A General Methodology Toward Drug/Dye Incorporated Living Copolymer−Protein Hybrids: (NIRF Dye-Glucose) Copolymer−Avidin/BSA Conjugates as Prototypes

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Azide-terminated poly(*tert*-butyl acrylate) was synthesized via atom transfer radical polymerization (ATRP). Subsequent deprotection was performed to yield poly(acrylic acid) (PAA) possessing a reactive chain-end. A one-pot sequential amidation of the PAA with the amine derivatives of a near-infrared fluorescent dye (ADS832WS) and glucose produced NIRF dye-incorporated water-soluble copolymers. End-group modifications were performed to produce alkyne/biotin-terminated copolymers which were further employed to generate dye-incorporated polymer−protein hybrids via the biotin−avidin interaction with avidin or "click" bioconjugation with azide-modified BSA. We have overcome two fundamental limitations in the synthesis of bioconjugates: (a) the basic restriction in the diversity of copolymers which can be synthesized for producing bioconjugates, (b) the limitation in the number of dyes/drug molecules that can be attached per protein molecule. The copolymers possessed enhanced optical properties compared to the dye due to increased solubility in water. Potential utility of these copolymers and conjugates in multiwell plate based assays, cell surface imaging and in vivo animal imaging were explored.

INTRODUCTION

Polymer−protein hybrids are a newly emerging class of bioconjugates with several applications in biotechnology, biopharmaceutical chemistry, and other life science areas (*1*−*4*). There are, however, some fundamental limitations in the current methodologies available for the synthesis of these hybrids: (a) Using current technologies, the number of copies of cytotoxic drugs/dyes that can be chemically conjugated to a protein (antibody, avidin/streptavidin, etc.) is limited. (b) There is a basic limitation in the diversity of copolymers that can be synthesized for bioconjugation. The former limitation arises from the fact that extensive modification of proteins with several copies of a drug/dye would cause deactivation of the active sites and because of the limited numbers of functional groups available per protein molecule for bioconjugation. The latter limitation (in the diversity of copolymers synthesized for bioconjugation) arises from (a) widely different reactivity between monomers and (b) the lack of reactivity of many biologically relevant acrylates (e.g., an acrylate derivative of the anticancer drug candidate curcumin can be synthesized but will not polymerize since the molecule is a radical quencher) (*5*). Biological properties (e.g., bioactivity and self-assembly) of polymer−protein hybrids demands the synthesis of well-defined polymers for bioconjugation (*6*−*8*). In the early embodiments, the polymer component of the conjugates was synthesized via uncontrolled free radical polymerization (*9*).

The synthesis of well-defined polymer−protein hybrids in which the polymers are synthesized via controlled radical polymerization methods (such as atom transfer radical polymerization [ATRP] or reversible addition−fragmentation polymerization [RAFT] is a rich newly emerging field of research (*10*−*19*). The synthesis of living polymer−protein hybrid materials has, however, been restricted to a few acrylate/methacrylate monomers such as poly(ethylene glycol) acrylate/methacrylate and poly(*N*-isopropyl) acrylamide; the recently reported streptavidin conjugates with biotinylated polymers serve as examples (*10, 12, 13*).

Herein, we present a general methodology for significantly increasing the number of dye/drug molecules that can be attached per protein molecule. The diversity of copolymers that can be synthesized for bioconjugation to proteins has also been considerably expanded. The synthesis of poly(acrylic acid)-based near infrared fluorescence (NIRF) dye and glucose incorporated novel copolymers that were further employed for bioconjugation to avidin and bovine serum albumen (BSA) demonstrates this breakthrough. It should be noted that near-infrared (NIR)-absorbing dyes have opened new avenues in optical imaging with direct applications in pharmacology, cellular biology, and diagnostics as living subjects can be monitored with safe, noninvasive optical imaging/contrasting techniques (*20*−*23*). In vitro and in vivo imaging with cyanine-based NIRF dyes are advantageous due to significant reduction of background absorption, enhanced fluorescence, the availability of low-cost sources of irradiation, the versatility of different reporter probes, large molar extinction coefficients, and moderate-to-high fluorescence quantum yields (*24*−*26*).

MATERIALS AND METHODS

Synthesis. Detailed information on the materials used, synthetic procedures, and compound characterization data are provided in the Supporting Information. A few representative experiments are presented here.

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Synthesis of Azide-Terminated Poly(*tert*-butyl acrylate) (2). A 100 mL flask fitted with a stopcock was flame-dried under vacuum and allowed to cool at ambient temperature under argon. The flask was charged with CuBr (115 mg, 0.8 mmol). Under positive pressure of argon, a solution of 2-bromo-2-methylpropionic acid 2-[2-(2-azidoethoxy)ethoxy] ethyl ester (118 mg, 0.36 mmol) dissolved in *tert*-butyl acrylate (4 mL, 27.55 mmol) was added via syringe, followed by the addition of 1,1,4,7,7-pentamethyldiethylenetriamine (PMDETA) (0.17 mL, 0.8 mmol). Following three freeze−pump−thaw degassing cycles, the reaction was allowed to stir for 2 h 10 min at 60 °C. The polymerization was quenched by submerging the flask in liquid nitrogen; the mixture was allowed to warm to ambient temperature and diluted with tetrahydrofuran. CupriSorb was added and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, deionized water solution, and a white solid product was precipitated. The polymer was further purified via reprecipitation and dried under vacuum; isolated yield was 2.18 g (47%). DPn = 64, M_n = 8586 g/mol, M_w/M_n = 1.21. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) 4.19 (s, 2H), 3.55−3.75 (m, 6H), 3.39 (t, 2H), 2.18−2.30 (bm), 1.85 (s), 1.28−1.62 (bm), 1.15 (s, 6H), 0.89 (m). FTIR (film, cm^{-1}): 3433, 2977, 2289, 2113 (azide), 1727, 1619, 1450, 1367, 1255, 1153, 845, 752.

