Monitoring Protein−Polymer Conjugation by a Fluorogenic Cu(I)-Catalyzed Azide−Alkyne 1,3-Dipolar Cycloaddition

A. (Ton) J. Dirks, Jeroen J. L. M. Cornelissen,*,† and Roeland J. M. Nolte

Institute for Molecules and Materials, Radboud University Nijmegen, Toernooiveld 1, 6525 ED, Nijmegen, The Netherlands.
Received October 27, 2008; Revised Manuscript Received March 25, 2009

The Cu(I)-catalyzed azide−alkyne cycloaddition (CuAAC) has recently proven to be a powerful synthetic tool in various fields of chemistry, including protein−polymer conjugation. In this article, we describe a fluorogenic CuAAC, which allows for efficient monitoring of protein−polymer conjugation. We show that profluorescent 3-azido coumarin-terminated polymers can be reacted with an alkyne-functionalized protein to produce a strongly fluorescent triazole-linked conjugate. Upon formation of the product, the evolution of fluorescence can accurately be determined, providing information about the course of the CuAAC. As a proof of concept, we synthesized several 3-azido coumarin terminated poly(ethylene glycol) (PEG) chains and investigated their conjugation with alkyne-functionalized bovine serum albumin (BSA) as a model protein. CuAAC conjugation was shown to be very efficient and proceeded rapidly. Conversion plots were constructed from measuring the fluorescence as function of reaction time. An additional benefit of the fluorogenic CuAAC is the *in situ* labeling of bioconjugates. We envision that the fluorogenic protein−polymer conjugation is not restricted to the reaction system reported in this work, but may also be ideal to screen for optimal reaction conditions of various other systems.

INTRODUCTION

The modification of proteins with synthetic polymers has been demonstrated to be beneficial for applications in medicine and biotechnology (*1*−*5*). A well-known example in this respect is the conjugation of proteins with poly(ethylene glycol) (PEG), termed PEGylation (*6*−*11*). In comparison to native proteins, PEGylated derivatives often exhibit improved biodistribution and pharmacokinetics, reduced immunogenicity, prolonged plasma half-life, and increased solubility (*12, 13*). As a result, several therapeutic protein−PEG conjugates are currently on the market or in clinical development (*10, 14*). Besides PEGylated proteins, other types of protein−polymer conjugates have also gained much interest. In particular, bioconjugates that comprise stimuli-responsive polymers (e.g., poly(*N*-isopropylacrylamide), pNIPAM) have been widely investigated for their reversible phase transition behavior (*3, 15*−*17*). Somewhat less deeply studied are the conjugates of hydrophobic polymers (e.g., polystyrene; PS) and proteins, which have been shown to act as giant amphiphiles and form bioactive assemblies in water (*18*−*24*).

As protein−polymer conjugates constitute a versatile and interesting class of materials, their synthesis has attracted a lot of attention. Several synthetic routes have now been established, mainly following two approaches: (i) the propagation of a polymer from a protein equipped with initiator moieties (i.e., grafting-from), and (ii) the coupling of an end-functionalized polymer to peripheral amino acid side chains of a protein (i.e., grafting-to) (*25*−*27*). The latter approach is most commonly applied and allows the polymer segment to be presynthesized under conventional, optimized conditions. Obviously, the success of the grafting-to method strongly relies on the selection of an efficient coupling strategy for the joining of bulky building blocks. In the choice of chemistry, it should furthermore be taken into consideration that site-specific conjugation may be required to retain maximum biological activity. Therefore, a frequently used strategy is to couple thiol-reactive polymers to unpaired cysteine residues, which are naturally very scarce (*23, 28, 29*). In fact, many proteins lack free thiol groups, but in those cases, genetic engineering can be used to introduce cysteines residues at specific positions (*30*). Another strategy to create a handle for site-specific conjugation is via the incorporation of non-natural amino acids (*31*). In this way, functional groups such as ketones, azides, and alkynes can be installed, facilitating a bioorthogonal derivatization by oxime ligation (*32*−*36*) or the recently developed Cu(I) catalyzed azide−alkyne 1,3-dipolar cycloaddition (CuAAC) (*37*−*40*). The latter can be considered as a prototypical click reaction (*41*) and has proven to be a powerful synthetic tool in the fields of biohybrid chemistry (*42*−*44*) and materials science (*45, 46*). In terms of protein−polymer conjugation, the CuAAC has successfully been employed in grafting-to approaches to prepare PEGylated enzymes (*47, 48*), glycopolymer−virus particles (*49*), stimuli-responsive bioconjugates (*50*), and giant amphiphiles (*22*).

Protein−polymer conjugation is generally monitored by characterization techniques that discriminate between the size of the starting materials and products, e.g., sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) or size exclusion chromatography (SEC). However, we realized that using the CuAAC would also allow for monitoring of the bioconjugation process by a fluorogenic assay. That is, exploiting the fact that profluorescent 3-azido coumarin derivatives become strongly fluorescent upon undergoing a cylcoaddition reaction, as depicted in Figure 1. 3-Azido coumarin derivatives have been developed by Wang and co-workers (*51*) and proved to be very useful to signal for an effective CuAAC with alkynes (*52*−*55*) and cycloaddition with oxanorbornadienes (*56*). Extending on the signaling purposes, it should be possible to determine the progress of coupling 3-azido coumarin-terminated polymers to alkyne-functionalized proteins by simply measuring the increase in fluorescence intensity. In addition, the newly formed bioconjugate would instantly be labeled with

* Corresponding author. Tel: (+31) 24 365 2381. Fax: (+31) 24 365 2929. E-mail: J.J.L.M.Cornelissen@tnw.utwente.nl.

† Present address: Laboratory for Biomolecular Nanotechnology, MESA+ Institute, University of Twente, Enschede, The Netherlands.

10.1021/bc8004667 CCC: $40.75
Published on Web 05/19/2009

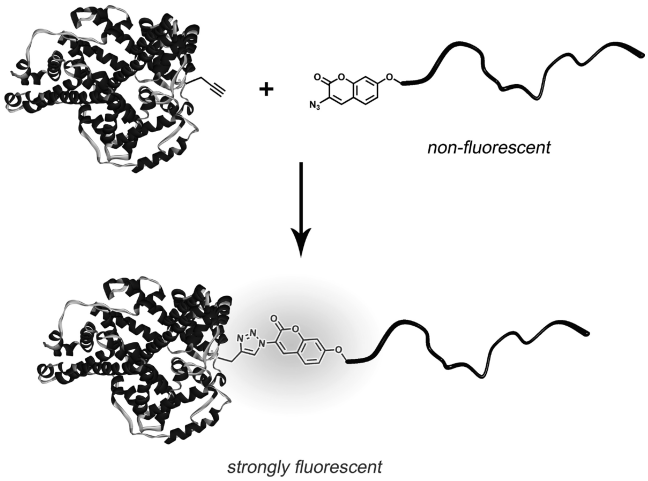


Figure 1. Schematic representation of a fluorogenic Cu(I)-catalyzed azide−alkyne 1,3-dipolar cycloaddition between a protein and a polymer.

a fluorescent probe, which is of great assistance in tracking the materials with established characterization techniques (e.g., SEC and SDS−PAGE). For the reasons described above, we decided to further develop the concept of monitoring protein−polymer conjugation by a fluorogenic CuAAC. Considering the great technological relevance of protein PEGylation, we focused in our proof of concept on the coupling of 3-azido coumarin terminated PEG to an alkyne-functionalized model protein. Bovine serum albumin (BSA) was selected as a model protein because it is readily available and easy to functionalize with a single alkyne group (*22*). In this article, we concentrate on 3-azido coumarin derivatives, but it should be noted that other azide/alkyne-bearing profluorescent probes also may be selected for the same purpose (*57, 58*).

RESULTS AND DISCUSSION

Synthesis of 3-Azido Coumarin Terminated PEG. Nowadays, a range of well-defined PEG derivatives is commercially available. Commonly, these polymers are equipped with hydroxyl end groups, which allow for their functionalization by straightforward modifications. We envisioned that a 3-azido coumarin compound carrying a carboxyl acid group (i.e., compound **3**) would be ideal for coupling to hydroxyl-terminated PEG as well as to other end-functional polymers in future studies. Therefore, acid derivative **3** was synthesized starting from 3-azido-7-hydroxy coumarin (**1**) reported by Wang and co-workers (Scheme 1) (*51*). First, a *tert*-butyl ester was introduced, which could subsequently be deprotected to a carboxylic acid under mild conditions, i.e., treatment with trifluoro acetic acid (TFA). Both reaction steps proceeded smoothly and in good yield. However, a similar route employing a methyl ester was found to be unsuccessful, and no azide-containing coumarin could be recovered after deprotection attempts with 1 M NaOH in THF.

