Bioorganic & Medicinal Chemistry 20 (2012) 4532–4539

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Covalent protein–oligonucleotide conjugates by copper-free click reaction

Santoshkumar L. Khatwani [a], Jun Sung Kang [a], Daniel G. Mullen [a], Michael A. Hast [b], Lorena S. Beese [b], Mark D. Distefano [a], T. Andrew Taton [a,*]

[a] Department of Chemistry, University of Minnesota, Minneapolis, MN 55455, USA
[b] Department of Biochemistry, Duke University, Durham, NC 27710, USA

ARTICLE INFO

Article history:
Received 29 January 2012
Revised 3 May 2012
Accepted 9 May 2012
Available online 17 May 2012

Keywords:
Click chemistry
Bioconjugation
Protein–oligodeoxynucleotide conjugates
Protein prenylation
Protein immobilization

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.

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 PCR[6] or mass spectroscopy.[7] Conjugating antisense ODNs to proteins has 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–streptavidin[5] and nickel–histidine[14]) 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 amine[15] or a cysteine thiol[16]—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] cycloaddition[22] (CuAAC, a 'click' reaction[23]) 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 molecules[10] 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

* Corresponding author. Tel.: +1 612 626 4681; fax: +1 612 626 7541.
E-mail address: taton@umn.edu (T.A. Taton).

0968-0896/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2012.05.017

protocols is known to oxidize amino acid sidechains[27] 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), $CuSO_4$ (Mallinckrodt Baker, Phillipsburg, NJ), tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA; Aldrich, Milwaukee, WI), and *N*-hydroxysuccinimidyl–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(C_{10})**,[51] and 1-azido-10,11-dihydrofarnesyl diphosphate **3(C_{15})**[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 C_{18} 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 H_2O and 95:5 CH_3CN:0.03 M TEAA-buffered H_2O as the mobile phase. All buffers, including 0.1 M NaCl phosphate buffer (50 mM NaH_2PO_4, 0.1 M NaCl, pH 7.3) and 1.0 M NaCl phosphate buffer (50 mM NaH_2PO_4, 1.0 M NaCl, pH 7.3) were prepared in ultrapure deionized water (R >18 MΩ).

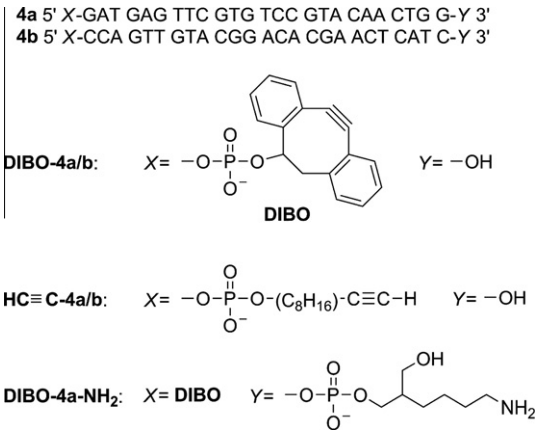
2.2. Construction, overexpression and purification of ScFTase

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 from 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′-AATTCGGATTGCACATATGCGACAGAGAGTAG GAAGGTCCATAGC-3′ (*Nde*I restriction site underlined) and 5′-TAGTAGGAACCTAGGTTAACTTGGAGAAGATAAATTGGATTTAAAG TAGTGG-3′ (*Avr*II restriction site underlined), and the α subunit was amplified using the primers 5′-AGCGATTGCAGGATCCAATG-GAGGAGTACGATTATTCAGACGTTAAACCTTTG-3′ (*Bam*HI restriction site underlined) and 5′-GTAGTAGGAAGCGGCCGCTCAGTTCAG ATTATTTATTTTGTGGTGCCACAAG-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, 6×His-containing tag MGSSHHHHHHSQDP 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 OD_{600} reached 1.2. The overexpression of 6×His-yPFTase was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) and $ZnSO_4$ (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 μM, λ_{ex} = 340 nm, λ_{em} = 505 nm).[26]

2.3. Synthesis of alkyne-funtionalized ODNs

DNA synthesis reagents were purchased from Glen Research (Sterling, VA). ODNs **4a** and **4b** were made by standard solid-phase oligonucleotide synthesis on a Perseptive Biosystems Expedite 8909 from controlled-pore glass (CPG, 500 Å, 1 μmol 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-NH_2**) and terminal-alkyne-modified ODNs (**HC≡C-4a** and **HC≡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



