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RAFT-synthesized Graft Copolymers that Enhance pH-dependent Membrane Destabilization and Protein Circulation Times

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Abstract

Here we describe a new graft copolymer architecture of poly(propylacrylic acid) (polyPAA) that displays potent pH-dependent, membrane-destabilizing activity and in addition is shown to enhance protein blood circulation kinetics. PolyPAA containing a single telechelic alkyne functionality was prepared via reversible addition-fragmentation chain transfer (RAFT) polymerization with an alkyne-functional chain transfer agent (CTA) and coupled to RAFT polymerized poly(azidopropyl methacrylate) (polyAPMA) through azide-alkyne [3+2] Huisgen cycloaddition. The graft copolymers become membrane destabilizing at endosomal pH values and are active at significantly lower concentrations than the linear polyPAA. A biotin terminated polyPAA graft copolymer was prepared by grafting PAA onto polyAPMA polymerized with a biotin functional RAFT CTA. The blood circulation time and biodistribution of tritium labeled avidin conjugated to the polyPAA graft copolymer was characterized along with a clinically utilized 40 kDa branched polyethylene glycol (PEG) also possessing biotin functionalization. The linear and graft polyPAA increase the area under the curve (AUC) over avidin alone by 9 and 12 times, respectively. Furthermore, polyPAA graft copolymer conjugates accumulated in tumor tissue significantly more than the linear polyPAA and the branched PEG conjugates. The collective data presented in this report indicate that the polyPAA graft copolymers exhibit robust pH-dependent, membrane-destabilizing activity, low cytotoxicity and significantly enhance blood circulation time and tumor accumulation.

Keywords

circulation half-life; pH-responsive polymer; protein therapeutics; drug delivery

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1. Introduction

Conjugation of proteins to water soluble polymers can prolong circulation half-life and promote tumor targeting [1–3], and polyethylene glycol (PEG) conjugates have successfully been used clinically for cytokine and enzyme delivery [4, 5]. The drug PEGASYS, for example, consists of Interferon α -2a conjugated to a branched 2×20 kDa (40 kDa total) polyethylene glycol PEG. This protein-polymer conjugate is approved to treat hepatitis C and is currently being tested in clinical trials for treatment of melanoma and chronic myelogenous leukemia [6–9]. Linear formulations of PEG have also been shown to enhance protein stability and circulation half-life [4] but have been found to be less effective than branched formulations [10]. Alternative polymers to PEG have also been used to impart protein stability and enhanced circulation time *in vivo* including N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers [11], poly-L-glutamic acid (PGA) [12], and styrene maleic anhydride copolymers [13].

There is still a need however for alternative polymer compositions and architectures that can provide circulation stability and introduce functionality not found in PEG. For example, inefficient escape from endo-lysosomal compartments is a primary limitation to therapeutic activity of intracellular-acting biomacromolecular drugs [14–17]. Our group has been developing pH-responsive, membrane-destabilizing polymers for the intracellular delivery of biologic drugs. To date, the carriers investigated have either been based on linear unimeric architectures or diblock copolymer micelle architectures containing the pH-responsive monomer propylacrylic acid (PAA) [18–21]. The PAA monomer is an amphiphilic structure containing a propyl tail and a carboxylic acid with a pKa just below physiologic pH. The increased level of protonation of the carboxylic acid that occurs in slightly acidic environments (i.e., endosomes) switches the net hydrophilic-hydrophobic character of the polymer. In the ionized state, poly(alkylacrylic acids) such as polyPAA are extended and soluble, but in the protonated state they transition into a more hydrophobic, globular conformation that can insert into and disrupt lipid bilayer membranes [22, 23]. In the current study, a new brush-like polyPAA architecture was synthesized to test whether pre-organizing the polyPAA chains via a grafting design would enhance its membrane destabilizing activity. At the same time, it was hypothesized that the brush architecture could enhance protein circulation time to a greater extent than a linear version. Pharmacokinetic and biodistribution properties of linear and graft copolymeric polyPAA were compared against a branched PEG that is clinically used to enhance circulation time of protein therapeutics [6, 8, 24]. Both architectures significantly enhanced circulation half-life of the model protein avidin, though not to the extent of the alternative branched and higher Mn PEG architecture. The brush-like polyPAA architecture produced significantly longer circulation half-life of the model protein avidin compared to linear polyPAA and produced higher tumor biodistribution relative to both linear polyPAA and even the branched PEG. These results suggest the brush polyPAA could provide an effective architecture for the delivery of intravenously-injected protein/peptide drugs with intracellular targets.