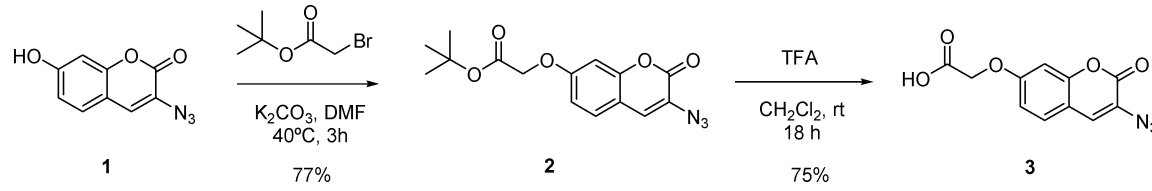
Coumarin **3** was coupled to hydroxyl-terminated PEG (M_n = 2000 and 5000 Da) using 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (EDCI) as a coupling reagent. This resulted in end-functional polymers **4** and **5** containing an ester linkage (Scheme 2). Additionally, hydroxyl PEG (M_n = 2000 Da) was used as a starting material for the preparation of amide-linked 3-azido coumarin terminated PEG **8**, which is expected to be more stable under physiological conditions. To this end, the PEG was first reacted with acrylonitrile to give polymer **6**. After reduction of the nitrile to the corresponding amine (**7**), the polymer was coupled to coumarin **3** using EDCI under conditions similar to those employed for the ester derivatives. All 3-azido coumarin terminated polymers were characterized by ^{1}H NMR spectroscopy and SEC. Figure 2 displays the ^{1}H NMR spectra of PEG **4** and **8** (both with M_n = 2000 Da), which clearly show the signals corresponding to the coumarin end groups. It should be noted that for the amide-linked PEG **8**, traces corresponding to ester-linked PEG **4** were observed in the ^{1}H NMR spectrum. This shows that not all chains of PEG **7** were amine terminated, which might be caused by an incomplete Michael addition with acrylonitrile or the occurrence of the reversed reaction. Integration of the α-terminal methoxy signal (*a*) and the protons neighboring the carbonyl (*f* and *h* for **4/5** and **8**, respectively) gave a ratio close to 3:2, pointing to quantitative functionalization. For all polymers, the covalent attachment of the coumarin moiety to PEG was confirmed further by SEC using UV detection at λ = 340 nm. The coupled products clearly showed a signal at the expected elution times, while the starting PEG gave no response. As expected, detection by refractive index (RI) afforded comparable results for PEG and the corresponding functionalized derivatives.

Synthesis of Alkyne-Functionalized BSA. To evaluate the concept of monitoring protein−polymer conjugation by a fluorogenic CuAAC, we selected alkyne-functionalized BSA **10** as a model protein. Following up on previous work (*22*), commercially available BSA was coupled to *N*-propargyl maleimide. A CuAAC with 3-azido-7-hydroxy-coumarin (**1**) was successfully conducted to check for functionalization of the protein (see Supporting Information).

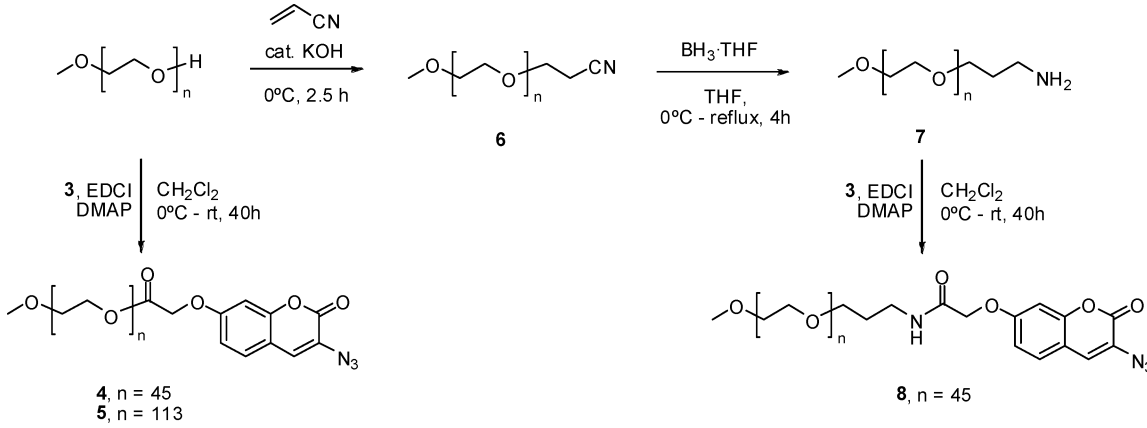
Fluorogenic PEGylation via the CuAAC. Before carrying out the PEGylation of BSA, we decided to examine the reactivity of the azido end group of PEG using low molecular weight alkyne **12**. This alkyne derivative was taken as a model for **10**, and was synthesized by reacting *N*-propargyl maleimide with β-mercapto ethanol (Scheme 3). With the help of $CuSO_4$/Na-ascorbate, alkyne **12** was reacted with PEG **4** using THF/water (3:2) as a solvent system. The reaction was accompanied by a clear evolution of fluorescence (when irradiated under a hand-held UV lamp, λ = 366 nm), indicating that the azide moiety was successfully converted into a triazole. Complete conversion of the reaction was confirmed by ^{1}H NMR spectroscopy, which showed a full shift of coumarin H_4 from δ = 7.16 ppm to δ = 8.49 ppm. Furthermore, a distinct signal of the triazole proton was detected at δ = 8.60 ppm. Analysis by matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry showed a single molecular weight distribution (Figure 3) corresponding to the expected mass of a sodium adduct of the clicked polymer **13** (calcd for $[M + Na]^+$ n = 45; 2492.3, found; 2492.1). Consequently, it was confirmed that the coumarin end group is accessible and participates in a fluorgenic CuAAC in aqueous media. The fluorescence properties of the clicked PEG were evaluated in a phosphate buffer (PB), comparable to the conditions of the bioconjugation experiments described below. Figure 4 displays the excitation and emission spectra of the polymer. The emission intensity at λ_{max} = 418 nm was found to depend linearly on the concentration in the range of 16−140 ng/mL. The excitation wavelength of λ = 340 nm was also used for analysis of the reported PEGylation experiments.

In bioconjugation experiments, typically an excess of reagents is used in order to drive the reaction to completion, especially if bulky substrates are to be conjugated. For our fluorogenic PEGylation experiments (Scheme 4), we therefore started off by using 20 equiv of 3-azido coumarin terminated PEG **4** compared to a 10 mg/mL solution of alkyne-functioanlized BSA **10** (0.15 mM) in the presence of 3 mM $CuSO_4$ and 4.5 mM Na-ascorbate. Under these conditions, a significant evolution of fluorescence was evident upon irradiation by a standard UV lamp (λ = 366 nm), within few minutes after the addition of

Scheme 1. Synthesis of an Acid Functionalized 3-Azido Coumarin Derivative 3



Scheme 2. Preparation of 3-Azido Coumarin Terminated PEG with Ester or Amide Linkage



the copper/Na-ascorbate mixture (Figure 5A). A control experiment in which no copper was added hardly showed fluorescence. The fluorescence intensity of the reaction mixture did not seem to increase further after 5 min, and at that point, the fluorescence emission spectrum was measured as depicted in Figure 5B.

The spectrum of the reaction mixture was comparable to that found for PEG **13**. Compared to the model compound PEG **13** (the emission intensity at $\lambda_{max} = 418$ nm was found to depend linearly on the concentration in the range of 16-140 ng/mL); however, the quantified intensity at $\lambda = 418$ nm was found to be approximately three times higher than expected. This implies that either PEG **13** is not a good model for the BSA−PEG conjugate or the number of alkyne moieties in the protein sample was higher than expected. The latter is unlikely because maleimides react ∼1000 times faster with free thiols compared to amines (*28*), and moreover, the excess of *N*-propargyl

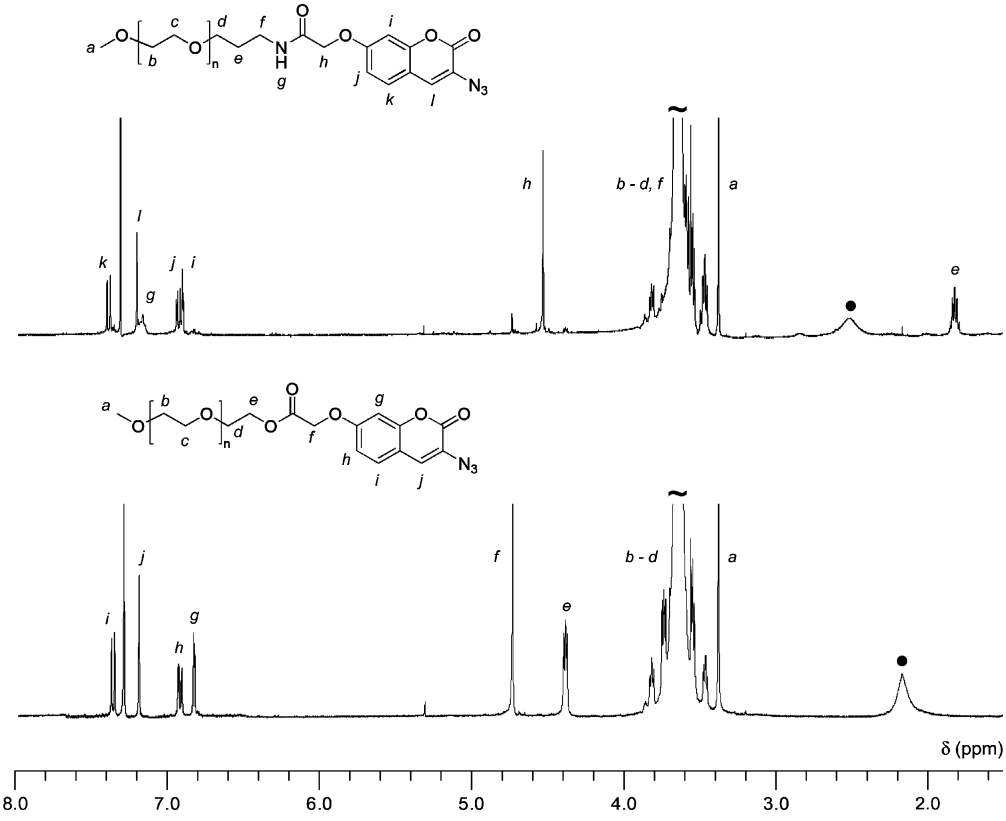


Figure 2. 1H NMR ($CDCl_3$, 400 MHz) spectra of 3-azido coumarin terminated PEG **4** and **8**. The broad signal indicated by ● is assigned to water.