Scheme 1.

deprotection, cleavage from support, and reverse-phase (C_{18}) 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 ($\varepsilon_{4a/b,260} = 2.4 \times 10^5\ M^{-1}\ cm^{-1}$).[52] All DNA samples (15 μL) were mixed with 5 μL of 6× loading buffer (0.25% bromophenol blue and 40% sucrose in deionized water) and analyzed using a 1.5% agarose gel in 1× 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 ($\varepsilon_{eGFP,488} = 55{,}000\ M^{-1}\ cm^{-1}$) for eGFP[54] and 587 nm ($\varepsilon_{mCherry,587} = 72{,}000\ M^{-1}\ cm^{-1}$) 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(C_{10})** or **3(C_{15})** as described previously.[26] To attach an azidogeranyl group, a 10 mL solution of either **eGFP-CVIA** or **mCherry-CVIA** (2.4 μM), DTT (5 mM), $MgCl_2$ (10 mM), $ZnCl_2$ (10 μM), Tris–HCl (50 mM, pH 7.5), **3(C_{10})** (40 μM) and ScFTase (150 nM) was incubated at 30 °C for 2 h. To attach an azidofarnesyl group, **3(C_{15})** was used in place of **3(C_{10})**, 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 μL 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-(C_{10})N_3**, **eGFP-(C_{15})N_3**, **mCherry-(C_{10})N_3**, **mCherry-(C_{15})N_3**, and **GIP-(C_{15})N_3**. 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-N_3**) 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 μL) 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 μL) 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.

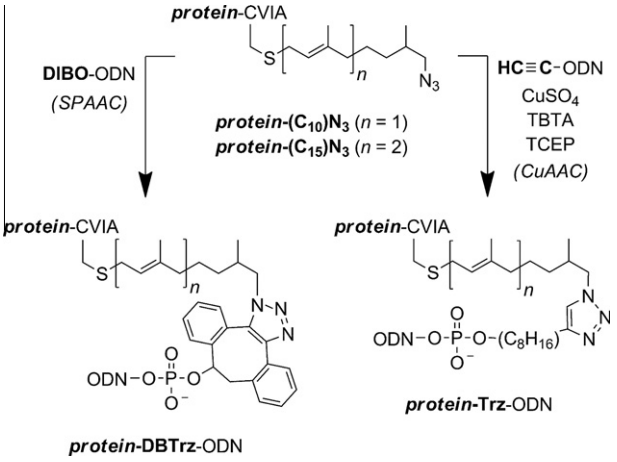
2.6. Synthesis of dibenzocycloctyltriazole (DBTrz)-linked protein–ODN conjugates by Cu-free SPAAC

Azide-functionalized protein (Scheme 2; 14 μM) was combined with a DIBO-modified ODN (**DIBO-4a** or **DIBO-4b**, respectively; 4.6 μM) 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 μL, 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 ($\lambda_{abs} = 260$ nm) and, in the case of eGFP and mCherry, protein ($\lambda_{abs} = 488$ nm for eGFP, $\lambda_{abs} = 587$ nm for mCherry) components. The fluorescence detector was set to monitor fluorescent protein ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 510$ nm for eGFP; $\lambda_{ex} = 588$ nm, $\lambda_{em} = 610$ 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-(C_{10})N_3** or **mCherry-(C_{10})N_3** (14 μM) was reacted with **HC≡C-4a** or **HC≡C-4b** (17 μM) 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 μL, passed through a NAP-5 column, and eluted with 0.1 M NaCl phosphate buffer. The sample was concentrated to ~100 μL using Centricon ultracentrifugal filters (MWCO 3000 Da). The



Scheme 2. Reaction scheme for synthesizing protein–DNA conjugates by Cu-free and Cu-catalyzed click reactions.