Synthesis of Biotin-Terminated Poly(glucosamine)-Poly-(NIRF dye) Copolymer (6). The biotinylated poly(acrylic acid) polymer (**5**) (76 mg, 1.055 mmol of COOH), Near-infrared absorption dye (ADS832WS) (200 mg, 0.213 mmol), EDC·HCl (213 mg, 1.11 mmol), and HOBt (153 mg, 1.148 mmol) were dissolved in DMF (1.5 mL) in a r.b. followed by the addition of triethylamine (0.15 mL, 1.08 mmol). After stirring for 2 days at room temperature, D-(+)-glucosamine (230 mg, 1.067 mmol) (in water (2 mL) and DMF (1 mL) mixture solution) was added. EDC·HCl (200 mg, 1.043 mmol) was also added, and the mixture was stirred for another 3 days at room temperature. After stopping the reaction, the solution was transferred into a dialysis bag (MWCO 3.5 KDa), and the solution was dialyzed against a continuous flow of deionized water for 24 h. The dialyzed reaction mixture was filtered and lyophilized to yield greenish-brown crude product. The crude product was further purified with Sephadex LH-20 size-exclusion column with deionized water as eluent and lyophilized. Isolated yield: 249 mg. GPC (H_2O): M_n = 23 259 g/mol, M_w/M_n = 1.48. ^{1}H NMR (600 MHz, CD_3OD) δ (ppm) 8.98 (d), 7.18−8.34 (bm), 6.68 (m), 6.37 (m), 5.27 (d), 4.63 (s), 4.30 (s), 3.31−3.96 (m), 3.19 (m), 3.12 (m), 2.99−3.02 (m), 2.86−2.91 (m), 2.79 (s), 1.35−2.22 (m), 1.61 (d), 1.19−1.32 (m), 1.08 (m).

Synthesis of Alkyne-Terminated Poly(glucosamine)-Poly-(NIRF dye) Copolymer (10). Poly(glucosamine)-poly(NIRF dye) copolymer (60 mg, 3 μmol) was dissolved in 3 mL of *t*-BuOH/THF/H_2O (1:1:1) and stirred with propargyl ether (9.14 mg, 10 μL, 31.5 equiv), $CuSO_4 \cdot 5H_2O$ (5 mg, 0.02 mmol), Sodium ascorbate (4 mg, 0.02 mmol) in a r.b. flask under N_2 atmosphere at room temperature for 24 h. The reaction was stopped, and the reaction mixture was dialyzed extensively in deionized water using 10 kD MWCO membrane. The dialyzed mixture was further purified via Sephadex-LH 20 size exclusion chromatography and lyophilized. Yield: 42 mg (70%). GPC (H_2O): M_n = 19 988, M_w/M_n = 1.40. ^{1}H NMR (600 MHz, D_2O) δ (ppm): 1.03 (bs), 1.52−1.75 (bd), 2.01 (bs), 2.58 (s), 2.81 (s), 2.94 (s), 3.36 (s), 3.66−3.73 (bd), 5.06−5.23 (bm), 7.15−8.33 (m). FT-IR (cm^{-1}): 3299, 2928, (No N_3 peak ~2100), 1648, 1536, 1429, 1389, 1351, 1232, 1155, 1113.

Conjugation of Biotin-Terminated Copolymer 6 and Avidin. Avidin (1 mg) was dissolved in 0.25 mL of PBS buffer, pH 7.4. Polymer **6** (5.5 mg) was dissolved in 0.65 mL PBS buffer and slowly added. After 1.5 h at room temperature, the solution was extensively dialyzed using a 50 KDa MWCO membrane in deionized water, and the modified protein was analyzed by fast protein liquid chromatography (FPLC) and SDS-PAGE.

Synthesis of Protein−Polymer Conjugate; BSA-"Clicked"-Poly(glucosamine)-Poly(NIRF dye) Copolymer (14). BSA-azide (5 mg, approximately 1.5 μmol in azide) was incubated with alkyne-poly(glucosamine)-poly(NIRF dye) polymer (20 mg, 1 μmol) in Tris buffer (1800 μL, 0.1 M, pH 8) in the presence of TCEP (4 mM), "click" ligand (4 mM, dissolved in 200 μL of DMF) and copper sulfate (2 mM) for 16 h at 4 °C. The ligand was added before the addition of copper sulfate. The reaction mixture was then dialyzed using Spectra Por 6 dialysis membrane (MWCO 50 KDa) in PBS pH 7.4 for 48 h to remove excess of copper and ligand. The conjugate was further characterized via SDS-PAGE and Fast Protein Liquid Chromatographic system (Akta Purifier, Amarsham Bio-Science's) using HiPrep 26/10 Sephacryl S-200HR (GE Healthcare) size-exclusion column in PBS pH 7.4 as eluent buffer.