2. Materials and Methods

2.1. Materials

All reagents were purchased from Sigma-Aldrich and Wako Chemicals and used without further purification unless specified otherwise. The trithiocarbonate CTA ethyl cyanovaleric trithiocarbonate (ECT) and propylacrylic acid (PAA) were synthesized as previously described.[25, 26] 2,2-azobisisobutyronitrile (AIBN), was recrystallized from methanol. A procedure described by Moad and coworkers was modified to synthesize 4-cyano-4-(ethylsulfanylthiocarbonyl)sulfanyl pentanoic acid (ECT).[27] Azidopropylmethacrylate (APMA) was synthesized using a method described by Sumerlin and co-workers.[28]

2.2. Chain Transfer Agent Synthesis

Synthesis of alkyne ECT—A solution of ECT (2 g, 7.59 mmol) and *N*-Hydroxysuccinimide (NHS) (2.6206 g, 22.77 mmol) in chloroform (200 mL) was purged by nitrogen for 1 hour and cooled over an ice bath. *N,N'*-Dicyclohexylcarbodiimide (6.2642 g, 30.36 mmol) was slowly added while vigorously stirring the mixture. The reaction remained in an ice bath for 1 hour and then was removed and allowed to react at room temperature for 22 hours. The reaction mixture was filtered and the organic solvent was removed by vacuum. The NHS activated ECT was redissolved in chloroform (200 mL) and propargylamine (0.1808 g, 3.28 mmol) was then slowly added. The reaction mixture was stirred at room temperature for 18 hours. Chloroform (100 mL) was added and the mixture was extracted with an aqueous solution of hydrochloric acid (1/10 v/v, 5 × 100 mL), 10 wt% sodium hydroxide (5 × 100 mL) and ddH₂O water (2 × 100 mL). The chloroform solution was dried over magnesium sulfate. After solvent removal by vacuum, three successive column chromatographies (Silica gel 60) were performed (ethyl acetate/hexanes (75%/25%), ethyl acetate (100%), chloroform/methanol (98%/2%). After drying under vacuum, a viscous orange-red oil was obtained (yield 92%). ¹H NMR 300MHz (CDCl₃, RT, ppm) 1.35 (t, 3H, CH₃); 1.89 (s, 3H, CH₃); 2.2–2.6 (m, 5H, CH₂CH₂, CH); 3.34 (q, 2H, CH₂); 4.00 (m, 2H, CH₂); 5.93 (b, 1H, NH). MS Calcd for C₁₂H₁₆N₂NaOS₃ (M+Na): 323.03; found m/z 323.1.

Synthesis of Biotin-PEO₃-ECT—The synthesis of the biotinylated RAFT chain transfer agent was accomplished according to a related procedure described by Bathfield et al [29]. Briefly Biotin-dPEGTM₃-NH₃⁺TFA[−] (2.0 g, 3.567 mmol) and triethylamine (720 mg, 7.1 mmol) in 200 mL chloroform was added dropwise in three equal portions 20 minutes apart to a stirred solution of *N*-hydroxy succinimide activated ECT (1.41 g, 3.92 mmol) in 350 mL chloroform. The reaction was allowed to stir at room temperature for 18 hours. The chloroform was then removed by rotary evaporation and the product isolated by column chromatography (silica gel stationary phase, 9:1 v/v CH₂Cl₂:CH₃OH). ¹H NMR: (CDCl₃) δ 1.38 t (SCH₂CH₃); δ 1.93 s (CCNCH₃); δ 2.3–2.65 m (CH₂CH₂); δ 3.35 q (SCH₂CH₃ convoluted); 4.35–4.6 (m, CHSCH₂); δ 2.75–2.9 (dd, CHCH); δ 5.15–5.79 (s, NHCONH); δ 2.85–2.91 (d, CHCHCH); δ 2.72–2.74 (dd, CHCHCH₂); δ 4.28–4.54 (SCH₂CH); δ 3.14 (dt, CH₂CHS); δ 2.23 (t, COCH₂CH₂CH₂CH₂); δ 1.44 (dt, CH₂CHS); δ 1.68 (overlapping multiplet, COCH₂CH₂CH₂CH₂); δ 3.5–3.6 (CH₂CH₂CH₂(OCH₂CH₂)₃, multiplets). MS Calcd for C₁₂H₁₆N₂NaOS₃ (M+Na): 323.03; found m/z 323.1.