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 μL) contained 1 μM 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 $MgCl_2$. 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×*g* for 20 min at room temperature, removing supernatant, re-suspending 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-NH$_2$** 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-(C$_{15}$)N$_3$**, **mCherry-(C$_{15}$)N$_3$**, 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 eGFP[54] and mCherry[55] 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(C$_{10}$)** or **3(C$_{15}$)** 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-(C$_{10}$)N$_3$**) 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 conjugates[60] 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-(C$_{10}$)N$_3$** 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-(C$_{10}$)N$_3$**. 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

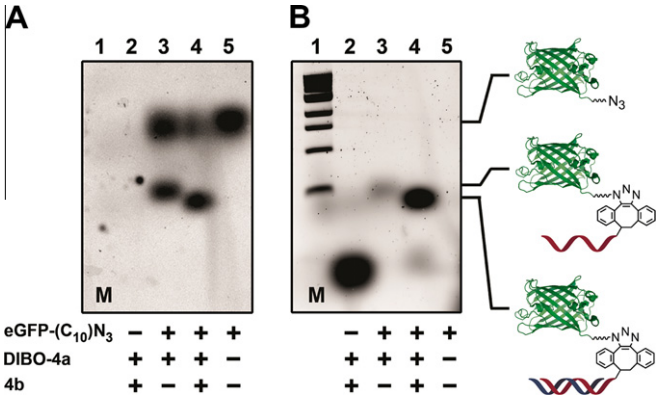


Figure 1. Agarose gel electrophoresis (using 1.5% agarose) of unpurified SPAAC reactions between **eGFP-(C$_{10}$)N$_3$** and ODN **DIBO-4a**. The reaction mixture was either loaded directly onto the gel (lane 3), or incubated with complementary ODN **4b** (lane 4); **eGFP-(C$_{10}$)N$_3$** alone (lane 5), the hybridized **DIBO-4a**/**4b** pair (lane 2), and a 1-kb 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.

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-(C_{10})N_3**, 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-(C_{10})N_3**, 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 geranyl (C_{10}), farnesyl (C_{15}), 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-(C_{15})N_3**, **mCherry-(C_{15})N_3**, **NC-N_3**, **GIP-(C_{15})N_3**, and a hexahistidine-tagged **His_6-eGFP-(C_{15})N_3**. 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 between **mCherry-(C_{15})N_3** and **DIBO-4a** over a range of pH values (from pH 3.5 to 12.3) yielded nearly identical conjugate product bands by gel electrophoresis (Fig. S1), 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 (0.9–2.3 M^{-1} 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-(C_{15})N_3** and **DIBO-4a** by reverse-phase HPLC, and tracked the amount of unreacted **eGFP-(C_{15})N_3** 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,[62] 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.

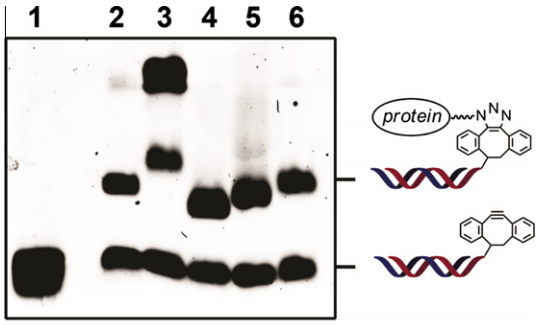


Figure 3. Agarose gel electrophoresis (using 1.5% agarose) of unpurified SPAAC reactions between ODN **DIBO-4a** and **eGFP-(C_{15})N_3** (lane 2), **mCherry-(C_{15})N_3** (lane 3), **NC-N_3** (lane 4), **GIP-(C_{15})Nb_3** (lane 5), and **His_6-eGFP-(C_{15})N_3** (lane 6). Each reaction also contained 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-(C_{15})N_3**, because mCherry fluoresces near the same wavelength as DNA-complexed EtBr.

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 λ = 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