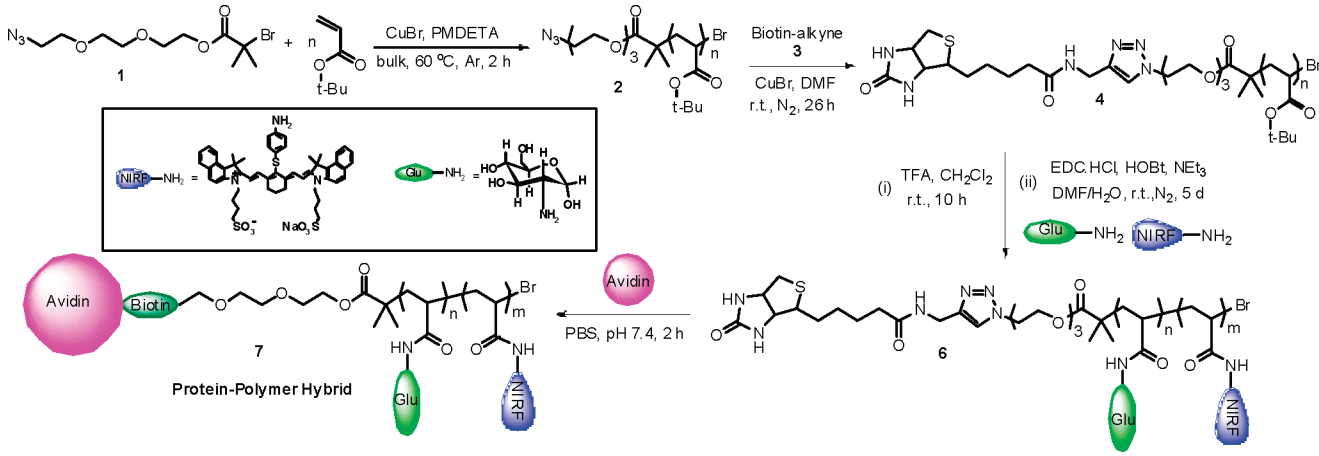
UV−vis−NIR and Fluorescence Spectroscopy of NIRF Dye and Polymer 9. NIRF dye (ADS832WS) (2.8 mg) was dissolved in 10 mL of deionized water to prepare a stock solution of 3 μM. Azide-terminated poly(glucosamine)-poly(NIRF dye) polymer **9** (11.7 mg; 5 dye molecule per polymer chain) was dissolved in 10 mL of deionized water to prepare a solution of 0.3 mM of effective dye concentration. The stock solution of NIRF dye was further diluted to 0.3 μM. The UV−vis−NIR spectra were obtained on an Agilent Technologies 845× UV−vis System using deionized water as blank. The fluorescence spectra of both the dye and the dye-incorporated polymer **9** were obtained on JobinYvon Horiba FluoroMax-3 instrument using the solutions containing 0.3 μM effective dye concentration.

Detection of Binding Affinity of Biotinylated Copolymers to Streptavidin-Coated Wells. Control poly(glucosamine)-poly(NIRF dye) polymer, biotin-terminated poly(acrylic acid)-poly (NIRF dye) polymer (**6a**), and biotin-terminated poly(glucosamine)-poly(NIRF dye) copolymer (**6**). All samples were prepared at a starting effective molar dye concentration of 0.2 mM in PBS (pH = 7.4); then, each of the three samples was successively diluted 10-fold to final concentrations of 0.02, 2.0 × 10^{-3}, and 2.0 × 10^{-4} mM. 50 μL of each solution was then added to the wells and incubated overnight at 4 °C. Then, the wells were washed four times with PBS containing 0.05% Tween 20. Finally, the wells were then visualized using the Odyssey imaging system (LI-COR, Lincoln, NE) at 800 nm.

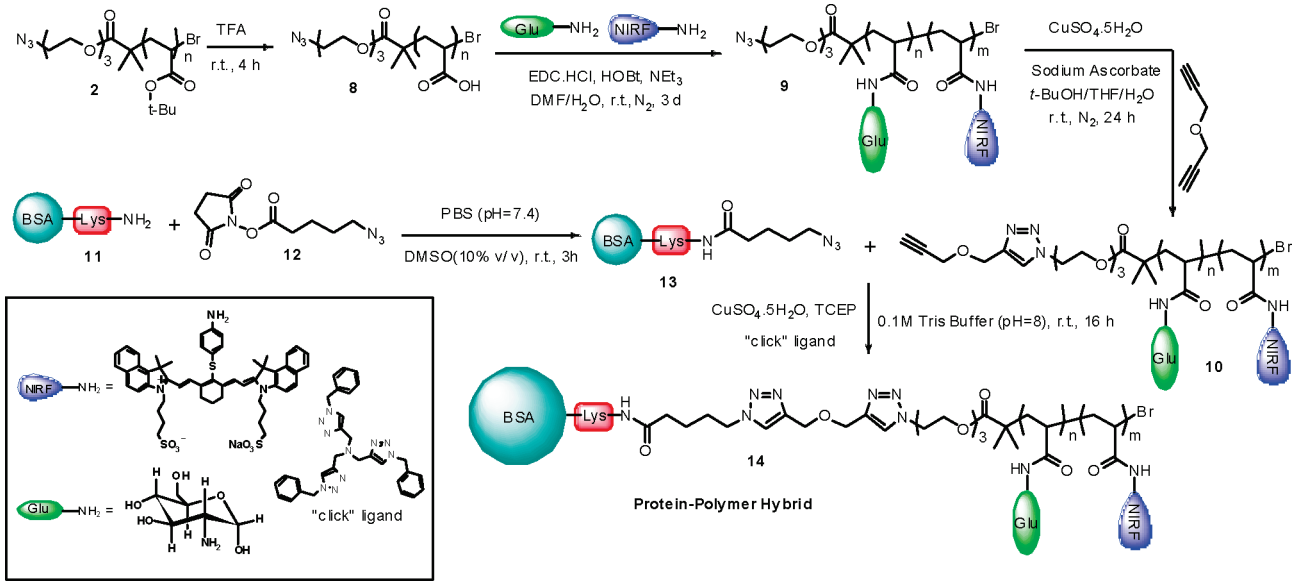
Test of the Utility of NIRF Dye-Incorporated Polymer in Noninvasive Imaging in Mice. Each of two female, six-month-old mice were injected with 200 μL of the NIRF dye, and the biotin-terminated poly(glucosamine)-poly(NIRF dye) polymer **6** (0.2 mM effective dye concentration in both cases) via the tail vein. Each mouse was then anaesthetized using a mixture of 15 μL Xylezene mixed with 54 μL Ketamine to reach a dose of 10 mg/kg body weight of Xylezene and 90 mg/kg bodyweight of Ketamine per mouse, and then scanned using a Odyssey scanner (Near IR) at wavelength 800 nm. To investigate the extent of fluorescence and the extent of catabolism of the polymer, the mice were scanned after 1 and 4 h. Another female six-month-old mouse (control mouse) was anaesthetized with the same mixture and then scanned to eliminate background fluorescence.