2.3 RAFT Polymerization of Poly(azidopropyl methacrylate)

APMA was RAFT polymerized employing the azo initiator 2,2-azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) and ECT as the CTA. Polymerizations were conducted at 30°C for 18 hours at 30 wt% monomer in dimethylformamide under a nitrogen atmosphere in septasealed vials. (*Caution:* special care should be taken not to heat the azide compound above 75–80 °C as it becomes shock-sensitive at elevated temperatures.) The initial chain transfer agent (CTA) to initiator ([CTA]₀/[I]₀) and monomer to CTA ([M]₀/[CTA]₀) were 10:1 and 60:1, respectively. The resultant polymer was isolated by precipitation in a 50/50 pentane/ether mixture followed by repeated cycles of dissolution in acetone and precipitation in a 50/50 pentane/ether mixture.

2.4. RAFT Polymerization of Alkyne-Terminated Poly(propylacrylic acid)

PAA was polymerized in bulk employing a conventional azoinitiator azobisisobutyronitrile (AIBN) as the source of primary radicals and the trithiocarbonate-based CTA, alkyne functional ECT. Polymerizations were conducted at 60°C for 48 hours under a nitrogen atmosphere in septasealed vials. The [CTA]₀/[I]₀ ratio was 1:1, and the monomer:CTA ratio

($[M]_0/[CTA]_0$) was such that the theoretical M_n at 100% conversion ranged between 4 600 and 17 100. The polymer was isolated by precipitation in ether followed by repeated cycles of dissolution in N,N'-dimethylformamide (DMF) and precipitation in ether.

2.5. Coupling of alkyne-functional polyPAA with polyAPMA via click chemistry

Huisgen alkyne-azide cycloaddition “click” reactions were employed to couple the alkyne terminated polyPAA chains onto the azide containing backbone using copper bromide (CuBr) and pentamethyl diethylenetriamine (PMDETA) as the catalyst and ligand, respectively. The reactions were conducted in DMF at 25°C under a nitrogen atmosphere at 10 wt% polymer in septasealed vials for 72 hours. The $[alkyne]_0/[azide]_0$ ratios, $[CuBr]_0/[azide]_0$ ratios and $[PMDETA]_0/[CuBr]_0$ ratios were 1:1, 5:1 and 25:1 respectively. The unreacted azides on the polymer backbones were capped with propargyl alcohol to eliminate the potential toxic effects of azides ($[propargyl\ alcohol]_0/[azide]_0 = 100:1$). Following addition of CuBr and PMDETA ($[CuBr]_0/[azide]_0 = 5:1$, $[PMDETA]_0/[CuBr]_0 = 1:1$), the capping reactions were conducted at 25°C under a nitrogen atmosphere for 72 hours. The resultant polymer was isolated by precipitation in ether and subsequently dissolved in 0.1 M PB, pH 7.4, 0.15 M NaCl. Residual copper was removed by treating the polymer solution with Chelex overnight at 4°C. Polymer solution was dialyzed for 4 days against 0.1 M PB, pH 7.4 and 0.15 M NaCl in a 100 kDa MWCO Float-a-Lyzer (Spectra/Por) with multiple buffer changes to remove any unreacted polyPAA followed by 24 hours of dialysis against ddH₂O to remove buffer salts. The final dry polymer was isolated by lyophilization.

2.6. Red Blood Cell Hemolysis Assay

Membrane disruptive behavior of the polyPAA brushes at physiologic and endosomal pH was characterized using a hemolysis assay. Hemolysis assays are employed to characterize the capacity of agents to disrupt lipid bilayer membranes at pH values representative of physiologic (pH 7.4) as well as early and late endosomal (pH 6.6 and pH 5.8) environments. For this assay, red blood cells were incubated at 37°C with the polyPAA graft copolymers and linear polyPAA ($M_n = 3.6$ kDa, PDI = 1.38) at concentrations of 20 µg/mL. Membrane disruptive activity was evaluated spectrophotometrically by measuring the release of hemoglobin ($\lambda = 451$ nm) from the lysis of red blood cells.

2.7. Cytotoxicity Assay

Cytotoxicity of the polyPAA graft copolymers was determined using a lactate dehydrogenase (LDH) cytotoxicity detection kit. HeLa cells were seeded in 96-well plates at a density of 100,000 cells/cm² and allowed to adhere for 24 hours. Polymer samples were added to wells in triplicate. After cells were incubated for 24 hours with the polymer-containing media, the media was removed, and the cells were washed with PBS. The cells were then lysed with lysis buffer (100 µL/well, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate 1 mM β-glycerophosphate, 1 mM sodiumorthovanadate) for 1 hour at 4 °C. After mixing by pipetting, 20 µL of lysate was diluted into 80 µL PBS and quantified for lactate dehydrogenase (LDH) by mixing with 100 µL of the LDH substrate solution. Following a 10 minute incubation, LDH was colorimetrically determined by measuring absorbance at 490 nm. Percent viability was expressed relative to samples receiving no treatment.