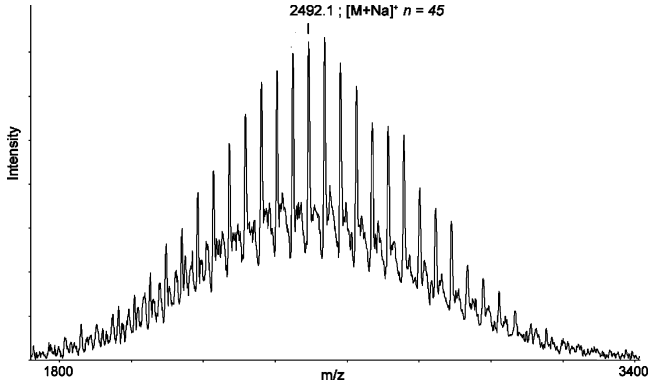


Figure 3. MALDI-ToF mass spectrum of the clicked PEG derivative **13**.

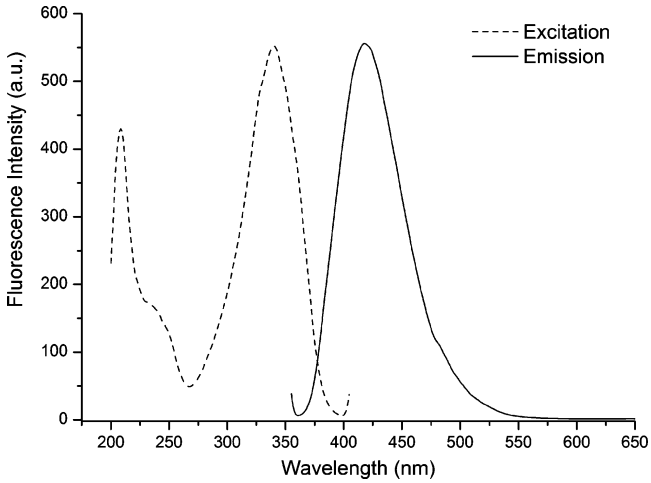
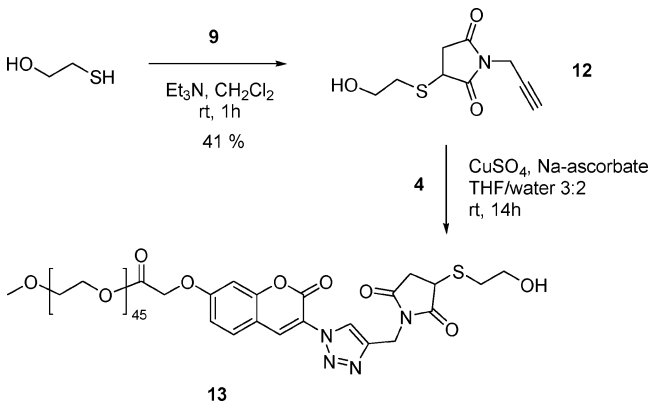


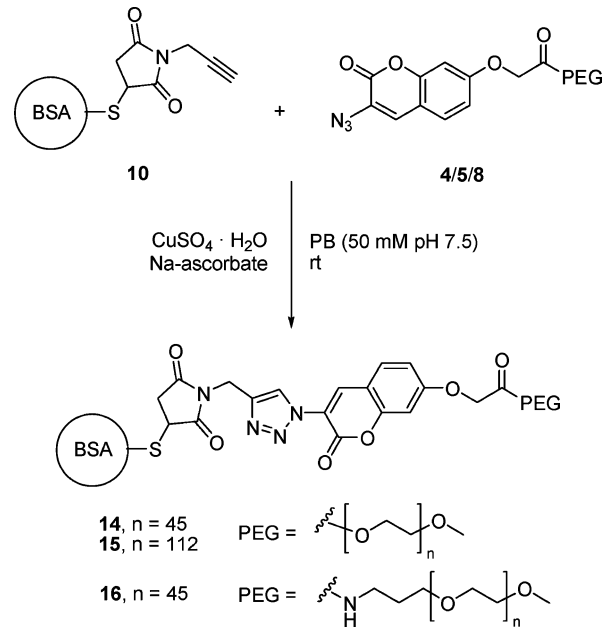
Figure 4. Fluorescence excitation (dashed) and emission (solid) spectra of clicked PEG **13** in PB (50 mM, pH 7.5); $\lambda_{ex.max} = 340$ nm, $\lambda_{em.max} = 418$ nm.

maleimide was removed by size exclusion chromatography (Sephadex G50). Furthermore, aqueous size exclusion does not point to the formation of higher molecular weight polymer−protein conjugates (see below). Another explanation for the difference in fluorescence intensity could be that the protein environment influences the photochemical properties of the coumarin, similar to effects caused by different solvents. Measuring the fluorescence spectra of PEG **13** in different solvents resulted in considerable differences in fluorescence intensity, with toluene giving a 2.3 times lower emission than ethanol or THF and 1.5 times lower than phosphate buffer. The

Scheme 3. Fluorogenic CuAAC at the 3-Azido Coumarin End Group of PEG 4



Scheme 4. CuAAC between 3-Azido Coumarin Terminated PEG (4/5/8) and Alkyne-Bearing BSA 10



polarity of the environment evidently influences the fluorescence properties of coumarin.

As a consequence, the fluorescence evolving from the protein−polymer conjugation cannot directly be quantified in terms of an absolute conversion. Nevertheless, a relative increase of intensity could be determined, which also provides information about the course of the reaction. The PEGylation reaction was therefore monitored by periodically removing samples from the reaction mixture for spectroscopic analysis. The excess of PEG was lowered to 10 equiv because the initial CuAAC appeared to be extremely fast. Again, a rapid increase in fluorescence intensity (at $\lambda = 418$ nm) was observed, and around 15 min, the measured emission was found not to increase further. Upon addition of an extra amount of catalyst, the fluorescence intensity remained constant, suggesting that the reaction was complete. Plotting the relative fluorescence intensity against time resulted in a curve comparable to a conversion plot (Figure 6). As can be seen from Figure 6, the CuAAC between PEG and BSA is very fast and reaches completion in approximately 15 min. Repeating the reaction with halved concentrations still resulted in a fast conjugation (Figure 6), which seemed to be complete after 40 min. To confirm the covalent attachment of PEG to BSA, the crude mixture at 60 min was subjected to aqueous SEC (Figure 7). This clearly showed the formation of a product with a higher molecular weight than that of starting BSA. Additionally, the newly formed compound showed UV absorbance at both $\lambda = 340$ nm and $\lambda = 280$ nm, whereas starting BSA only shows absorbance at the lower wavelength, confirming the presence of the coumarin moiety. The SEC trace of the conjugate showed some tailing, which may be caused by a residual amount of unreacted BSA; this is inevitable because in commercial batches of BSA, not all thiol groups are available for functionalization (*28*).