In Vitro Imaging Using Retinal Epithelial Cells. Retinal pigment epithelial cells were divided onto four wells and then allowed to grow for three days with regular feeding; the cells were then fixed using 2% PFA, then rinsed three times for five minutes each using PBS (pH = 7.4). The nonspecific binding sites were then blocked using a blocking buffer and incubated overnight. The blocking buffer was then removed, and well (**a**)

Scheme 1. Schematic Synthesis of NIRF Dye Incorporated Synthetic Polymer−Protein Hybrid via Biotin−Avidin Interaction



Scheme 2. Synthetic Representation of Protein−Polymer Hybrid via Bio-Orthogonal "Click" Reaction with NIRF Dye Incorporated Living Copolymer and BSA



only was incubated with the primary antibody and incubated overnight at 4 °C. The primary antibody was then rinsed 3 × 5 min each, and then the biotinylated secondary antibody was added to wells (a,b) and incubated overnight at 4 °C. Rinsing was performed for the excess secondary antibody using the same technique used for the primary antibody; then, 500 μL of Avidin (1 mg/mL) was added to each of wells (a,b,c) and incubated overnight at 4 °C, and excess avidin was washed off with PBS (pH = 7.4) 3 × 5 min each. Finally, 500 μL of poly(glucosamine)-poly(NIRF dye) copolymer **6** (0.1 mg/mL) was added to each of the wells (a,b,c,d) and incubated 2 h at 37 °C in a controlled temperature chamber. Excess polymer was washed off using PBS (pH = 7.4) 3 × 5 min each; fresh PBS was then added to the wells and scanned using Odyssey imaging system (LI-COR, Lincoln, NE) with detection in the NIR region at 800 nm.

RESULTS AND DISCUSSION

Our approach to these polymer−protein conjugates involved three steps: (1) the synthesis of well-defined living polymers containing reactive chain end and functional side chain pendant groups in which the chain end and side chain possesses orthogonal reactivity, (2) the attachment of a number of water-soluble, biocompatible moieties and imaging/therapeutic agents to the functional polymer side chains, and (3) the attachment of the polymers (via the reactive polymer chain end) with proteins to produce the final bioconjugates. Two parallel synthetic designs were employed to establish the feasibility of this concept. In the first design, poly(acrylic acid) with a single reactive biotin chain end was synthesized; straightforward post-polymerization modification of the polymer yielded the final NIRF dye-glucose copolymers for bioconjugation (Scheme 1). The biotinylated copolymers were employed to produce conjugates with avidin via the biotin−avidin interaction ($K_d \sim 10^{15}$) (*27*). In the second embodiment, an alkyne-terminated NIRF dye-glucose copolymer was synthesized and further conjugated to azide linker-modified BSA via the azide−alkyne triazole forming "click" bioconjugation reaction (*28*). The representation of both the copolymer-avidin (Scheme 1) and the copolymer-BSA (Scheme 2) hybrids portray the synthetic breakthrough that has been achieved: a copolymer composed of several copies of an imaging agent/drug displayed in a side chain polymer architecture is attached via a single link to avidin or BSA. Hence, on a per protein molecule basis a larger number of dyes/drugs are being attached using this strategy. In this Article, we also present the potential applications of representative polymers and conjugates in multiwell plate-based bioassays and in in vitro and in vivo imaging. To the best of our knowledge, this is the

first report describing the synthesis of well-defined NIRF dye incorporated living copolymer−protein hybrids.

Polymer−Protein Hybrids via Biotin−Avidin Interaction. The synthesis involved the polymerization of *tert*-butyl acrylate via ATRP (*29*) using an azide-incorporated initiator **1** to produce poly(*tert*-butyl acrylate) **2** (Scheme 1). The intact nature of the azide group in the polymer was confirmed by the presence of the azide peak at 2113 cm^{-1} in the IR spectrum of the polymer (see Supporting Information). The resulting polymer was reacted with an alkyne derivative of biotin **3** under [3 + 2] azide−alkyne triazole forming "click" conditions to produce a polymer with a single biotin chain end **4** (Scheme 1). Peaks arising from the biotin moiety (*14*) at 4.48 ppm and 4.31 ppm were observed in the ^{1}H NMR of **4**; peaks at 7.68 (s) and 7.44 (s) arising from the triazole protons (*30*) were also observed (see Supporting Information). Polymer **4** was treated with trifluoroacetic acid (TFA) to produce poly(acrylic acid) (PAA) with a single biotin chain end **5**. The rationale for synthesizing PAA with a single chain end is based on the facts that (a) PAA is FDA approved and is generally regarded as safe (GRAS); (b) amine derivatives of a broad spectrum of dyes and a wide range of therapeutic agents are readily available commercially and can be employed using amidation chemistry with PAA to produce copolymers; and (c) azidotriethylene glycol and the ATRP initiator with the azide group employed for the polymerizations are safe (many short chain azides are explosive). The amine derivatives, NIRF-NH_2 (ADS832WS) and Glu-NH_2 (D-(+)-glucosamine) were grafted to the PAA polymer **5** using standard amide coupling reagents 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC·HCl) and *N*-hydroxybenzotriazole (HOBt) in DMF to produce **6**. The polymers synthesized were characterized via ^{1}H NMR, gel permeation chromatography (GPC), and FT-IR spectroscopy (see Supporting Information).