2.8. Synthesis of Biotin-Terminated Poly(propylacrylic acid) (Linear and Graft copolymer)

Biotin functional polymers were synthesized to enable facile attachment to avidin, which was utilized as a model protein drug. Biotin-terminated linear PolyPAA was RAFT polymerized in bulk employing a conventional azoinitiator azobisisobutyronitrile (AIBN) as the source of primary radicals and a biotin terminated trithiocarbonate-based CTA ECT.

Polymerizations were conducted at 60°C for 48 hours under a nitrogen atmosphere in septasealed vials. The $[CTA]_0/[I]_0$ and monomer:CTA ($[M]_0/[CTA]_0$) ratios were 1:1 and 100:1, respectively. The polymer was isolated by repeated precipitation in ether from dimethylsulfoxide (DMSO). To prepare a biotin terminated backbone for the polyPAA graft copolymer, polyAPMA was RAFT polymerized employing the azo initiator 2,2-azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) and biotin functional ECT as the CTA. Polymerizations were conducted at 30°C for 18 hours under a nitrogen atmosphere in septasealed vials. The initial chain transfer agent (CTA) to initiator ($[CTA]_0/[I]_0$) and monomer to CTA ($[M]_0/[CTA]_0$) were 10:1 and 60:1, respectively. The resultant polymer was isolated by precipitation in a 50/50 pentane/ether mixture followed by repeated cycles of dissolution in acetone and precipitation in a 50/50 pentane/ether mixture. Click coupling of biotin terminated polyAPMA and alkyne polyPAA was completed as described for non-biotin terminated polyAPMA.

2.9. Preparation of tritium-labeled avidin

A 6.6- μ g quantity of [3 H]succinimidylpropionate (1×10^8 mCi/mol) (JenkemUSA) was added to 3.3 mg avidin (Sigma-Aldrich) in 50 mM phosphate buffer, pH 8.0, 10 mM NaCl. The solution was stirred for 2 hours at room temperature and loaded onto BioRad Bio-spin 30 chromatography columns (732–6006) coated with 0.5% BSA and 10% acetonitrile and equilibrated with 50 mM phosphate buffer, pH 8.0, 10 mM NaCl. Protein recovery achieved was 94%, and the tritium labeling resulted in 923,139 cpm/ μ g avidin.

2.10. Preparation of avidin-polymer conjugates

The molar excess of the biotinylated polymers necessary to achieve 2 polymers binding per molecule of avidin was determined using a colorimetric assay based on 4'-hydroxybenzene-2-carboxylic acid (HABA). HABA binds to avidin but is displaced from this binding site in the presence of biotin, and this displacement from avidin shifts the absorption of HABA at 500 nm. Changes in this absorption shift were measured with known quantities of free biotin to establish a standard curve. Then, the biotinylated polymers used in this study were added at a range of molar ratios to HABA bound avidin and the absorbance changes at 500 nm were monitored. The acquired data were utilized to estimate the molar excess of polymer required to achieve 2 polymers per avidin. All HABA assays were performed in Dulbecco's PBS, pH 7.4.

2.11. Pharmacokinetics and biodistribution

Subcutaneous injections of 1,000,000 EG.7 OVA cells in 100 μ L Dulbecco's PBS, were administered subcutaneously on the left flank of each mouse and allowed to grow into tumors approximately 1200 mm³ in size. A 100- μ L volume of solution containing 20 μ g [3 H]avidin or [3 H]avidin conjugated to polymers in Dulbecco's PBS (pH 7.4) was injected into the circulation via the tail vein. At 2 min, 15 min, 30 min, 60 min, 240 min, and 1440 min, n=8 blood samples were isolated in each test group. At 1440 min, all animals were sacrificed and the tumor was removed. Tumors were carefully washed with saline, weighed, and homogenized in 10 mL ddH₂O per gram of tissue. 200 μ L of the homogenized tissue solution was transferred to a scintillation vial and 500 μ L Solvable (Perkin Elmer) was added. The samples were incubated at 60 degrees Celsius for 2 hours until complete tissue dissolution. 50 μ L of 200 mM EDTA and 200 μ L 30% hydrogen peroxide were added, and the samples were incubated overnight at room temperature. 25 μ L 10 N HCl and 10 mL of Ultima Gold scintillation fluid were subsequently added to each sample. The samples were vortexed, allowed to sit overnight, and then run on a scintillation counter. Scintillation counter data were utilized to calculate the % injected dose per gram of each tissue and blood sample. Semi-log plots were created with the data collected from the blood samples to determine the relevant time points to include for pharmacokinetic analysis. Pharmacokinetic

parameters were subsequently determined by computer elaboration using WinNonlin software based on a noncompartmental model.