Next, the fluorogenic PEGylation of BSA **10** was carried out with PEG **5** and **8**, using the same conditions as discussed above. Plotting the fluorescence emission against reaction time resulted in similar curves (Figure 8), despite the differences in molecular weight between **5** and **8/4**. The conjugation product of the higher molecular weight PEG was analyzed by aqueous SEC, and as expected, a peak at higher molecular weight than those of BSA **10** and BSA−PEG **14** was observed. Like conjugate **14**, the peak of **15** showed UV absorbance at both $\lambda = 340$ nm and λ

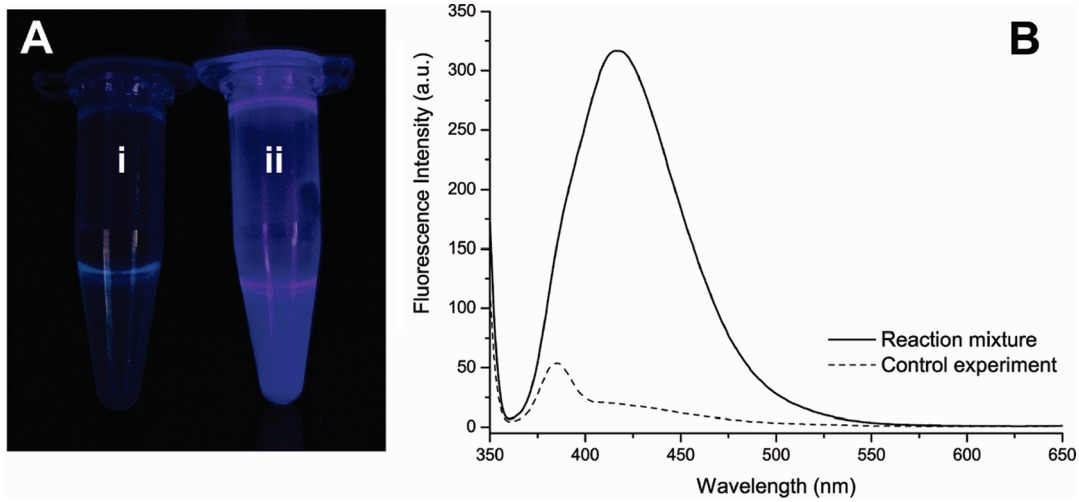


Figure 5. (A) Image taken under UV irradiation (λ = 366 nm) of fluorogenic CuAAC between PEG **4** and BSA **10**; i, control experiment without copper catalyst; ii, conjugation experiment at equal concentration of the other reactants. (B) Fluorescence emission spectrum of the conjugation reaction between **4** and **10** after 5 min (λ_{exc} = 340 nm).

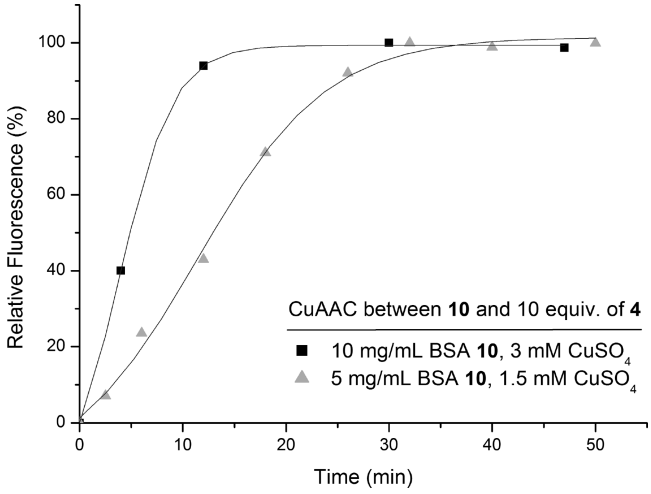


Figure 6. Increase of the fluorescence emission ($\lambda_{em.}$ = 418 nm) during the CuAAC between **4** and **10**, λ_{ex} = 340 nm. Ten equiv of PEG were used. The molar ratio of $CuSO_4$/Na-ascorbate was 1:1.5. Fluoresence is reported relative to the intensity at maximum conversion.

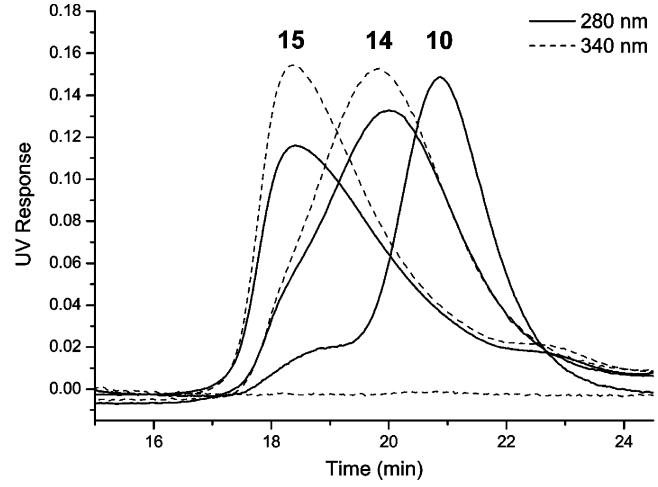


Figure 7. FPLC traces of alkyne-functionalized BSA (**10**) and crude mixtures of BSA−PEG conjugates **14** and **15** after 60 min of reaction time. Phosphate buffer (20 mM, pH 7.5) was used as the eluent in combination with UV detection at λ = 340 nm (dashed lines) and at λ = 280 nm (solid lines).

= 280 nm. The SEC results depicted in Figure 7 demonstrate that the PEGylation had effectively occurred when the fluorescence emission reached a constant level. Accordingly, the fluorescence−time plots (Figures 6 and 8) are considered to be representative for the progression of the bioconjugation reaction.

CONCLUSIONS

A fluorogenic PEGylation reaction via the CuAAC has been described. Several 3-azido coumarin terminated PEG derivatives were synthesized by coupling an acid-functionalized coumarin to a hydroxyl or amine terminus of PEG. The resulting 3-azido coumarin end group was demonstrated to participate in the CuAAC with a low molecular weight alkyne, under clear evolution of fluorescence. Likewise, the polymers were coupled to alkyne-bearing BSA, serving as a model protein. Upon bioconjugation, a clear increase in fluorescence emission was observed. Determination of the fluorescence intensity as a function of reaction time resulted in a conversion plot of the PEGylation process. Completion of the reaction could accurately be determined from the conversion curve and the covalent attachment of PEG to BSA was confirmed by aqueous SEC. Because of the presence of the coumarin moiety, the bioconjugates could easily be traced by SEC.

In summary, the CuAAC of proteins and 3-azido coumarin terminated polymers offers the possibility to monitor the bioconjugation process by a quick fluorogenic assay that is based on the progression of the reaction. This is a procedure that is fast compared to, for example, size exclusion or gel electrophoresis and furthermore allows for a visual check. Although, strictly speaking, the reported procedure at this point does not provide a tool to quantify polymer conjugation, in combination with the highly orthogonal character of the CuAAC it is reasonable to assume that polymers only couple to the acetylene groups on the protein. This is particularly the case when the acetylene groups are incorporated in the aimed protein by residue-specific introduction of noncanonical amino acids (*48*). We believe that the concept is not limited to the PEGylation example used as a proof of principle in this research. In fact, the reported concept may be ideal to screen for optimal reaction conditions in various systems, even in the more laborious coupling of immiscible polymer segments such as in the formation of protein−polymer biohybrid amphiphiles (*18*).

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise stated, all chemicals were obtained from commercial sources and used without further

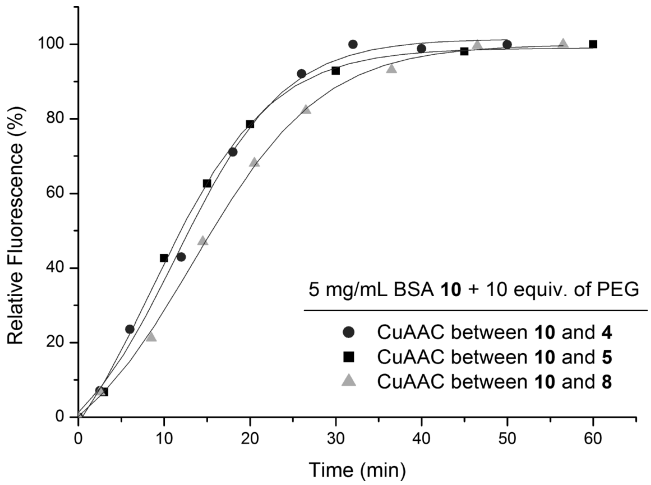


Figure 8. Increase of the fluorescence emission ($\lambda_{em.} = 418$ nm) during the CuAAC between **10** and **4/5/8**, $\lambda_{em.} = 340$ nm. The concentration of protein and $CuSO_4$ were 5 mg/mL and 1.5 mM, respectively. Ten equiv of PEG were used. The molar ratio of $CuSO_4$/Na-ascorbate was 1:2. Fluoresence is reported relative to the intensity at maximum conversion.

purification. Analytical thin layer chromatography (TLC) was performed on Merck precoated silica gel 60 F254 plates (layer thickness 0.25 mm) with visualization by ultraviolet (UV) irradiation at $\lambda = 254$ nm and/or $\lambda = 366$ nm and/or staining with $KMnO_4$ or Ninhydrin. Purifications by silica gel chromatography were performed using Acros (0.035−0.070 mm, pore diameter ca. 6 nm) silica gel. The water used in the experiments with bovine serum albumin (BSA) was deionized using a Labconco Water Pro PS purification system. THF and Et_2O were distilled under nitrogen from sodium/benzophenone. CH_2Cl_2 was distilled under nitrogen from CaH_2. Styrene was distilled under reduced pressure. Anhydrous DMF was obtained from Biosolve. BSA was obtained from Sigma.