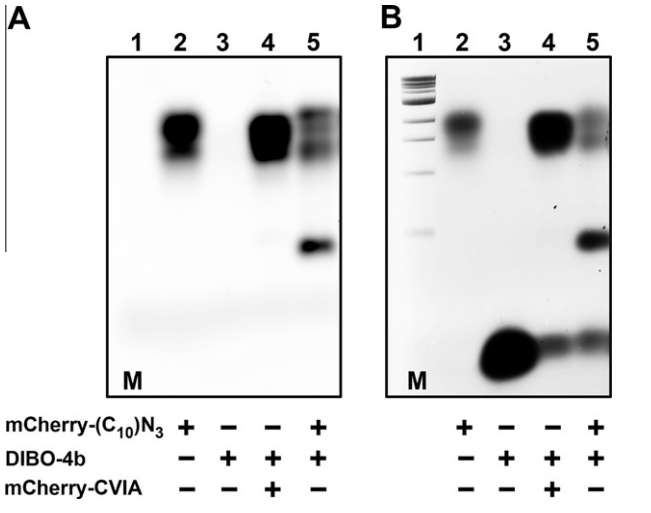


Figure 2. Agarose gel electrophoresis (1.5% agarose) of an unpurified SPAAC reaction between **mCherry-(C_{10})N_3** and ODN **DIBO-4b**. Wells were loaded with the reaction mixture (lane 5), a negative control mixture of **mCherry-CVIA** and **DIBO-4b** (lane 4), **DIBO-4b** alone (lane 3), **mCherry-(C_{10})N_3** alone (lane 2), and a 1-kb 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).

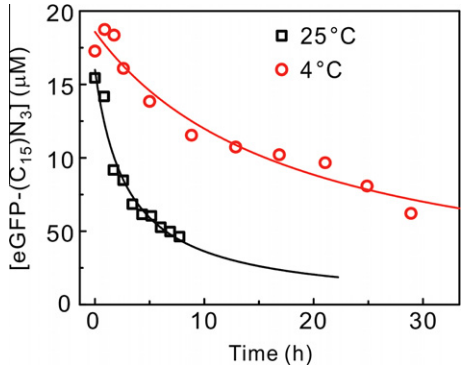


Figure 4. Reaction of a 1:1 molar ratio of **eGFP-(C_{15})N_3** and **DIBO-4a**, monitored by RP-HPLC, at different temperatures. Each dataset was fit to a second-order kinetic decay function (solid lines). for these fits, $k_{25\ °C}$ = 6 M^{-1} s^{-1}, and $k_{4\ °C}$ = 0.8 M^{-1} s^{-1}. Although 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.

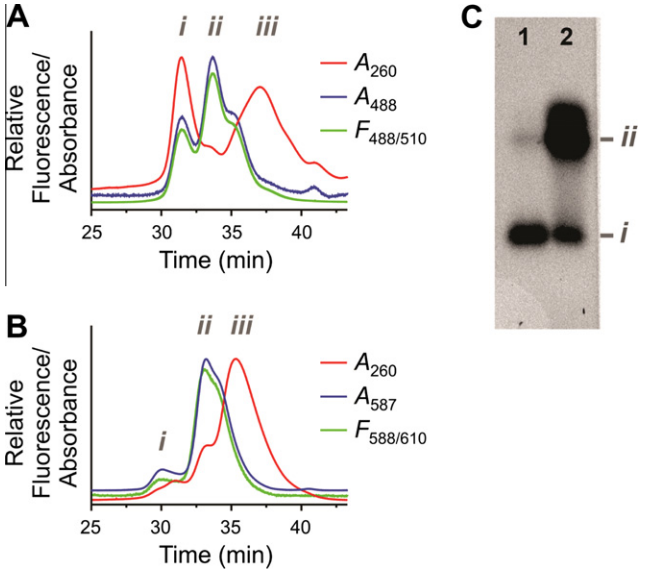


Figure 5. Purification of SPAAC-generated protein–ODN conjugates. (A) SEC of a crude reaction mixture intended to generate **eGFP-DBTrz-4a** product (peak *i*) from **eGFP-(C_{10})N_3** (peak *ii*) and **DIBO-4a** (peak *iii*) starting materials. (B) SEC of a crude reaction mixture intended to generate **mCherry-DBTrz-4b** product (peak *i*) from **mCherry-(C_{10})N_3** (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-(C_{10})N_3** (lane 2), imaged as green fluorescence.