On the basis of the GPC results, comparing the molecular weights of **4** and **6**, the percentage of the NIRF dye in the polymer **6** was determined to be ~17% per polymer chain (see Supporting Information). Polymers with higher dye loading numbers and a control polymer **6a** (PAA with dye alone) were also synthesized; however, those polymers were not readily soluble in water. Therefore, copolymer **6** was chosen for further studies due to its better solubility in water. It should be noted that, recently, side chain poly(alkyne) polymers were synthesized and various azide molecules were attached to the polymer backbone via "click" chemistry (*31*). The approach is restricted to a few azides because many small azides are potentially explosive (*31*). The copolymer−protein hybrid **7** was synthesized by incubating the copolymer **6** with avidin. The formation of a conjugate was indicated by higher molecular weight bands in SDS PAGE; the conjugate band which glowed when imaged using a NIRF imager was also visible following Coomassie staining indicating the presence of both polymer and protein at the same position (Figure 1B). The formation of the conjugate was further confirmed via size exclusion fast protein liquid chromatography (FPLC) where conjugate **7** eluted earlier (due to its higher molecular weight) in comparison to the synthetic polymer **6** and the control avidin sample (Figure 1A).

Polymer−Protein Hybrids via "Click" Reaction. In the second synthetic methodology (Scheme 2), the azide-terminated polymer **2** was treated with TFA to produce PAA with azide chain end **8**. The pendant carboxylic acid side chains of polymer **8** were grafted with NIRF-NH_2 and Glu-NH_2 via amide-coupling (EDC·HCl and HOBt) in DMF to produce poly(glucosamine)-poly(NIRF dye) copolymer **9**. In a separate reaction, BSA was modified by incubating with NHS-azide or NHS-alkyne heterobifunctional linkers to produce either azide-labeled BSA **13** or alkyne-labeled BSA **13a**, respectively. MALDI-TOF spectroscopy was used to estimate the average number of lysine residue modifications of the BSA (please note that BSA contains 65 lysine groups). Results indicate that on an average BSA was modified with ~35 alkyne or ~45 azide groups (see Supporting Information). Polymer **9** with a reactive azide chain end was incubated with alkyne-modified BSA **13a** under "click" bioconjugation conditions (*11*) but unfortunately without any success (lack of reaction confirmed via SDS-PAGE; data not shown). Earlier reports support the reduced/lack of reactivity of alkyne modified proteins in "click" bioconjugation reactions (*12*). To circumvent the above-mentioned problem, copolymer **9** was reacted with a large excess of dipropargyl ether under "click" condition to convert the azide-terminated copolymer to an alkyne-terminated poly(glucosamine)-poly(NIRF dye) copolymer **10**. The absence of the azide peak (~2100 cm^{-1}) in the FT-IR spectra confirmed the conversion of all the azide end groups to alkyne (see Supporting Information). The incubation of azide-linker modified BSA **13** with alkyne-terminated copolymer **10** finally produced the desired polymer−protein hybrid **14**. The formation of the conjugate was confirmed via FPLC (Figure 2) and SDS-PAGE (Figure 3). The conjugate **14** eluted earlier than both copolymer **10** and modified BSA **13** in the FPLC experiment. The unmodified BSA **11** eluted at the same volume as the modified BSA **13**; hence, it was not shown in the chromatogram (Figure 2). The SDS-PAGE gels were consistent with the FPLC results; the bands which glowed at 800 nm (lanes 4 and 8) also appeared when stained with

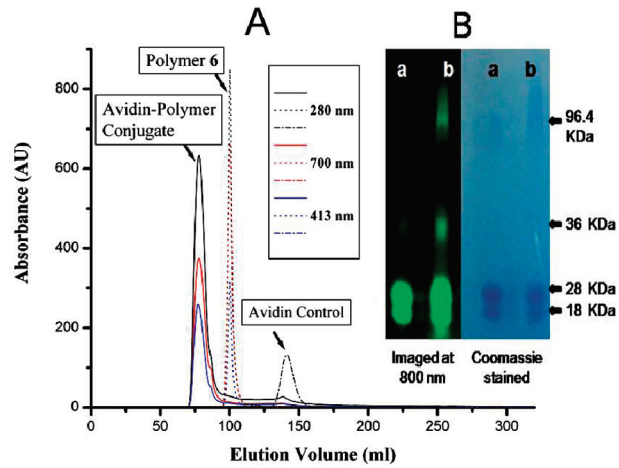


Figure 1. A. Size-exclusion FPLC (Hiprep 26/60 Sephacryl S-200 HR column) of avidin (dashed line), copolymer **6** (dotted line), and conjugate **7** (solid line). B. SDS-PAGE of avidin (lane a) and conjugate **7** (lane b).