General Instrumentation. NMR spectra were recorded on Bruker DMX300 (300 and 75 MHz for ^1H and ^{13}C, respectively) and Varian Inova 400 (400 MHz) spectrometers. ^1H NMR chemical shifts (δ) are reported in parts per million (ppm) relative to a residual proton peak of the solvent, $\delta = 7.26$ for $CDCl_3$, and $\delta = 2.50$ for DMSO-d_6. Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), dt (doublet of triplets), dq (double quartet), ddd (double, double doublet), ddt (double, double triplet), or m (multiplet). Broad peaks are indicated by the addition of br. Coupling constants are reported as a J value in hertz (Hz). The number of protons (n) for a given resonance is indicated as nH and is based on spectral integration values. ^{13}C NMR chemical shifts (δ) are reported in ppm relative to DMSO-d_6 ($\delta = 39.5$) or $CDCl_3$ ($\delta = 77.0$). IR spectra were recorded on an ATI Matson Genesis Series FTIR spectrometer fitted with an ATR cell. The vibrations (ν) are given in cm^{-1}. Molecular weight distributions were measured with a Shimadzu SEC, equipped with a guard column and a PL gel 5 μm mixed D column (Polymer Laboratories) with differential refractive index (RI) and UV ($\lambda = 254$ nm and $\lambda = 340$ nm) detection using THF as an eluent (1 mL/min at 35 °C). Poly(ethylene glycol) (PEG) standards were used for calibration. Matrix assisted laser desorption/ionization time-of-flight (MALDI-ToF) spectra were measured on a Bruker Biflex III spectrometer, and samples were prepared from THF solutions using indoleacrylic acid (IAA) (20 mg/mL) as a matrix. High resolution mass spectra were recorded on a JEOL AccuToF (ESI) or a MAT900 (EI, CI, and ESI). UV−vis spectra were measured on a Varian Cary 50 spectrophotometer equipped with a RM6 Lauda temperature controller using a 1 cm path-length quartz cuvette. Protein conjugates mixtures were analyzed with a Pharmacia SMART high performance liquid chromatography (HPLC) system, equipped with a Superdex 75 PC 3.2/30 column (optimal separation 3−70 kDa) (Pharmacia) using phosphate buffer (20 mM, pH 7.5) as an eluent (50 μL/min at room temperature) in combination with UV detection ($\lambda = 280$ nm and $\lambda = 340$ nm). Prior to injection, crude reaction mixtures were diluted to a protein concentration of 1.25 mg/mL and filtered over a 0.2 μm nylon filter. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed using 10% polyacrylamide gels. To denaturate the samples, β-mercapto ethanol was added, and the samples were heated at 95 °C for 10 min. Staining was accomplished using Coomassie blue.

Fluorescence Experiments. Fluorescence measurements were performed on a Perkin-Elmer luminescence spectrometer LS 55 equipped with a RM6 Lauda temperature controller fixed at 23 °C. A 1 cm path-length quartz cuvette was used, and after measurements of protein containing samples, the cuvette was washed thoroughly with deionized water and EtOH. Samples from the bioconjugation experiments were prepared by withdrawing 40 μL of reaction medium followed by dilution in the reaction buffer to a protein concentration of 0.59 μg/mL. The excitation wavelength was set at 340 nm, and the spectra were recorded using excitation and emission slits of 10 nm.

3-Azido-7-hydroxy Coumarin (1). This compound was prepared according to a literature procedure and was obtained as a light brown solid (12% yield). ^1H NMR (DMSO-d_6, 400 MHz): $\delta = 10.53$ (s, 1 H, O*H*), 7.60 (s, 1 H, coumarin H_4), 7.48 (d, $J = 8.5$ Hz, 1 H, coumarin H_5), 6.81 (dd, $J = 8.5$, 2.3 Hz, 1 H, coumarin H_6), 6.76 (d, $J = 2.3$ Hz, 1 H, coumarin H_8). ^{13}C NMR (DMSO, 75 MHz): $\delta = 160.3$, 157.3, 152.7, 129.1, 127.8, 121.1, 113.8, 111.3, 102.0. FT-IR (ATR): 3291, 2115 (νN_3), 1679, 1616, 1303.

3-Azido-7-(*tert*-butylcarboxy methoxy) Coumarin (2). 3-Azido-7-hydroxy-coumarin (803 mg; 3.95 mmol) and K_2CO_3 (580 mg; 4.20 mmol) were suspended in anhydrous DMF (15 mL) under an argon atmosphere and stirred for 10 min at room temperature. The mixture was heated to 40 °C, and *tert*-butyl-2-bromoacetate (584 μL; 3.96 mmol) was added. The mixture was stirred for 3.5 h at 40 °C, after which completion of the reaction was determined by TLC (EtOAc/heptane; 1:1 v/v). After dilution with EtOAc (200 mL), the mixture was washed with water (2 × 150 mL). The aqueous phases were extracted with EtOAc (100 mL), and the combined organic phases were dried over anhydrous Na_2SO_4 and filtered. Upon concentration under reduced pressure, the crude product was obtained as a brown oil. The oil was dissolved in CH_2Cl_2 (10 mL), and this solution was added dropwise to *n*-pentane (400 mL) under stirring, forming a brown precipitate and a yellow solution. After filtration, the precipitation procedure was repeated for the residue. The filtrates were combined and concentrated under reduced pressure, affording the product as a yellowish-brown solid (960 mg, 77%) $R_f = 0.7$ (EtOAc/heptane; 1/1), ^1H NMR (DMSO-d_6, 300 MHz): $\delta = 7.63$ (s, 1H, coumarin H_4), 7.58 (d, $J = 8.6$ Hz, 1H, coumarin H_5), 7.02 (d, $J = 2.4$ Hz, 1H, coumarin H_8), 6.97 (dd, $J = 2.5$, 8.6 Hz, 1H, coumarin H_6), 4.8 (s, 2H, OC*H*$_2$CO$_2$), 1.43 (s, 9H, O^{t}Bu). ^{13}C NMR (DMSO-d_6, 75 MHz): $\delta = 167.3$, 159.7, 157.1, 152.4, 128.8, 127.1, 122.6, 113.2, 101.4, 81.7, 65.3, 27,7. FT-IR (ATR): 2120 (νN_3), 1744, 1722, 1618, 1139.

3-Azido-7-(carboxy methoxy) Coumarin (3). To a solution of 3-azido-7-(*tert*-butylcarboxy methoxy) coumarin (**2**; 700 mg, 2.21 mmol) in CH_2Cl_2 (30 mL) was added trifluoro acetic acid (TFA; 1.5 mL, 20 mmol), and the mixture was stirred at room temperature for 18 h. The reaction was determined to be almost complete by TLC (EtOAC/heptane, 1/1). An extra amount of TFA (1.5 mL, 20 mmol) was added, and the mixture was

allowed to stir for an additional 3 h. The reaction mixture was diluted with CH_2Cl_2 (150 mL) and washed with water (75 mL) and a 10% aqueous solution of citric acid (75 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo*, yielding an brownish-orange solid (434 mg, 75%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 13.13 (br s, 1H, COO*H*), 7.64 (s, 1H, coumarin H_4), 7.58 (d, J = 8.6 Hz, 1H, coumarin H_5), 7.03 (d, J = 2.5 Hz, 1H, coumarin H_8), 6.98 (dd, J = 2.5, 8.6 Hz, 1H, coumarin H_6), 4.81 (s, 2H, OC*H*$_2$COOH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 169.6, 159.8, 157.1, 152.4, 128.8, 127.2, 122.5, 113.2, 112.9, 101.4, 64.9. FT-IR (ATR): 2152 (νN$_3$), 1748, 1725, 1621, 1245, 1221, 1156. HRMS (ESI-) calcd for $C_{11}H_6N_3O_5$ (M − H)$^-$: 260.03075; found, 260.03172.

Ester-Linked 3-Azido-coumarin Terminated Poly(ethylene glycol). Polymers **4** and **5** were prepared following a similar procedure. Procedure for **4**: To a flame-dried Schlenk vessel were added α-methoxy poly(ethylene glycol) (mPEG, M_n = 2000 g/mol; 259 mg, 0.130 mmol), 3-azido-7-(carboxy methoxy) coumarin (**3**; 51 mg, 0.20 mmol), 4-(dimethylamino)-pyridine (DMAP; 9 mg, 0.07 mmol), and anhydrous CH_2Cl_2 (4 mL) under an argon atmosphere. Next, the mixture was cooled to 0 °C and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl; 38 mg, 0.20 mmol) was added. The mixture was stirred at 0 °C for 1 h followed by 40 h at room temperature. After dilution with CH_2Cl_2 (150 mL), the mixture was washed with a saturated aqueous solution of $NaHCO_3$ (2 × 100 mL) and a saturated aqueous solution of NH_4Cl (100 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure until approximately 5 mL of the mixture remained. Upon precipitation in Et_2O (450 mL), the product was obtained as a yellow−orange solid (216 mg, 73%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.35 (d, J = 8.7 Hz, 1H, coumarin H_5), 7.18 (s, 1H, coumarin H_4), 6.92 (dd, J = 2.5, 8.7 Hz, 1H, coumarin H_6), 6.82 (d, J = 2.4 Hz, 1H, coumarin H_8), 4.73 (s, 2H, OC*H*$_2$CO$_2$), 4.41−4.36 (m, 2H, CH$_2$C*H*$_2$OCO), 3.64 (br s, 180H, PEG backbone), 3.38 (s, 3H, OC*H*$_3$). FT-IR (ATR): 2883, 2123 (νN$_3$), 1960, 1725, 1619, 1466, 1341, 1102. SEC (THF): M_n = 1830 g/mol; M_n/M_w = 1.22.