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-(C_{10})N_3**, and 9% of the **mCherry-(C_{10})N_3**, 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-(C_{10})N_3** than for **eGFP-(C_{10})N_3**, 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-(C_{10})N_3** (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-(C_{10})N_3** and **mCherry-(C_{10})N_3**, 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 $CuSO_4$ 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

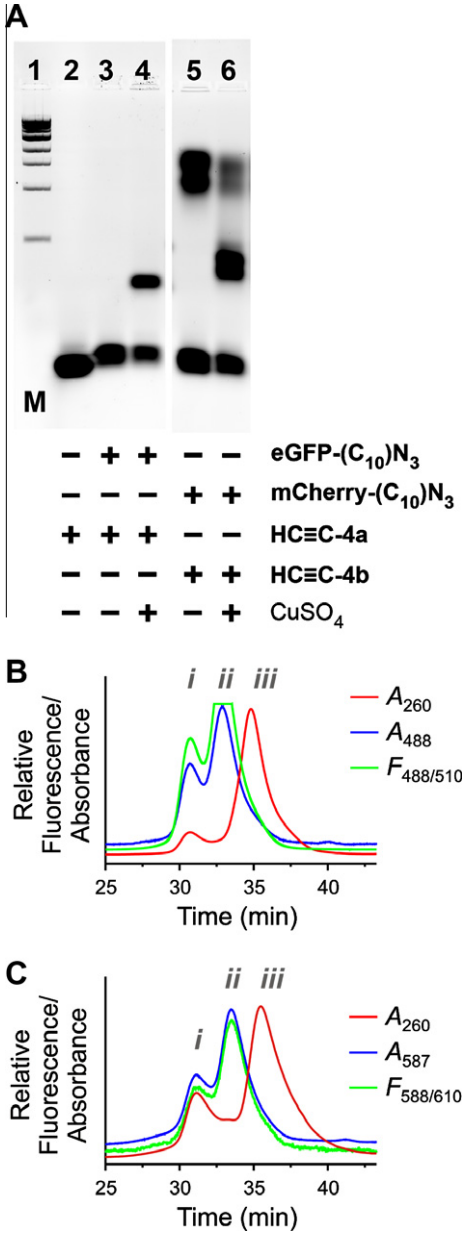


Figure 6. Analysis and purification of CuAAC-generated protein–ODN conjugates. (A) Agarose gel electrophoresis (1.5% agarose) of CuAAC reaction mixtures containing **eGFP-(C_{10})N_3** and **HC≡C-4a** (lanes 3, 4) or **mCherry-(C_{10})N_3** and **HC≡C-4b** (lanes 5, 6); reactions either contained $CuSO_4$ (lanes 4, 6) or were run in the absence of $CuSO_4$ as a negative control (lanes 3, 5). A 1-kb 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-(C_{10})N_3 (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-(C_{10})N_3** (peak *ii*) and **HC≡C-4b** (peak *iii*) starting materials.

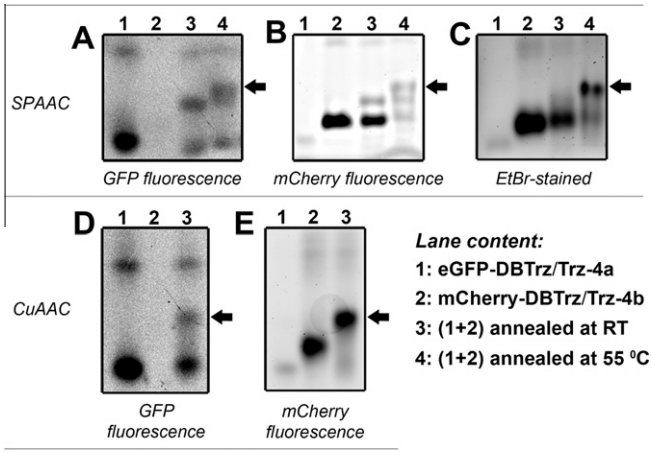


Figure 7. Agarose gel electrophoresis (1.5% agarose) of **eGFP-mCherry** heterodimers, formed via hybridization of complementary ODN sequences **4a** and **4b**. (**A**–**C**) Agarose gel of combinations of SPAAC bioconjugates **eGFP-DBTrz-4a** and **mCherry-DBTrz-4b**, imaged for green fluorescence (**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.

times for CuAAC-generated conjugates were similar to those prepared by SPAAC. From the SEC chromatograms, we estimate that CuAAC reaction of **eGFP-(C_{10})N_3** went 90% to completion after 24 h, and reaction of **mCherry-(C_{10})N_3** 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 high-temperature (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 non-specific 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-NH_2** 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-(C_{15})N_3** or **mCherry-(C_{15})N_3** 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