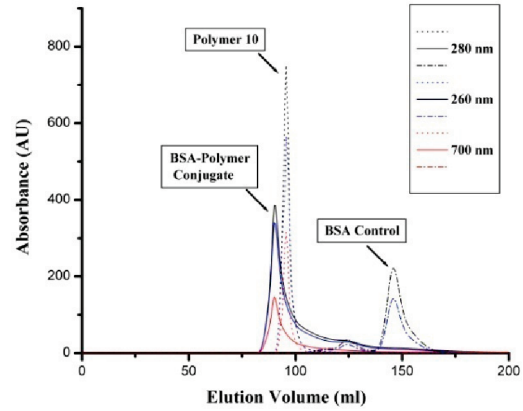


Figure 2. Size-exclusion FPLC (Hiprep 26/60 Sephacryl S-200 HR column) of azide-modified BSA **13** (dashed line), copolymer **10** (dotted line), and conjugate **14** (solid line).

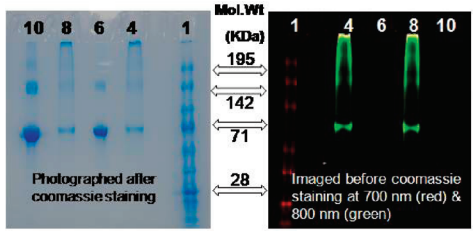


Figure 3. SDS-PAGE of conjugate **14** (lanes 4,8), mixture of azide modified BSA **13** and copolymer **10** without "click" reagents (lane 6), and unmodified BSA (lane 10).

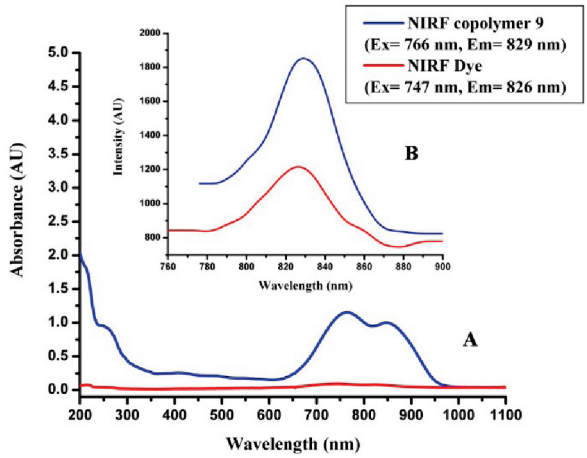


Figure 4. UV/vis/NIR absorption spectra (A) and fluorescence emission spectra (B) of NIRF copolymer **9** (blue line), NIRF dye (red line) in water at 25 °C. The effective dye concentrations in both the cases are identical (0.3 μM).

Coomassie (but the control lanes 6 and 10 did not glow at 800 nm). This confirmed the presence of both protein and polymer at the same ordinate in the conjugate samples. To confirm that copolymer **10** was indeed chemically bonded to the protein, we incubated the mixture of copolymer **10** and modified BSA **13** in the same ratio but without the "click" reagents and dialyzed the mixture with a 50 KDa MWCO membrane; in this case, no higher molecular weight conjugate bands were observed (lane 6).

UV and Fluorescence Study. We have investigated the absorbance and fluorescence emission profiles for the NIRF dye (ADS832WS) and the poly(glucosamine)-poly(NIRF dye) copolymer **9** (Figure 4). Copolymer **9** had superior solubility in water compared to the dye molecule. As a result, higher absorbance values were observed in the case of the polymer sample even though both solutions contained the same effective dye concentration of 0.3 μM (Figure 4A). A red shift of 19 nm (from 747 to 766 nm) in the absorbance maxima (λ_{max}) of the dye was also observed. Although the fluorescence emission intensity of copolymer **9** was higher than that of the dye as expected, the dye displayed larger Stokes shift (79 nm) compared to copolymer **9** (63 nm) (Figure 4B).

Multiwell Plate Binding Assay. Immobilization of the synthesized biotinylated neoglycopolymer was performed on streptavidin-coated multiwell plates where the plates were incubated with a 10-fold serial dilution of (a) nonbiotinylated poly(glucosamine)-poly(NIRF dye) copolymer control (b) polymer **6a** and (c) copolymer **6**. The wells were washed with PBS buffer to avoid nonspecific binding and scanned using an NIRF imaging system; see Figure 5A. The result indicated that the control polymer did not bind to the plates (low fluorescence intensity), whereas the biotin-terminated polymers remained bound to the streptavidin-coated plates, thereby resulting in

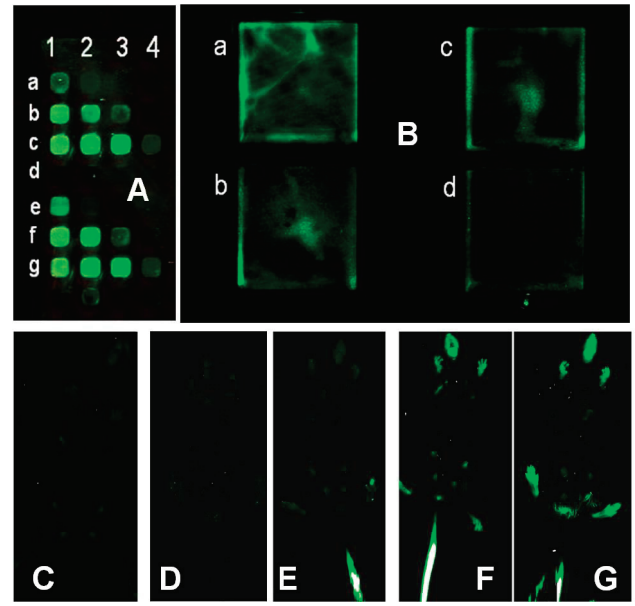


Figure 5. A. Streptavidin-coated multiwell plates treated with nonbiotinylated copolymer control (a,e), polymer **6a** (b,f), and copolymer **6** (c,g). Wells 1−4 correspond to a 10-fold serial dilution of the polymers. B. Fixed retinal pigment epithelial cells (ARPE-19) labeled using copolymer **6**. Slide a was incubated with primary, biotinylated secondary antibody, avidin, and copolymer **6** and imaged. Primary antibody was omitted in slide b; in slide c, the primary and biotinylated secondary antibodies were omitted; in slide d, the primary, biotinylated secondary antibodies and avidin were omitted prior to the copolymer **6** treatment step. C. The control mouse without an imaging agent. D,E. Mouse scanned 1 and 4 h after tail-vein injection with the NIRF dye, respectively. F,G. Mouse scanned 1 and 4 h after tail-vein injection with copolymer **6**, respectively. All the images were scanned using an odyssey NIRF imager at 800 nm.

