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.
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-aminoethoxy)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, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. After filtration, the mixture was poured into a 15% methanol, and stirred ca. 30 min to remove copper complex. 

Synthesis of Biotin-Terminated Poly(glucoasamine)-Poly(NIRF dye) Copolymer (10). 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 HOBr (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) was dissolved in 3 mL of deionized water solution, and a white solid product was precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. The polymer was further purified via reprecipitation with deionized water solution, and a white solid product was isolated yield was 2.18 g (47%). DPn precipitated. 

Synthesis of Alkyne-Terminated Poly(glucoasamine)-Poly(NIRF dye) Copolymer (10). Poly(glucoasamine)-poly(NIRF dye) copolymer (60 mg, 3 μmol) was dissolved in 3 mL of t-BuOH/THF/H2O (1:1:1) and stirred with propargyl ether (9.14 mg, 10 μL, 31.5 equiv), CuSO4 (115 mg, 0.8 mmol), Sodium ascorbate (4 mg, 0.02 mmol) in a r.b. flask under N2 care) size-exclusion column in PBS pH 7.4 as eluent buffer. The nonspecific binding sites were then blocked using a blocking buffer and incubated overnight. The blocking buffer was then removed, and well (a)
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(gluco-samine)-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 (Kd ~ 1015) (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 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 1H 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-NH₂ (ADS832WS) and Glu-NH₂ (D-((+)-glucosamine) were grafted to the PAA polymer (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). Polymer 5 was reacted with an alkyne derivative of biotin under “click” conditions to produce a polymer with a single biotin chain end 6 (Scheme 1). Peaks arising from the biotin moiety (14) at 4.48 ppm and 4.31 ppm were observed in the 1H NMR of 6; peaks at 7.68 (s) and 7.44 (s) arising from the triazole protons (30) were also observed (see Supporting Information). Polymer 6 was treated with trifluoroacetic acid (TFA) to produce poly(acrylic acid) (PAA) with a single chain end 7. 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-NH₂ (ADS832WS) and Glu-NH₂ (D-((+)-glucosamine) were grafted to the PAA polymer 5 using standard amide coupling reagents 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDC·HCl) and HOBt in DMF to produce 6. The polymers synthesized were characterized via 1H 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(alkylamine) 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-NH₂ and Glu-NH₂ via amidate-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 azide or ~45 azide groups (see Supporting Information). Polymer 9 with a reactive azide chain end was incubated with azide-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 azide-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 azide-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
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<sub>max</sub>) 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 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 neoglyco- polymers 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, 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.
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(glu-)number of dyes/drug molecules can be attached per protein molecule. To demonstrate our synthetic strategy, poly(glu-)

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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


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