α-Methoxy ω-(2-Cyanoethyl) Poly(ethylene glycol) (6). A solution of α-methoxy poly(ethylene glycol) (mPEG, M_n = 2000 g/mol; 4.0 g, 2.0 mmol) in acrylonitrile (20 mL) was cooled to 0 °C before adding KOH (10 mg, 0.18 mmol). The mixture was stirred at 0 °C for 2.5 h, after which the mixture turned slightly yellow. At this point, the reaction was quenched by the addition of a drop of concentrated HCl. Subsequently, the mixture was concentrated under reduced pressure resulting in an off-white solid (3.94 g, 96%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 3.73 (t, J = 6.4 Hz, 2H, overlap with signal at 3.64 ppm, OC*H*$_2$CH$_2$CN), 3.64 (br s, 180H, PEG backbone), 3.38 (s, 3H, OC*H*$_3$), 2.64 (t, J = 6.4 Hz, 2H, OCH$_2$C*H*$_2$CN). FT-IR (ATR): 2883, 2249, 1960, 1466, 1340, 1106.

α-Methoxy ω-(3-Aminopropyl) Poly(ethylene glycol) (7). To a cooled (0 °C) solution of borane−THF complex (5 mL 1 M in THF, 5 mmol) in anhydrous THF (15 mL) was added a solution of α-methoxy ω-(2-cyanoethyl) PEG (**6**; 2.01 g, 0.979 mmol) in anhydrous THF (10 mL) under an argon atmosphere. The mixture was stirred at 0 °C for 30 min, after which it was heated to reflux for 4 h. The mixture was cooled again to 0 °C, and MeOH (5 mL) was added dropwise (resulting in the evolution of hydrogen gas). Next, HCl (0.25 mL) was added slowly, and the mixture was stirred for 1 h, after which it was concentrated under reduced pressure. Trimethyl borate was removed by coevaporation with MeOH (3 × 50 mL). An aqueous solution of NaOH (1 M, 50 mL) was added to the residue, and the resulting mixture was extracted with CH_2Cl_2 (2 × 100 mL). The organic phases were dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo*, yielding a white solid (1.94 g, 96%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 3.64 (br s, 180H, PEG backbone), 3.55 (m, 2H, overlap with signal at 3.64 ppm, OC*H*$_2$CH$_2$CH$_2$), 3.38 (s, 3H, OC*H*$_3$), 2.80 (t, J = 6.2 Hz, 2 H, CH$_2$C*H*$_2$NH$_2$), 1.73 (m, 2H, OCH$_2$C*H*$_2$CH$_2$). FT-IR (ATR): 2882, 1960, 1466, 1340, 1101.

Amide-Linked 3-Azido Coumarin Terminated Poly(ethylene glycol) (8). To a flame-dried Schlenk vessel were added α-methoxy ω-(3-aminopropyl) poly(ethylene glycol) (**7**; 252 mg, 0.122 mmol), 3-azido-7-(carboxy methoxy) coumarin (**3**; 48 mg, 0.18 mmol), 4-(dimethylamino)-pyridine (DMAP; 38 mg, 0.31 mmol), and anhydrous CH_2Cl_2 (4 mL) under an argon atmosphere. Next, the mixture was cooled to 0 °C and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl; 36 mg, 0.19 mmol) was added. The mixture was stirred at 0 °C for 1 h followed by 40 h at room temperature. After dilution with CH_2Cl_2 (150 mL), the mixture was washed with a saturated aqueous solution of $NaHCO_3$ (2 × 100 mL) and a 10% aqueous solution of citric acid (100 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure, affording a yellow−orange solid (249 mg, 88%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.39 (d, J = 8.6 Hz, 1H, coumarin H_5), 7.2 (s, 1H, coumarin H_4), 7.16 (br s, 1H, CH$_2$N*H*CO), 6.93 (dd, J = 2.5, 8.6 Hz, 1H, coumarin H_6), 6.90 (d, J = 2.4 Hz, 1H, coumarin H_8), 4.53 (s, 2H, OC*H*$_2$CONH), 3.64 (br s, 180H, PEG backbone), 3.60−3.53 (m, overlaps with signal at 3.64 ppm), 3.38 (s, 3H, OC*H*$_3$), 1.83 (q, J = 5.9 Hz, 2H, OCH$_2$C*H*$_2$CH$_2$). FT-IR (ATR): 2883, 2125 (νN$_3$), 1964, 1714, 1666, 1619, 1466, 1341. 1104. SEC (THF): M_n = 1830 g/mol; M_n/M_w = 1.24.

***N*-Propargyl Maleimide (9).** Propargylamine (330 μL, 4.84 mmol) was dissolved in a saturated aqueous solution of $NaHCO_3$ (40 mL) and cooled on an ice bath. Then *N*-(methoxy carbonyl) maleimide (751 mg, 4.81 mmol) was added in portions over 20 min under vigorous stirring. The mixture was stirred for 30 min at 0 °C, followed by 45 min at room temperature. After extraction with CH_2Cl_2 (3 × 50 mL), the organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo*. Purification by silica gel column chromatography (EtOAc/heptane, 1:2) yielded the product as a colorless oil after coevaporation with CH_2Cl_2 and would eventually lead to white crystals (291 mg, 45% yield). R_f = 0.56 (EtOAc/heptane, 1:1 v/v) ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 6.76 (s, 2H, *H*C=C*H*); 4.30 (d, J = 2.5 Hz, 2H, C*H*$_2$C'CH); 2.22 (t, J = 2.5 Hz, 1H, C'C*H*) ^{13}C NMR (75 MHz, $CHCl_3$): δ = 169.2, 134.4, 76.9, 71.5, 26.8. FT-IR (ATR): 3272, 3100, 2972, 2127, 1698, 1430, 1402, 1315, 1348, 1154 cm^{-1}. HRMS (EI+) *m/z* calcd for $C_7H_5NO_2$: 135.0320; found, 135.0324.

3-(2-Hydroxy ethylsulfanyl)-*N*-propargyl-succinimide (18). 2-Mercaptoethanol (25 μL, 0.36 mmol) was added to a solution of *N*-propargyl-maleimide (40 mg, 0.30 mmol) and Et_3N (50 μL, 0.36 mmol) in anhydrous CH_2Cl_2 (2 mL), under an argon atmosphere. The mixture was stirred at room temperature for 1 h. After dilution with CH_2Cl_2 (10 mL), the mixture was washed with 1 M aqueous HCl (2 × 10 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure, affording the crude product as a colorless oil. Purification by preparative TLC (EtOAc/heptane, 4:1 v/v) yielded the desired compound as a colorless oil (26 mg, 41% yield). R_f = 0.41 (EtOAc/heptane, 4:1 v/v) ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 4.28 (d, J = 2.5 Hz, 2H, C*H*$_2$C'CH), 3.91 (dd, J = 4.1, 9.3 Hz, 1H, CH$_2$C*H*S), 3.92−3.87 (m, 2H, CH$_2$C*H*$_2$OH), 3.22 (dd, J = 9.3, 18.8 Hz, 1H, C*H*$_2$CHS), 3.16 (ddd, J = 4.8, 6.1, 14.4 Hz, 1H, SC*H*$_2$), 2.91 (ddd, J = 4.9, 6.4, 14.4 Hz, 1H, SC*H*$_2$), 2.60 (dd, J = 4.1, 18.8 Hz, 1H, C*H*$_2$CHS) 2.63−2.58 (br m, 1H, O*H*), 2.22 (t, J = 2.5 Hz, 1H,

C'C*H*). ^{13}C NMR (75 MHz, $CHCl_3$): δ = 176.2, 173.0, 76.1, 71.8, 61.8, 39.5, 36.3, 35.7, 28.2. FT-IR (ATR): 3421, 3282, 2925, 2850, 2565, 2250, 2127, 1703, 1422, 1392, 1343, 1174, 1060 cm^{-1}. HRMS (ESI+) *m/z* calcd for $C_7H_{11}NO_3SNa$: 236.03544 $(M + Na)^+$; found, 236.03573.