higher fluorescence intensity. In the case of **6**, the fluorescence intensity was significantly higher than that of **6a** presumably because of the superior water/buffer solubility of **6**. The resulting immobilized neoglycopolymers can interact with complementary cell surface receptors/lectins (*32*). Hence, one can potentially create a convenient platform to study carbohydrate−receptor interactions; by varying the carbohydrates in the polymer and by using dye-modified lectins/cell receptors, which are fluorescence resonance energy transfer (FRET) (*33*) partners for the NIRF dye on the polymers, a range of lectins/cell receptor interactions can be explored.

In Vitro Imaging Study. The utility of copolymer **6** in in vitro imaging cells was also evaluated using retinal pigment epithelial cells. The cells were fixed and treated with primary antibody (2B-α5 rabbit polyclonal IgG), biotinylated secondary antibody, avidin, and poly(glucosamine)-poly(NIRF dye) copolymer **6** (Figure 5B). Slide a was incubated with primary, secondary antibodies, avidin, and copolymer **6**; in slide b, the primary antibody was omitted; in slide c, the primary and secondary antibodies were omitted; in slide d, the primary, secondary antibodies, and avidin were omitted prior to the copolymer **6** treatment step. Slides b, c, and d serve as controls. The results indicate that only slide a showed maximum fluorescence, while control slides show minimal fluorescence.

In Vivo Imaging Study. Polymer **6** also showed considerable promise in noninvasive in vivo imaging of mice: mice injected with the polymer could be imaged more efficiently than mice injected with the small molecule NIRF-NH_2 dye (Figure 5C−G; please note that since the mice were not shaved only the hairless body parts of mice were clearly visible). The polymer is superior for imaging mice compared to the low molecular weight dye probably because of the superior solubility of poly(glucosamine)-

poly(NIRF dye) copolymer **6** in water/blood/serum compared to the dye and because it is not cleared from the blood rapidly via kidney filtration. Employing the copolymer to produce conjugates (with antibodies) for tissue-specific targeted imaging is being currently pursued; this is beyond the scope of the current article.

CONCLUSION

In this study, we have overcome two fundamental limitations in the synthesis of bioconjugates: (a) the basic restriction in the diversity of copolymers which can be synthesized for producing bioconjugates and (b) the limitation that only a small number of dyes/drug molecules can be attached per protein molecule. To demonstrate our synthetic strategy, poly(glucosamine)-poly(NIRF dye) copolymers with reactive chain ends were synthesized via a one-pot amidation of poly(acrylic acid) with NIRF-NH_2 and Glu-NH_2. The copolymers were further employed to produce bioconjugates via (a) biotin−avidin interaction and (b) covalent linkage with BSA (triazole link, "click" bioconjugation reaction) and successfully characterized via NMR, FT-IR, GPC, FPLC, and SDS-PAGE techniques. The synthesized NIRF dye-based copolymers possessed enhanced optical properties compared to their monomeric counterparts. The copolymers were successfully immobilized for potential multiwell plate based assay experiments and utilized for in vitro and in vivo imaging. We are currently exploiting this general synthetic methodology to produce a range of bioactive copolymers and protein/antibody conjugates for applications ranging from tissue-specific imaging to targeted drug delivery.

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Supporting Information Available: Experimental details and characterization data of synthesized compounds and bioconjugates. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Hermenson, G. T. (2008) *Bioconjugate Techniques*, 2nd ed., Academic Press, San Diego, CA.

(2) Wu, A. M., and Senter, P. D. (2005) Arming antibodies: prospects and challenges for immunoconjugates. *Nat. Biotechnol. 23*, 1137–1146.

(3) Raja, K., McDonald, R., Tuck, S., Rodriguez, R., Milley, B., and Traquina, P. (2007) One-pot synthesis, purification, and formulation of bionanoparticle-CpG oligodeoxynucleotide hepatitis B surface antigen conjugate vaccine via tangential flow filtration. *Bioconjugate Chem. 18*, 285–288.

(4) Raja, K. S., Wang, Q., Gonzalez, M. J., Manchester, M., Johnson, J. E., and Finn, M. G. (2003) Hybrid virus-polymer materials. 1. Synthesis and properties of PEG-decorated cowpea mosaic virus. *Biomacromolecules 4*, 472–476.

(5) Shi, W., Dolai, S., Rizk, S., Hussain, A., Tariq, H., Averick, S., L'Amoreaux, W., El Idrissi, A., Banerjee, P. and, and Raja, K. S. (2007) Synthesis of monofunctional curcumin derivatives, clicked curcumin dimer, and a PAMAM dendrimer curcumin conjugate for therapeutic applications. *Org. Lett. 9*, 5461–5464.

(6) Hannink, J. M., Cornelissen, J. J., Farrera, J. A., Foubert, P., De Schryver, F. C., Sommerdijk, N. A., and Nolte, R. J. (2001) Protein-polymer hybrid amphiphiles. *Angew. Chem., Int. Ed. Engl. 40*, 4732–4734.