2.12. Polymer Characterization

The polymers in this study were characterized by gel permeation chromatography using three Tosoh TSK-GEL columns (TSK- α 3000, α 3000, α 4000) (Tosoh Bioscience, Montgomeryville, PA) connected in series to a MiniDAWN TREOS multi-angle light scattering detector and Optilab rEX RI detector (Wyatt, Santa Barbara, CA). The mobile phase was HPLC-grade DMF containing 0.1 wt% LiBr. NMR and mass spectroscopy analysis were performed on a Bruker AVance AV300 (300 MHz) spectrometer and Bruker Esquire Liquid Chromatograph Ion Trap Mass Spectrometer, respectively.

3. Results and Discussion

3.1. RAFT polymerization of propylacrylic acid grafts

The polymerization of sterically bulky α -alkyl substituted acrylic acid monomers is generally difficult, and these reactions often exhibit slow polymerization rates and low monomer conversion[30]. For this reason, the RAFT polymerization of polyPAA was conducted under bulk monomer conditions with a low initial chain transfer to initiator ratio ($[CTA]_0/[I]_0 = 1$). In order to introduce telechelic alkyne functionality suitable for click reactions onto the α chain terminus, a RAFT CTA containing an alkyne functional R group was synthesized. Polymerizations were conducted with a $[M]_0/[CTA]_0$ ratio such that the theoretical molecular weight at 100 % monomer conversion was 2 500 g/mol and 11 500 g/mol for polymer 1 and 2, respectively (Table 1.). These molecular weights were selected so that polymer brushes with long and short grafts could be prepared. From Table 1, it can be seen that both polymers are in relatively good agreement between the theoretical and experimentally derived molecular weights ($M_n = 3\ 600$ g/mol, PDI = 1.38; $M_n = 18\ 500$ g/mol, PDI = 1.52). For both polymers the PDI values are somewhat higher than those typically observed for a pure living RAFT polymerizations (PDI = 1.38 – 1.53), however, these values are significantly lower than those observed for a conventional radical polymerization of PAA (PDI > 2.0).

Shown in Figure 1a is a ^1H NMR spectrum of polymer 1 in D₆ DMSO. By comparing the polyPAA carboxyl proton (11.5 – 12.5 ppm) to the methylene protons adjacent to the alkyne (3.78 – 3.8 ppm), it was possible to determine the percentage of chains containing an alkyne functionality. For polymer 1 this ratio was close to unity (alkyne/polyPAA = 1.1) and polymer 2 had an alkyne to polyPAA ratio of 0.92. This ratio is consistent with the presence of a low percentage of initiator-derived polymer chains. Because the primary radical source used in these studies (AIBN) does not contain an alkyne functionality, these chains are not capable of participating in click reactions. The number of nonfunctional chains was observed to be low and did not hinder the formation of graft copolymers. Furthermore, any unreacted polyPAA chains were successfully removed to below detection limits by dialysis.

3.2 RAFT polymerization of azidopropyl methacrylate

Azide-functional polymers that served as the graft copolymer backbone were prepared by polymerizing APMA in the presence of the RAFT chain transfer agent ECT at 30 °C with V-70 as the primary radical source [27]. Using these conditions, excellent control over the polymerization was observed as evidenced by the close agreement between the theoretical and experimentally ($M_{\text{theory}} = 9.4$ kDa, $M_{\text{exp}} = 10.4$ kDa) derived molecular weights as well as the low polydispersity (PDI = 1.14). The resultant molecular weight distribution (Figure 1b.) is unimodal and shows no evidence of polymer coupling or crosslinking. It should be noted that some crosslinking of polyAPMA was observed over time when the

material was stored at room temperature, but this was avoided by storing the polymer at -20°C .