CuAAC between 3-(2-Hydroxy ethylsulfanyl)-*N*-propargyl-succinimide (12) and 3-Azido-coumarin Terminated Poly(ethylene glycol) (4). To a solution of 3-(2-hydroxy ethylsulfanyl)-*N*-propargyl-succinimide (12.5 mg, 58.6 μmol) and 3-azido coumarin terminated PEG **4** (50 mg, 22 μmol) in THF (1.5 mL) was added a mixture of $CuSO_4 \cdot 5H_2O$ (7.0 mg, 28 μmol) and Na-ascorbate (8.3 mg, 42 μmol) in water (1 mL). Upon stirring for 14 h at room temperature, the mixture showed a strong fluorescence when irradiated with UV light (λ = 366 nm). The mixture was diluted with an aqueous solution of ethylenediaminetetraacetic acid (EDTA; 25 mL, 0.065 M) and extracted with CH_2Cl_2 (3 × 25 mL). The combined organic layers were washed with water (25 mL) and dried over anhydrous Na_2SO_4. After filtering and drying *in vacuo*, the crude product was taken up in CH_2Cl_2 (1 mL) and precipitated in Et_2O (15 mL). Upon centrifugation, a yellowish pellet was obtained, which was washed with Et_2O and dried *in vacuo* to yield an off-white to yellowish solid (26 mg, 47% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.60 (s, 1H, triazole H), 8.49 (s, 1H, coumarin H_4), 7.59 (d, J = 8.8 Hz, 1H, coumarin H_5), 7.01 (dd, J = 2.5, 8.7 Hz, 1H, coumarin H_6), 6.89 (d, J = 2.4 Hz, 1H, coumarin H_8), 4.91 (s, 1H, OC*H*$_2$CO$_2$), 4.78 (s, 1H, NC*H*$_2$C=CH), 4.41−4.38 (m, 2H, CH_2C*H*$_2$OCO), 3.91 (dd, J = 4.0, 9.3 Hz, 1H, CH_2C*H*S), 3.89−3.83 (m, 2H, CH_2C*H*$_2$OH), 3.65 (br s, PEG backbone), 3.37 (s, 3H, OC*H*$_3$), 3.22 (dd, J = 9.3, 18.8 Hz, 1H, C*H*$_2$CHS), 3.09 (ddd, J = 4.7, 6.1, 14.4 Hz, 1H, SC*H*$_2$), 2.89 (ddd, J = 4.9, 6.4, 14.4 Hz, 1H, SC*H*$_2$), 2.67 (dd, J = 4.0, 18.8 Hz, 1H, C*H*$_2$CHS). MALDI-ToF MS (IAA): calcd for $[M + Na]^+$ n = 45; 2492.3, *m/z* found = 2492.1.

Alkyne-Functionalized Bovine Serum Albumin (10). BSA (66 mg, 1.0 μmol) was placed in a Greiner tube and dissolved in phosphate buffer (PB) (1.5 mL; 50 mM, pH 7.5) by gently mixing for 30 min. Next, a solution of *N*-propargyl maleimide (8 mg, 59 μmol) in THF (0.5 mL) was added, and the mixture was gently mixed on a roller bank for 1.5 h, with exclusion of light. Excess *N*-propargyl maleimide was removed by passing the mixture through a sephadex (G50) column using phosphate buffer (50 mM, pH 7.5) as the eluent. The fractions containing BSA (judged by UV−vis spectroscopy) were combined and stored at 4 °C until further use (typically within a few days). The final concentration was determined by UV−vis spectroscopy using a molar extinction coefficient of 42316 M^{-1} cm^{-1}.

CuAAC of Alkyne-Functionalized BSA (10) and 3-Azido-7-hydroxy-coumarin (1). To an Eppendorf tube was added a solution of alkyne-functionalized BSA **10** (2.1 mg, $3.2 \cdot 10^{-2}$ μmol) in PB (250 μL, 50 mM, pH 7.5) and a solution of 3-azido-7-hydroxy-coumarin (0.18 mg, 0.89 μmol) in THF (54 μL). After the addition of a catalyst mixture (54 μL) containing $CuSO_4 \cdot 5H_2O$ (0.16 mg, 0.63 μmol) and Na-ascorbate (0.19 mg, 0.95 μmol) in PB (50 mM, pH 7.5), the reaction vial was placed on a roller bank for 18 h. The mixture was analyzed by fluorescence spectroscopy and SDS−PAGE without further purification.

Typical Procedure for CuAAC between Alkyne-Functionalized BSA (10) and 3-Azido-coumarin Terminated PEGs. A Greiner tube was loaded with an argon-purged solution of alkyne-functionalized BSA **10** (2.5 mg, $3.8 \cdot 10^{-2}$ μmol) in PB (300 μL, 50 mM, pH 7.5) and a solution of PEG **4** (0.93 mg, 0.41 μmol) in argon-purged PB (100 μL). After the addition of a catalyst mixture (100 μL) containing $CuSO_4 \cdot 5H_2O$ (0.19 mg, 0.76 μmol) and Na-ascorbate (0.29 mg, 1.46 μmol) in argon-purged PB (50 mM, pH 7.5), the reaction mixture was gently shaken, and samples were withdrawn periodically for spectroscopic analysis. Reactions between alkyne-functionalized BSA **10** and PEG **5** or **6** were carried out using a comparable protocol.

ACKNOWLEDGMENT

We thank Eva Sans i Atxer and Eline Mol for their assistance at the initial stage of this research. This work was financially supported by the research school NRSC-Catalysis, the Chemical Council of The Netherlands Organization for Scientific Research (CW-NWO), the European Science Foundation (ESF), and the Royal Netherlands Academy of Arts and Sciences (KNAW).

Supporting Information Available: Fluorogenic conjugation to BSA. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Duncan, R. (2003) The dawning era of polymer therapeutics. *Nat. Rev. Drug Discovery 2*, 347–360.
(2) Duncan, R. (2006) Polymer conjugates as anticancer nanomedicines. *Nat. Rev. Cancer 6*, 688–701.
(3) Hoffman, A. S., and Stayton, P. S. (2004) Bioconjugates of smart polymers and proteins: Synthesis and applications. *Macromol. Symp. 207*, 139–151.
(4) Thordarson, P., Le Droumaguet, B., and Velonia, K. (2006) Well-defined protein-polymer conjugates: synthesis and potential applications. *Appl. Microbiol. Biotechnol. 73*, 243–254.
(5) Vandermeulen, G. W. M., and Klok, H.-A. (2004) Peptide/protein hybrid materials: Enhanced control of structure and improved performance through conjugation of biological and synthetic polymers. *Macromol. Biosci. 4*, 383–398.
(6) Abuchowski, A., Vanes, T., Palczuk, N. C., and Davis, F. F. (1977) Alteration of Immunological properties of bovine serum-albumin by covalent attachment of polyethylene-glycol. *J. Biol. Chem. 252*, 3578–3581.
(7) Abuchowski, A., McCoy, J. R., Palczuk, N. C., Vanes, T., and Davis, F. F. (1977) Effect of covalent attachment of polyethyleneglycol on immunogenicity and circulating life of bovine liver catalase. *J. Biol. Chem. 252*, 3582–3586.
(8) Veronese, F. M., and Harris, J. M. (2002) Preface: Introduction and overview of peptide and protein pegylation. *Adv. Drug Delivery Rev. 54*, 453–456.
(9) Roberts, M. J., Bentley, M. D., and Harris, J. M. (2002) Chemistry for peptide and protein PEGylation. *Adv. Drug Delivery Rev. 54*, 459–476.
(10) Pasut, G., and Veronese, F. M. (2007) Polymer-drug conjugation, recent achievements and general strategies. *Prog. Polym. Sci. 32*, 933–961.
(11) Pasut, G., Veronese, F. M. (2006) PEGylation of Proteins As Tailored Chemistry for Optimized Bioconjugates, in *Polymer Therapeutics I: Polymers as Drugs, Conjugates and Gene Delivery Systems*, Vol. 192, pp 95−134, Springer Publishing, New York.
(12) Veronese, F. M. (2001) Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials 22*, 405–417.
(13) Harris, J. M., and Chess, R. B. (2003) Effect of pegylation on pharmaceuticals. *Nat. Rev. Drug Discovery 2*, 214–221.
(14) Veronese, F. M., and Harris, J. M. (2008) Peptide and protein PEGylation III: advances in chemistry and clinical applications. *Adv. Drug Delivery Rev. 60*, 1–2.
(15) Hoffman, A. S., and Stayton, P. S. (2007) Conjugates of stimuli-responsive polymers and proteins. *Prog. Polym. Sci. 32*, 922–932.
(16) Maynard, H. D., Heredia, K. L., Li, R. C., Parra, D. P., and Vazquez-Dorbatt, V. (2007) Thermoresponsive biohybrid materials synthesized by ATRP. *J. Mater. Chem. 17*, 4015–4017.
(17) Heredia, K. L., Bontempo, D., Ly, T., Byers, J. T., Halstenberg, S., and Maynard, H. D. (2005) In situ preparation of protein:

