Fields GB. *Int. J. Pept. Protein Res.* 1990; 36:255. [PubMed: 2279849]
59. Zhong M, Fang J, Wei Y. *Bioconjugate Chem.* 2010; 21:1177.
60. Lovrinovic M, Niemeyer CM. *Biochem. and Biophys. Res. Commun.* 2005; 335:943. [PubMed: 16102730]
61. Hellman LM, Fried MG. *Nat. Protocols.* 2007; 2:1849.
62. Schaeffer PM, Dixon NE. *Aust. J. Chem.* 2009; 62:1328.
63. Dahdah DB, Morin I, Moreau MJJ, Dixon NE, Schaeffer PM. *Chem. Commun.* 2009:3050.
64. Feldkamp U, Niemeyer CM. *Angew. Chem. Int. Ed.* 2006; 45:1856.
65. Sawoo S, Dutta P, Chakraborty A, Mukhopadhyay R, Bouloussa O, Sarkar A. *Chem. Comm.* 2008:5957. [PubMed: 19030551]
66. Zimmermann JL, Nicolaus T, Neuert G, Blank K. *Nat. Protocols.* 2010; 5:975.
67. Boozer C, Ladd J, Chen S, Jiang S. *Anal. Chem.* 2006; 78:1515. [PubMed: 16503602]

Figure 1.

Agarose gel electrophoresis (using 1.5% agarose) of unpurified SPAAC reactions between **eGFP-(C₁₀)N₃** and ODN **DIBO-4a**. The reaction mixture was either loaded directly onto the gel (lane 3), or incubated with complementary ODN **4b** (lane 4); **eGFP-(C₁₀)N₃** alone (lane 5), the hybridized **DIBO-4a/4b** pair (lane 2), and a 1-kilobase DNA marker (lane 1) were also loaded onto the gel for comparison. The gel was imaged for green (protein) fluorescence (**A**), and then stained with ethidium bromide (EtBr) and imaged for DNA-bound EtBr fluorescence (**B**). Staining in lanes 2 and 4 of (**B**) is darker than in other lanes because EtBr is selective for dsDNA.

Figure 2.

Agarose gel electrophoresis (1.5% agarose) of an unpurified SPAAC reaction between **mCherry-(C₁₀)N₃** and ODN **DIBO-4b**. Wells were loaded with the reaction mixture (lane 5), a negative control mixture of **mCherry-CVIA** and **DIBO-4b** (lane 4), **DIBO-4b** alone (lane 3), **mCherry-(C₁₀)N₃** alone (lane 2), and a 1-kilobase DNA marker (lane 1). The gel was imaged for red fluorescence (**A**), and then stained with ethidium bromide (EtBr) and imaged for DNA-bound EtBr fluorescence (**B**). Because the fluorescence spectra of mCherry and EtBr overlap, both components are observed in (**B**).

Figure 3.

Agarose gel electrophoresis (using 1.5% agarose) of unpurified SPAAC reactions between ODN **DIBO-4a** and **eGFP-(C₁₅)N₃** (lane 2), **mCherry-(C₁₅)N₃** (lane 3), **NC-N₃** (lane 4), **GIP-(C₁₅)N₃** (lane 5), and **His₆-eGFP-(C₁₅)N₃** (lane 6). Each reaction also contained complementary ODN **4b**, which hybridizes to **4a** and enhances EtBr staining. Lane 1 contained only ODNs **DIBO-4a** and **4b** as a negative indicator. Lane 3 shows an additional slow-migrating band due to unreacted **mCherry-(C₁₅)N₃**, because mCherry fluoresces near the same wavelength as DNA-complexed EtBr.

Figure 4.

Reaction of a 1:1 molar ratio of **eGFP-(C₁₅)N₃** and **DIBO-4a**, monitored by RP-HPLC, at different temperatures. Each dataset was fit to a second-order kinetic decay function (solid lines). for these fits, $k_{25^\circ C} = 6 \text{ M}^{-1} \text{ s}^{-1}$, and $k_{4^\circ C} = 0.8 \text{ M}^{-1} \text{ s}^{-1}$. Although the reaction product (**eGFP-DBTrz-4a**) was not observed by RP-HPLC, agarose gel electrophoresis of the reaction mixture confirmed that product was formed, and that the reactions went to completion.