(7) Velonia, K., Rowan, A. E., and Nolte, R. J. (2002) Lipase polystyrene giant amphiphiles. *J. Am. Chem. Soc. 124*, 4224–4225.

(8) Kochendoerfer, G. G., Chen, S.-Y., Mao, F., Cressman, S., Traviglia, S., Shao, H., 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.

(9) Lewis, J. D., Kovar, M., Strohalm, J., Etrych, T., Ulbrich, K., and Rihova, B. (2002) Star structure of antibody-targeted HPMA copolymer-bound doxorubicin: a novel type of polymeric conjugate for targeted drug delivery with potent antitumor effect. *Bioconjugate Chem. 13*, 206–215.

(10) Kulkarni, S., Schilli, C., Grin, B., Mueller, A. H. E., Hoffman, A. S., and Stayton, P. S. (2006) Controlling the aggregation of conjugates of streptavidin with smart block copolymers prepared via the RAFT copolymerization technique. *Biomacromolecules 7*, 2736–2741.

(11) Sengupta, 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. 34*, 4315–4317.

(12) Sengupta, S., Kuzelka, J., Singh, P., Lewis, W. G., Manchester, M., and Finn, M. G. (2005) Accelerated bioorthogonal conjugation: a practical method for the ligation of diverse functional molecules to a polyvalent virus scaffold. *Bioconjugate Chem. 16*, 1572–1579.

(13) Hou, S., Sun, X. L., Dong, C. M., and Chaikof, E. L. (2004) Facile synthesis of chain-end functionalized glycopolymers for site-specific bioconjugation. *Bioconjugate Chem. 15*, 954–959.

(14) Vazquez-Dorbatt, V., and Maynard, H. D. (2006) Biotinylated glycopolymers synthesized by atom transfer radical polymerization. *Biomacromolecules 7*, 2297–2302.

(15) Lele, B. S., Murata, H., Matyjaszewski, K., and Russell, A. J. (2005) Synthesis of uniform protein-polymer conjugates. *Biomacromolecules 6*, 3380–3387.

(16) De, P., Li, M., Gondi, S. R., and Sumerlin, B. S. (2008) Temperature-regulated activity of responsive polymer-protein conjugates prepared by grafting-from via RAFT polymerization. *J. Am. Chem. Soc. 130*, 11288–11289.

(17) 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.

(18) Bontempo, D., and Maynard, H. D. (2005) Streptavidin as a macroinitiator for polymerization: in situ protein-polymer conjugate formation. *J. Am. Chem. Soc. 127*, 6508–6509.

(19) Li, M., De, P., Gondi, S. R., and Sumerlin, B. S. (2008) Responsive polymer-protein bioconjugates prepared by RAFT polymerization and copper-catalyzed azide-alkyne click chemistry. *Macromol. Rapid Commun. 29*, 1172–1176.

(20) Fabian, J., Nakazumi, H., and Matsuoka, M. (1992) Near-infrared absorbing dyes. *Chem. Rev. (Washington, DC, U. S.) 92*, 1197–1226.

(21) Mishra, A., Behera, R. K., Behera, P. K., Mishra, B. K., and Behera, G. B. (2000) Cyanines during the 1990s: a review. *Chem. Rev. (Washington, DC, U. S.) 100*, 1973–2012.

(22) Law, K.-Y. (1993) Organic photoconductive materials: recent trends and developments. *Chem. Rev. (Washington, DC, U. S.) 93*, 449–486.

(23) Zhao, W., and Carreira, E. M. (2005) Conformationally restricted aza-bodipy: a highly fluorescent, stable, near-infrared-absorbing dye. *Angew. Chem., Int. Ed. Engl. 44*, 1677–1679.

(24) Patonay, G., Salon, J., Sowell, J., and Strekowski, L. (2004) Noncovalent labeling of biomolecules with red and near-infrared dyes. *Molecules 9*, 40–49.

(25) Tung, C.-H. (2004) Fluorescent peptide probes for in vivo diagnostic imaging. *Biopolymers 76*, 391–403.

(26) Zaheer, A., Lenkinski, R. E., Mahmood, A., Jones, A. G., Cantley, L. C., and Frangioni, J. V. (2001) In vivo near-infrared fluorescence imaging of osteoblastic activity. *Nat. Biotechnol. 12*, 1148–1154.
(27) Weber, P. C., Ohlendorf, D. H., Wendoloski, J. J., and Salemme, F. R. (1989) Structural origins of high-affinity biotin binding to streptavidin. *Science 243*, 85–88.
(28) 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. Engl. 41*, 2596–2599.
(29) Wang, J. S., and Matyjaszewski, K. (1995) Controlled/"living" radical polymerization. atom transfer radical polymerization in the presence of transition-metal complexes. *J. Am. Chem. Soc. 117*, 5614–5615.
(30) Luxenhofer, R., and Jordan, R. (2006) Click chemistry with poly(2-oxazoline)s. *Macromolecules 39*, 3509–3516.
(31) Geng, J., Mantovani, G., Tao, L., Nicolas, J., Chen, G., Wallis, R., Mitchell, D. A., Johnson, B. R. G., Evans, S. D., and Haddleton, D. M. (2007) Site-directed conjugation of "clicked" glycopolymers to form glycoprotein mimics: binding to mammalian lectin and induction of immunological function. *J. Am. Chem. Soc. 129*, 15156–15163.
(32) Kiessling, L. L., Gestwicki, J. E., and Strong, L. E. (2006) Synthetic ligands as probes of signal transduction. *Angew. Chem., Int. Ed. Engl. 45*, 2348–2368.
(33) Andrews, D. L. (2008) Mechanistic principles and applications of resonance energy transfer. *Can. J. Chem. 86*, 855–870.

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