3.3. Synthesis of poly(APMA-graft-polyPAA)

The copper catalyzed Huisgen 1,3-dipolar cycloaddition click reaction was employed to assemble the polymeric components as shown in Scheme 1c. It has been previously found that the polymerization of propargyl methacrylate can yield polymers with high PDIs and poorly defined molecular weights.[28] For this reason azidopropyl methacrylate, which has been shown to polymerize well by ATRP, was selected as the backbone component. PolyPAA containing a single CTA-derived alkyne functionality was then grafted to this backbone. Shown in Figure 1b are the molecular weight distributions for polyAPMA, polyPAA, and the corresponding polyPAA graft copolymer. Proof of the formation of graft copolymers is provided by the clear shift in polymer GPC elution time. The molecular weight of polyAPMA and polyPAA 2 were determined to be 10.4 kDa and 18.5 kDa. In comparison, the graft copolymer was determined to have a molecular weight of 142.1 kD. Azides have been shown to exhibit high cytotoxicity and the presence of unreacted azides along the polymer backbone could result in significant cell death even at low concentrations. In order to minimize any potential for cytotoxicity, residual azides were reacted with excess propargyl alcohol to yield triazole rings containing a hydrophilic hydroxyl group. FTIR experiments (Figure 1c.) confirm the disappearance of the sharp azide peak at 2100 cm^{-1} .

Based on total absolute graft copolymer molecular weight, the average number of polyPAA grafts per polyAPMA was determined by subtracting the molecular weight of polyAPMA backbone from that of the brush and then dividing by the molecular weight of the alkyne functional polyPAA used in fabrication of the graft. Polymer 3 ($M_n = 68.3\text{ kDa}$), which is composed of polyPAA chains with a molecular weight of 3.6 kDa, was determined to have 16 grafts on average per polymer backbone. Polymer 4 ($M_n = 142.1\text{ kDa}$), which was prepared from the longer polyPAA 3, showed a lower graft density of 7. When evaluated as a percentage of the total number of available grafting sites on the backbone (number of azides per polyAPMA) the average percent grafting was determined to be 31 % for polymer 3 and 14 % for polymer 4. These results correspond to 3.2 APMA repeat units per polyPAA graft for polymer 3 and 7.1 APMA repeat units per polyPAA graft for polymer 4.

3.4. Red blood cell hemolysis and cytotoxicity assays

Shown in Figure 2a are the results of the red blood cell hemolysis assay for both graft copolymers as well as a linear control. Linear polyPAA produced minimal hemolysis at physiological pH (7.4). However, as the pH is lowered to values approximating the early and late endosomes, there is a sharp increase in red blood cell lysis. For linear polyPAA greater than 90 % hemolysis is observed at a pH 6.6 with complete lysis at pH 5.8. Both polymer grafts also show the expected low hemolysis at pH 7.4 and a comparably sharp increase in hemolysis at pH 5.8, although they were lower at pH 6.6. Furthermore, the graft copolymers exhibited this pH-dependent membrane disruptive behavior at significantly lower molar concentrations relative to linear polyPAA. At concentrations as low as $0.14\text{ }\mu\text{M}$ ($10\text{ }\mu\text{g/mL}$), graft copolymers containing seven 18.5 kDa grafts ($M_n = 142.1\text{ kDa}$) exhibited 100% red blood cell lysis at endosomal pH and graft copolymers with sixteen 3.6 kDa grafts ($M_n = 68.3\text{ kDa}$) achieved this at concentrations of $0.29\text{ }\mu\text{M}$ ($20\text{ }\mu\text{g/mL}$). Similar concentrations of the linear polyPAA did not exhibit equivalent levels of hemolysis at the endosomal pH values.

The concentration-dependent cell cytotoxicity of the polyPAA graft copolymers was evaluated in HeLa cells using a lactate dehydrogenase (LDH) assay (Figure 2b). The extent of cell survival was determined after 24 h of incubation with copolymer solutions at

concentrations of 10 and 80 $\mu\text{g/mL}$. Minimal cytotoxicity was expected as FTIR analysis confirmed the disappearance of the sharp azide peak at 2100 cm^{-1} (Figure 1c). Over 80% cell survival was observed for polymer 3 graft copolymer and 100% cell survival was observed for polymer 4 graft copolymer even at the highest polymer concentration of 80 $\mu\text{g/mL}$. This result is consistent with the biocompatibility previously observed for other delivery systems based on linear analogs of polyPAA.

3.5. Synthesis of biotinylated graft copolymer and linear polyPAA polymers

By employing a biotin functionalized chain transfer agent [26], biotin-terminated polyPAA was successfully polymerized. Incorporation of biotin was verified with NMR analysis. By comparing the integration of the peak associated with the carboxyl protons in the propyl monomer (11.8–12.2 ppm) to the integration of the multiplet peak (6.25–6.32 ppm) associated with protons in the biotin group, it was determined that ca. 100% of the polyPAA chains possessed biotin end groups. PolyAPMA was also polymerized using the biotin functionalized chain transfer agent and coupled to alkyne polyPAA to yield a graft copolymeric system, $M_n = 84.1\text{ kDa}$, $\text{PDI} = 1.29$ with twenty 3.6 kDa polyPAA grafts on each backbone.