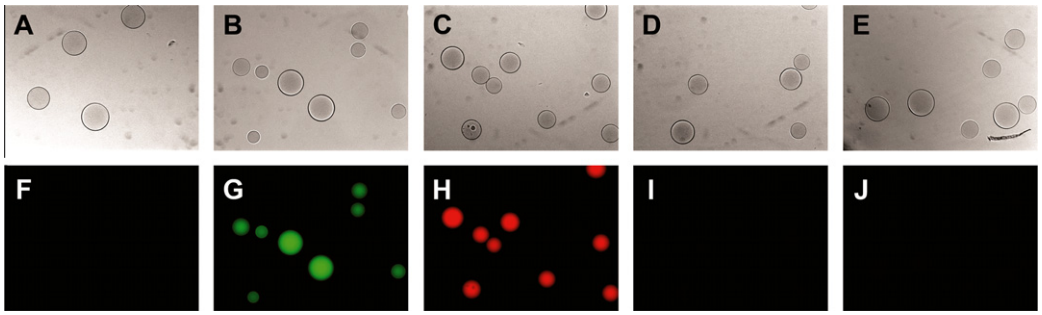


Figure 8. Immobilization of **eGFP**-(C_{15})N_3 and **mCherry**-(C_{15})N_3 on **DIBO-4a**-Sepharose beads via SPAAC, imaged by brightfield (**A**–**E**) and fluorescence (**F**–**J**) optical microscopy. (**A**,**F**) Unmodified NHS-Sepharose; (**B**,**G**) **eGFP**-(C_{15})N_3 + **DIBO-4a**-Sepharose; (**C**,**H**) **mCherry**-(C_{15})N_3 + **DIBO-4a**-Sepharose; (**D**,**I**) **eGFP**-(C_{15})N_3 + **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.

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.

Acknowledgments

We thank Jonathan Dozier for providing 1-azido-6,7-dihydrogeranyl diphosphate **3** (C_{10}), James Wollack for 1-azido-10,11-dihydrofarnesyl diphosphate **3** (C_{15}), Professor Karin Musier-Forsyth for helpful discussions concerning NC protein, and Professor George Barany for use of his automated peptide synthesizer. This work was supported by the NIH (CA122603 and GM084152).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.05.017.