"Smart" polymer conjugates with retention of bioactivity. *J. Am. Chem. Soc. 127*, 16955–16960.
(18) Dirks, A. J., Nolte, R. J. M., and Cornelissen, J. J. L. M. (2008) Protein-polymer hybrid amphiphiles. *Adv. Mater. 20*, 3953–3957.
(19) Reynhout, I. C., Cornelissen, J. J. L. M., and Nolte, R. J. M. (2007) Self-assembled architectures from biohybrid triblock copolymers. *J. Am. Chem. Soc. 129*, 2327–2332.
(20) Le Droumaguet, B., Mantovani, G., Haddleton, D. M., and Velonia, K. (2007) Formation of giant amphiphiles by post-functionalization of hydrophilic protein-polymer conjugates. *J. Mater. Chem. 17*, 1916–1922.
(21) Boerakker, M. J., Botterhuis, N. E., Bomans, P. H. H., Frederik, P. M., Meijer, E. M., Nolte, R. J. M., and Sommerdijk, N. A. J. M. (2006) Aggregation Behavior of giant amphiphiles prepared by cofactor reconstitution. *Chem.−Eur. J. 12*, 6071–6080.
(22) Dirks, A. J., van Berkel, S. S., Hatzakis, N. S., Opsteen, J. A., van Delft, F. L., Cornelissen, J. J. L. M., Rowan, A. E., van Hest, J. C. M., Rutjes, F. P. J. T., and Nolte, R. J. M. (2005) Preparation of biohybrid amphiphiles via the copper catalysed Huisgen [3 + 2] dipolar cycloaddition reaction. *Chem. Commun.* 4172–4174.
(23) Velonia, K., Rowan, A. E., and Nolte, R. J. M. (2002) Lipase polystyrene giant amphiphiles. *J. Am. Chem. Soc. 124*, 4224–4225.
(24) Boerakker, M. J., Hannink, J. M., Bomans, P. H. H., Frederik, P. M., Nolte, R. J. M., Meijer, E. M., and Sommerdijk, N. A. J. M. (2006) Giant amphiphiles by cofactor reconstitution. *Angew. Chem., Int. Ed. 41*, 4239–4241.
(25) Gauthier, M. A., and Klok, H.-A. (2008) Peptide/protein-polymer conjugates: synthetic strategies and design concepts. *Chem. Commun.* 2591–2611.
(26) Heredia, K. L., and Maynard, H. D. (2007) In situ preparation of protein: "Smart" polymer conjugates with retention of bioactivity. *Org. Biomol. Chem. 5*, 45–53.
(27) Nicolas, J., Mantovani, G., and Haddleton, D. M. (2007) Living radical polymerization as a tool for the synthesis of polymer-protein/peptide bioconjugates. *Macromol. Rapid Commun. 28*, 1083–1111.
(28) Mantovani, G., Lecolley, F., Tao, L., Haddleton, D. M., Clerx, J., Cornelissen, J. J. L. M., and Velonia, K. (2005) Design and synthesis of N-maleimido-functionalized hydrophilic polymers via copper-mediated living radical polymerization: A suitable alternative to PEGylation chemistry. *J. Am. Chem. Soc. 127*, 2966–2973.
(29) Bontempo, D., Heredia, K. L., Fish, B. A., and Maynard, H. D. (2004) Cysteine-reactive polymers synthesized by atom transfer radical polymerization for conjugation to proteins. *J. Am. Chem. Soc. 126*, 15372–15373.
(30) Rosendahl, M. S., Doherty, D. H., Smith, D. J., Carlson, S. J., Chlipala, E. A., and Cox, G. N. (2005) A long-acting, highly potent interferon alpha-2 conjugate created using site-specific PEGylation. *Bioconjugate Chem. 16*, 200–207.
(31) Connor, R. E., and Tirrell, D. A. (2007) Non-canonical amino acids in protein polymer design. *Polym. Rev. 47*, 9–28.
(32) Rose, K. (1994) Facile Synthesis of Homogeneous Artificial Proteins. *J. Am. Chem. Soc. 116*, 30–33.
(33) Kochendoerfer, G. G., Chen, S. Y., Mao, F., Cressman, S., Traviglia, S., Shao, H. Y., Hunter, C. L., Low, D. W., Cagle, E. N., Carnevali, M., Gueriguian, V., Keogh, P. J., Porter, H., Stratton, S. M., Wiedeke, M. C., Wilken, J., Tang, J., Levy, J. J., Miranda, L. P., Crnogorac, M. M., Kalbag, S., Botti, P., Schindler-Horvat, J., Savatski, L., Adamson, J. W., Kung, A., Kent, S. B. H., and Bradburne, J. A. (2003) Design and chemical synthesis of a homogeneous polymer-modified erythropoiesis protein. *Science 299*, 884–887.
(34) Schlick, T. L., Ding, Z. B., Kovacs, E. W., and Francis, M. B. (2005) Dual-surface modification of the tobacco mosaic virus. *J. Am. Chem. Soc. 127*, 3718–3723.
(35) Heredia, K. L., Tolstyka, Z. P., and Maynard, H. D. (2007) Aminooxy end-functionalized polymers synthesized by ATRP for chemoselective conjugation to proteins. *Macromolecules 40*, 4772–4779.
(36) Christman, K. L., Broyer, R. M., Tolstyka, Z. P., and Maynard, H. D. (2007) Site-specific protein immobilization through N-terminal oxime linkages. *J. Mater. Chem. 17*, 2021–2027.
(37) Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise Huisgen cycloaddition process: Copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem., Int. Ed. 41*, 2596–2599.
(38) Tornøe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem. 67*, 3057–3064.
(39) Bock, V. D., Hiemstra, H., and van Maarseveen, J. H. (2005) Cu-I-catalyzed alkyne-azide "click" cycloadditions from a mechanistic and synthetic perspective. *Eur. J. Org. Chem.* 51–68.
(40) Wu, P., and Fokin, V. V. (2007) Catalytic azide-alkyne cycloaddition: Reactivity and applications. *Aldrichimica Acta 40*, 7–17.
(41) Kolb, H. C., Finn, M. G., and Sharpless, K. B. (2001) Click chemistry: Diverse chemical function from a few good reactions. *Angew. Chem., Int. Ed. 40*, 2004–2021.
(42) Kolb, H. C., and Sharpless, K. B. (2003) The growing impact of click chemistry on drug discovery. *Drug Discovery Today 8*, 1128–1137.
(43) Dirks, A. J., Cornelissen, J. J. L. M., van Delft, F. L., van Hest, J. C. M., Nolte, R. J. M., Rowan, A. E., and Rutjes, F. P. J. T. (2007) From (bio)molecules to biohybrid materials with the click chemistry approach. *QSAR Comb. Sci. 26*, 1200–1210.
(44) Breinbauer, R., and Kohn, M. (2003) Azide-alkyne coupling: A powerful reaction for bioconjugate chemistry. *ChemBioChem 4*, 1147–1149.
(45) Binder, W. H., and Sachsenhofer, R. (2007) 'Click' chemistry in polymer and materials science. *Macromol. Rapid Commun. 28*, 15–54.
(46) Lutz, J.-F. (2007) 1,3-dipolar cycloadditions of azides and alkynes: A universal ligation tool in polymer and materials science. *Angew. Chem., Int. Ed. 46*, 1018–1025.
(47) Deiters, A., Cropp, T. A., Summerer, D., Mukherji, M., and Schultz, P. G. (2004) Site-specific PEGylation of proteins containing unnatural amino acids. *Bioorg. Med. Chem. Lett. 14*, 5743–5745.
(48) Schoffelen, S., Lambermon, M. H. L., van Eldijk, M. B., and van Hest, J. C. M. (2008) Site-specific modification of candida antartica lipase B via residue-specific incorporation of a non-canonical amino acid. *Bioconjugate Chem. 19*, 1127–1131.
(49) Sen Gupta, S., Raja, K. S., Kaltgrad, E., Strable, E., and Finn, M. G. (2005) Virus-glycopolymer conjugates by copper(I) catalysis of atom transfer radical polymerization and azide-alkyne cycloaddition. *Chem. Commun.* 4315–4317.
(50) Li, M., De, P., Gondi, S. R., and Summerlin, B. S. (2008) responsive polymer-protein bioconjugates perpared by RAFT polymerization and copper-catalyzed azide-alkyne click chemistry. *Macromol. Rapid Commun. 29*, 1172–1176.
(51) Sivakumar, K., Xie, F., Cash, B. M., Long, S., Barnhill, H. N., and Wang, Q. (2004) A fluorogenic 1,3-dipolar cycloaddition reaction of 3-azidocoumarins and acetylenes. *Org. Lett. 6*, 4603–4606.
(52) O'Reilly, R. K., Joralemon, M. J., Hawker, C. J., and Wooley, K. L. (2006) Fluorogenic 1,3-dipolar cycloaddition within the hydrophobic core of a shell cross-linked nanoparticle. *Chem.−Eur. J. 12*, 6776–6786.
(53) Beatty, K. E., Liu, J. C., Xie, F., Dieterich, D. C., Schuman, E. M., Wang, Q., and Tirrell, D. A. (2006) Fluorescence visualization of newly synthesized proteins in mammalian cells. *Angew. Chem., Int. Ed. 45*, 7364–7367.
(54) Beatty, K. E., Xie, F., Wang, Q., and Tirrell, D. A. (2005) Selective dye-labeling of newly synthesized proteins in bacterial cells. *J. Am. Chem. Soc. 127*, 14150–14151.
(55) Zeng, Q. B., Li, T., Cash, B., Li, S. Q., Xie, F., and Wang, Q. (2007) Chemoselective derivatization of a bionanoparticle by click reaction and ATRP reaction. *Chem. Commun.* 1453–1455.

(56) van Berkel, S. S., Dirks, A. J., Debets, M. F., van Delft, F. L., Cornelissen, J. J. L. M., Nolte, R. J. M., and Rutjes, F P. J. T. (2007) Metal-free triazole formation as a tool for bioconjugation. *ChemBioChem 8*, 1504–1508.

(57) Xie, F., Sivakumar, K., Zeng, Q. B., Bruckman, M. A., Hodges, B., and Wang, Q. (2008) A fluorogenic 'click' reaction of azidoanthracene derivatives. *Tetrahedron 64*, 2906–2914.

(58) Zhou, Z., and Fahrni, C. J. (2004) A fluorogenic probe for the copper(I)-catalyzed azide-alkyne ligation reaction: Modulation of the fluorescence emission via $3(n,\pi^*)$-$^1(\pi, \pi^*)$ inversion. *J. Am. Chem. Soc. 126*, 8862–8863.

BC8004667