Figure 5. Purification of SPAAC-generated protein-ODN conjugates. (A) SEC of a crude reaction mixture intended to generate **eGFP-DBTrz-4a** product (peak *i*) from **eGFP-(C₁₀)N₃** (peak *ii*) and **DIBO-4a** (peak *iii*) starting materials. (B) SEC of a crude reaction mixture intended to generate **mCherry-DBTrz-4b** product (peak *i*) from **mCherry-(C₁₀)N₃** (peak *ii*) and **DIBO-4b** (peak *iii*) starting materials. (C) Agarose gel electrophoresis (1.5% agarose) of SEC-purified **eGFP-DBTrz-4a** conjugate (lane 1) and unreacted **eGFP-(C₁₀)N₃** (lane 2), imaged as green fluorescence.

Figure 6.

Analysis and purification of CuAAC-generated protein-ODN conjugates. (A) Agarose gel electrophoresis (1.5% agarose) of CuAAC reaction mixtures containing **eGFP-(C₁₀)N₃** and **HC≡C-4a** (lanes 3, 4) or **mCherry-(C₁₀)N₃** and **HC≡C-4b** (lanes 5, 6); reactions either contained CuSO₄ (lanes 4, 6) or were run in the absence of CuSO₄ as a negative control (lanes 3, 5). A 1-kilobase DNA marker (lane 1) and a mixture of **HC≡C-4a** and **4b** (lane 2) were also loaded onto the gel as references. (B) SEC of a crude reaction mixture intended to generate **eGFP-Trz-4a** product (peak *i*) from **eGFP-(C₁₀)N₃** (peak *ii*) and **HC≡C-4a** (peak *iii*) starting materials. (C) SEC of a crude reaction mixture intended to generate **mCherry-**

Trz-4b product (peak *i*) from **mCherry-(C₁₀)N₃** (peak *ii*) and **HC≡C-4b** (peak *iii*) starting materials.

Figure 7.

Agarose gel electrophoresis (1.5% agarose) of eGFP-mCherry heterodimers, formed via hybridization of complementary ODN sequences **4a** and **4b**. (A-C) Agarose gel of combinations of SPAAC bioconjugates **eGFP-DBTrz-4a** and **mCherry-DBTrz-4b**, imaged for green fluorescence (A) and red fluorescence (B), and then stained with ethidium bromide (EtBr) and imaged for DNA-bound EtBr fluorescence (C). Lane 1: **eGFP-DBTrz-4a** monomer alone; lane 2: **mCherry-DBTrz-4b** monomer alone; lanes 3, 4: hybridization mixture of **eGFP-DBTrz-4a** and **mCherry-DBTrz-4b**, annealed at room temperature (lane 3) or at 55 °C (lane 4). (D, E) Agarose gel of combinations of CuAAC bioconjugates **eGFP-Trz-4a** and **mCherry-Trz-4b**, imaged for green fluorescence (D) and red fluorescence (E). Lane 1: **eGFP-Trz-4a** monomer alone; lane 2: **mCherry-Trz-4b** monomer alone; lane 3: hybridization mixture of **eGFP-Trz-4a** and **mCherry-Trz-4b**, annealed at room temperature. Arrows indicate bands corresponding to the eGFP-mCherry heterodimer product.

Figure 8.

Immobilization of **eGFP-(C₁₅)N₃** and **mCherry-(C₁₅)N₃** on **DIBO-4a-Sepharose** beads via SPAAC, imaged by brightfield (**A-E**) and fluorescence (**F-J**) optical microscopy. (**A,F**) Unmodified NHS-Sepharose; (**B,G**) **eGFP-(C₁₅)N₃ + DIBO-4a-Sepharose**; (**C,H**) **mCherry-(C₁₅)N₃ + DIBO-4a-Sepharose**; (**D,I**) **eGFP-(C₁₅)N₃ + 4a-Sepharose** (negative control); (**E,J**) **eGFP-CVIA + DIBO-4a-Sepharose** (negative control). Fluorescence images were collected using a green fluorescence filter set, except for image **H**, which was imaged for red fluorescence.

4a 5' X-GAT GAG TTC GTG TCC GTA CAA CTG G-Y 3'

4b 5' X-CCA GTT GTA CGG ACA CGA ACT CAT C-Y 3'

Scheme 1.

Scheme 2.

Reaction scheme for synthesizing protein-DNA conjugates by Cu-free and Cu-catalyzed click reactions.