References and notes

1. Niemeyer, C. M. *Angew. Chem., Int. Ed.* **2010**, *49*, 1200.
2. Niemeyer, C. M. *Trends Biotechnol.* **2002**, *20*, 395.
3. Bailey, R. C.; Kwong, G. A.; Radu, C. G.; Witte, O. N.; Heath, J. R. *J. Am. Chem. Soc.* **1959**, *2007*, 129.
4. Niemeyer, C. M.; Boldt, L.; Ceyhan, B.; Blohm, D. *Anal. Biochem.* **1999**, *268*, 54.
5. Niemeyer, C. M.; Sano, T.; Smith, C. L.; Cantor, C. R. *Nucleic Acids Res.* **1994**, *22*, 5530.
6. Sano, T.; Smith, C.; Cantor, C. *Science* **1992**, *258*, 120.
7. Zhang, L.; Kasif, S.; Cantor, R. C. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 3061.
8. Rajur, S. B.; Roth, C. M.; Morgan, J. R.; Yarmush, M. L. *Bioconjugate Chem.* **1997**, *8*, 935.
9. Diezmann, F.; Seitz, O. *Chem. Soc. Rev.* **2011**, *40*, 5789.
10. Duckworth, B. P.; Zhang, Z.; Hosokawa, A.; Distefano, M. D. *ChemBioChem* **2007**, *8*, 98.
11. Park, S. H.; Yin, P.; Liu, Y.; Reif, J. H.; LaBean, T. H.; Yan, H. *Nano Lett.* **2005**, *5*, 729.
12. Yan, H.; Park, S. H.; Finkelstein, G.; Reif, J. H.; LaBean, T. H. *Science* **1882**, *2003*, 301.
13. Chhabra, R.; Sharma, J.; Ke, Y.; Liu, Y.; Rinker, S.; Lindsay, S.; Yan, H. *J. Am. Chem. Soc.* **2007**, *129*, 10304.
14. Goodman, R. P.; Erben, C. M.; Malo, J.; Ho, W. M.; McKee, M. L.; Kapanidis, A. N.; Turberfield, A. J. *ChemBioChem* **2009**, *10*, 1551.
15. Jablonski, E.; Moomaw, E. W.; Tullis, R. H.; Ruth, J. L. *Nucleic Acids Res.* **1986**, *14*, 6115.
16. Corey, D.; Schultz, P. *Science* **1987**, *238*, 1401.
17. Ou, W.; Uno, T.; Chiu, H.; Grünewald, J.; Cellitti, S. E.; Crossgrove, T.; Hao, X.; Fan, Q.; Quinn, L. L.; Patterson, P.; Okach, L.; Jones, D. H.; Lesley, S. A.; Brock, A.; Geierstanger, B. H. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 10437.
18. Lovrinovic, M.; Seidel, R.; Wacker, R.; Schroeder, H.; Seitz, O.; Engelhard, M.; Goody, R. S.; Niemeyer, C. M. *Chem. Commun.* **2003**, 822.
19. Takeda, S.; Tsukiji, S.; Nagamune, T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2407.
20. Jongsma, M. A.; Litjens, R. H. *Proteomics* **2006**, *6*, 2650.
21. Tominaga, J.; Kemori, Y.; Tanaka, Y.; Maruyama, T.; Kamiya, N.; Goto, M. *Chem. Commun.* **2007**, 401.
22. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596.
23. Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2004**, *2001*, 40.
24. Baskin, J. M.; Bertozzi, C. R. *QSAR Comb. Sci.* **2007**, *26*, 1211.
25. Best, M. D. *Biochemistry* **2009**, *48*, 6571.
26. Duckworth, B. P.; Xu, J.; Taton, T. A.; Guo, A.; Distefano, M. D. *Bioconjugate Chem.* **2006**, *17*, 967.
27. Requena, J. R.; Chao, C.; Levine, R. L.; Stadtman, E. R. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 69.
28. Burrows, C. J.; Muller, J. G. *Chem. Rev.* **1998**, *98*, 1109.
29. Sigman, D. S.; Mazumder, A.; Perrin, D. M. *Chem. Rev.* **1993**, *93*, 2295.
30. Becer, C. R.; Hoogenboom, R.; Schubert, U. S. *Angew. Chem., Int. Ed.* **2009**, *48*, 4900.
31. Kele, P.; Mezö, G.; Achatz, D.; Wolfbeis, O. S. *Angew. Chem., Int. Ed.* **2009**, *48*, 344.
32. Beatty, K. E.; Fisk, J. D.; Smart, B. P.; Lu, Y. Y.; Szychowski, J.; Hangauer, M. J.; Baskin, J. M.; Bertozzi, C. R.; Tirrell, D. A. *ChemBioChem* **2010**, *11*, 2092.
33. Jewett, J. C.; Bertozzi, C. R. *Chem. Soc. Rev.* **2010**, *39*, 1272.
34. Baskin, J. M.; Prescher, J. A.; Laughlin, S. T.; Agard, N. J.; Chang, P. V.; Miller, I. A.; Lo, A.; Codelli, J. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 16793.