3.6. Polymer avidin conjugations

The HABA assay was used to determine the molar excess of the biotin-terminated polymers necessary to achieve biotin-mediated attachment of 2 polymers per avidin. As shown in Figure 3, the polymer-avidin conjugates had equivalent 2 branched-PEG, 2 linear PPAA, or 2 graft copolymer PPAA molecules per protein. Native polyacrylamide gel electrophoresis was performed to further verify successful polymer conjugation. Significant increases in molecular weight were observed upon polymer conjugation to avidin, and, as expected, the most significant shift was seen with the polyPAA graft copolymer conjugate.

3.7. Pharmacokinetic and biodistribution properties of polymer protein conjugates

The pharmacokinetics and biodistribution of tritium-labeled native and polymer-conjugated avidin were evaluated by administering tail vein injections in mice with subcutaneous tumors. The aim was to assess whether the linear polyPAA and/or brush-like polyPAA graft copolymers could enhance circulation time and tumor accumulation of the model protein avidin. The 40 kDa branched PEG was used as a “gold standard” reference, although the different architectures studied preclude direct comparison of PAA vs. PEG. As shown in Figure 4, less than 10% of the injected dose of avidin per gram of blood was detected at 2 minutes, and by 15 minutes avidin levels were undetectable.

The fast half-life of all the avidin samples precluded PK analysis of the initial distribution phase, and avidin also displayed fast elimination kinetics with a circulation $t_{1/2}$ that could only be estimated as $< 2\text{ min}$. The reference branched PEG substantially enhanced circulation of avidin in the blood, increasing $t_{1/2}$ to 81 min. Both linear and graft copolymer architectures of polyPAA graft copolymers were found to extend the circulation time of avidin in the blood relative to the free protein. The difference between the linear and graft forms of polyPAA was particularly apparent at 30 minutes post injection, when nearly 20% of the injected dose per gram of blood of the avidin polyPAA graft copolymer conjugate remained while less than 5% of the avidin linear polyPAA conjugate was detected. The polyPAA graft copolymer exhibited an elimination $t_{1/2}$ of 19 min and the corresponding linear polymer exhibited a $t_{1/2}$ of 13 min. These results were further corroborated by area under the curve (AUC) analysis, where the polyPAA graft copolymer and linear polyPAA displayed increases in AUC of 12-fold and 9-fold over avidin, respectively, with the PEG providing a 106-fold increase as reference. A biodistribution analysis was also completed to profile the effects of polymer conjugation on avidin accumulation in solid tumors. As shown

in Figure 5, significantly more polyPAA graft copolymer avidin conjugates accumulated in the tumor tissue compared to linear polyPAA-avidin and even the branched-PEG avidin conjugate. While the polyPAA based systems are yet to be optimized based on molecular weight and architecture, these initial results are promising and show the multi-functionality of the conjugates in providing stealth-like properties in the blood together with their ability to enhance intracellular delivery [31–33].

Conclusions

The pH-dependent membrane disruptive activity of polyPAA materials coupled with their low cytotoxicity has been previously found to correlate to efficient delivery of biotherapeutics aimed at intracellular targets [25, 31–33]. Here, it has been shown for the first time that PPAA can also be utilized to extend blood circulation half-life in tests using the model protein drug avidin. Furthermore, conjugation to graft architectures of polyPAA produced favorable effects on the biodistribution and *in vivo* circulation time of avidin relative to linear polyPAA. This finding was analogous to the significant enhancement of circulation time that has been previously observed for proteins delivered using branched PEGs.[8, 10, 34] The biodistribution of brached PEG-, linear polyPAA-, and polyPAA graft copolymer-avidin conjugates were evaluated in a mouse EG.7 tumor model, and although these polyPAA conjugates did not perform as well as the brached PEG, the graft and linear polyPAA were found to prolong circulation time of the model protein avidin significantly, with a 12-fold and 9-fold higher area under the blood concentration time curve, respectively. Furthermore, polyPAA graft copolymer avidin conjugates accumulated in tumor tissue significantly more than the branched PEG and linear polyPAA avidin conjugates. Based upon the robust hemolytic activity, low cytotoxicity, and significant circulation time and tumor accumulation enhancement observed in these studies, these polyPAA graft copolymers hold great promise as carriers for systemically-delivered biotherapeutics aimed at intracellular targets.