35. Dommerholt, J.; Schmidt, S.; Temming, R.; Hendriks, L. J. A.; Rutjes, F. P. J. T.; van Hest, J. C. M.; Lefeber, D. J.; Friedl, P.; van Delft, F. L. *Angew. Chem., Int. Ed.* **2010**, *49*, 9422.
36. Chang, P. V.; Prescher, J. A.; Sletten, E. M.; Baskin, J. M.; Miller, I. A.; Agard, N. J.; Lo, A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **1821**, *2010*, 107.
37. Neef, A. B.; Schultz, C. *Angew. Chem., Int. Ed.* **2009**, *48*, 1498.
38. Laughlin, S. T.; Baskin, J. M.; Amacher, S. L.; Bertozzi, C. R. *Science* **2008**, *320*, 664.
39. Dieterich, D. C.; Hodas, J. J. L.; Gouzer, G.; Shadrin, I. Y.; Ngo, J. T.; Triller, A.; Tirrell, D. A.; Schuman, E. M. *Nat. Neurosci.* **2010**, *13*, 897.
40. DeForest, C. A.; Sims, E. A.; Anseth, K. S. *Chem. Mater.* **2010**, *22*, 4783.
41. Ornelas, C.; Broichhagen, J.; Weck, M. *J. Am. Chem. Soc.* **2010**, *132*, 3923.
42. Bernardin, A.; Cazet, A. I.; Guyon, L.; Delannoy, P.; Vinet, F.; Bonnaffé, D.; Texier, I. *Bioconjugate Chem.* **2010**, *21*, 583.
43. Canalle, L. A.; van Berkel, S. S.; de Haan, L. T.; van Hest, J. C. M. *Adv. Funct. Mater.* **2009**, *19*, 3464.
44. Kuzmin, A.; Poloukhtine, A.; Wolfert, M. A.; Popik, V. V. *Bioconjugate Chem.* **2010**, *21*, 2076.
45. Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046.
46. Codelli, J. A.; Baskin, J. M.; Agard, N. J.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2008**, *130*, 11486.
47. Jewett, J. C.; Sletten, E. M.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2010**, *132*, 3688.
48. Ning, X.; Guo, J.; Wolfert, M. A.; Boons, G.-J. *Angew. Chem., Int. Ed.* **2008**, *47*, 2253.
49. van Delft, P.; Meeuwenoord, N. J.; Hoogendoorn, S.; Dinkelaar, J.; Overkleeft, H. S.; van der Marel, G. A.; Filippov, D. V. *Org. Lett.* **2010**, *12*, 5486.
50. Marks, I. S.; Kang, J. S.; Jones, B. T.; Landmark, K. J.; Cleland, A. J.; Taton, T. A. *Bioconjugate Chem.* **2011**, *22*, 1259.
51. Xu, J.; DeGraw, A. J.; Duckworth, B. P.; Lenevich, S.; Tann, C.-M.; Jenson, E. C.; Gruber, S. J.; Barany, G.; Distefano, M. D. *Chem. Biol. Drug Des.* **2006**, *68*, 85.
52. Tataurov, A. V.; You, Y.; Owczarzy, R. *Biophys. Chem.* **2008**, *133*, 66.
53. Chung, J. A.; Wollack, J. W.; Hovlid, M. L.; Okesli, A.; Chen, Y.; Mueller, J. D.; Distefano, M. D.; Taton, T. A. *Anal. Biochem.* **2009**, *386*, 1.
54. Heim, R.; Cubitt, A. B.; Tsien, R. Y. *Nature* **1995**, *373*, 663.
55. Shaner, N. C.; Campbell, R. E.; Steinbach, P. A.; Giepmans, B. N. G.; Palmer, A. E.; Tsien, R. Y. *Nat. Biotech.* **2004**, *22*, 1567.
56. Rashidian, M.; Song, J. M.; Pricer, R. E.; Distefano, M. D. J. *Am. Chem. Soc.* **2012**, *134*, 8455.
57. Liu, H.-W.; Cosa, G.; Landes, C. F.; Zeng, Y.; Kovaleski, B. J.; Mullen, D. G.; Barany, G.; Musier-Forsyth, K.; Barbara, P. F. *Biophys. J.* **2005**, *89*, 3470.
58. King, D. S.; Fields, C. G.; Fields, G. B. *Int. J. Pept. Protein Res.* **1990**, *36*, 255.
59. Zhong, M.; Fang, J.; Wei, Y. *Bioconjugate Chem.* **2010**, *21*, 1177.
60. Lovrinovic, M.; Niemeyer, C. M. *Biochem. Biophys. Res. Commun.* **2005**, *335*, 943.
61. Hellman, L. M.; Fried, M. G. *Nat. Protocols* **1849**, *2007*, 2.
62. Schaeffer, P. M.; Dixon, N. E. *Aust. J. Chem.* **2009**, *62*, 1328.
63. Dahdah, D. B.; Morin, I.; Moreau, M. J. J.; Dixon, N. E.; Schaeffer, P. M. *Chem. Commun.* **2009**, 3050.
64. Feldkamp, U.; Niemeyer, C. M. *Angew. Chem., Int. Ed.* **1856**, *2006*, 45.
65. Sawoo, S.; Dutta, P.; Chakraborty, A.; Mukhopadhyay, R.; Bouloussa, O.; Sarkar, A. *Chem. Commun.* **2008**, 5957.
66. Zimmermann, J. L.; 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.