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Figure 1. Representative characterization of polymer components utilized to construct the polyPAA graft copolymers

(A) ^1H NMR spectra of polymer 1 ($M_n = 3.6$ kDa, PDI = 1.38) was analyzed to determine the conservation of the alkyne functionality introduced through the RAFT CTA post-polymerization. Peak integrations indicated approximately 100% polymer chain-end alkyne functionalization. (B) GPC spectra illustrating relatively monodispersed alkyne-functionalized PPAA (polymer 2, $M_n = 18.5$ kDa, PDI = 1.53) and polyAPMA ($M_n = 10.4$ kDa, PDI = 1.14). “Click” reactions between these precursors yielded the polyPAA graft copolymer (polymer 4, 7 polyPAA grafts/polyAPMA, $M_n = 142.1$ kDa, PDI = 1.22). (C) FTIR spectra of polyPAA graft copolymer before and after capping of unreacted azides in the polyAPMA backbone with PgOH. PgOH capping resulted a complete disappearance of the azide peak at 2100 cm^{-1} , indicating removal of potentially cytotoxic azide molecules.

Figure 2. Enhanced potency of pH-dependent hemolysis and cytocompatibility of polyPAA graft copolymers

(A) pH-dependent hemolysis of linear polyPAA (polymer 1, $M_n = 3.6$ kDa), a graft copolymer with short, dense grafts (polymer 3, $M_n = 68.3$ kDa, 19 polyPAA grafts of 3600 Da per polyAPMA backbone) and a graft copolymer with longer, less dense grafts (polymer 4, $M_n = 142.1$ kDa, 8 polyPAA grafts of 18.5 kDa per polyAPMA backbone) were determined relative to positive control 1% v/v Triton X-100. Data indicated higher hemolytic potency at pH 5.8 at lower concentrations for the graft architectures relative to linear polyPAA. (B) Cytotoxicity of graft copolymers (polymer 3 and polymer 4) in HeLa cells after 24 h of exposure indicated cytocompatibility of the graft copolymer architectures analogous to that previously shown for linear polyPAA. For both experiments, $n=4$, and data are presented as mean $\pm$ standard deviation.

Figure 3. Biotin-functionalized polymer architectures utilized to synthesize conjugates for the study of the polyPAA graft copolymer effects on biodistribution and pharmacokinetics of tritium-labeled avidin

(A) Biotinylated 40 kDa Y-PEG represents a current gold standard in the pharmaceutical industry. (B) A biotin-functional RAFT CTA was used to polymerize end-functionalized linear polyPAA ($M_n = 7.8$ kDa, $PDI = 1.67$). (C) The biotin RAFT CTA was also used to synthesize biotin end-functionalized polyAPMA, which was subsequently utilized to fabricate polyPAA graft copolymers via click chemistry ($M_n = 84.1$ kDa, $PDI = 1.29$, 19 PPAA grafts of 3.6 kDa per backbone).

Figure 4. PolyPAA graft Copolymer enhancement of avidin blood circulation time
 Following tail vein injection, the persistence avidin in the circulation was assessed at multiple timepoints for free avidin, avidin conjugated to Y-PEG, avidin conjugated to linear polyPAA (7.8 kDa), and avidin conjugated to polyPAA graft copolymer (81.4 kDa consisting of 19×3.8 kDa polyPAA grafts). Two polymers were conjugated per avidin for all groups. Data indicated that both linear and graft copolymer polyPAA architectures extended avidin circulation time, with the graft architecture outperforming linear polyPAA. Industry gold-standard Y-PEG extended avidin circulation time more than all other formulations. (%ID/gram – percent injected dose of avidin per gram of blood, data presented as mean + SEM)

Figure 5. Poly(PAA) graft copolymer enhancement of tumor biodistribution

At 24 hours after intravenous injection, mice were euthanized, and biodistribution of avidin to tumor tissues was assessed for free avidin, avidin conjugated to Y-PEG, avidin conjugated to linear polyPAA (7.8 kDa), and avidin conjugated to polyPAA graft copolymer (81.4 kDa consisting of 19×3.8 kDa polyPAA grafts). Two polymers were conjugated per avidin for all groups. Conjugation to Y-PEG increased tumor biodistribution relative to free protein, and the polyPAA graft copolymer produced the highest tumor biodistribution relative to all other groups tested. (%ID/gram – percent injected dose of avidin per gram of blood, data presented as mean + SEM)

Scheme 1.

Synthetic pathway for the preparation of polyPAA graft copolymers from a polyAPMA polymer backbone. **(A)** polyPAA and **(B)** polyAPMA were polymerized using an alkyne- and carboxyl-functional RAFT CTA, respectively. The resultant alkyne- and azide-functional polymers were then assembled via a copper catalyzed Huisgen 1,3-dipolar cycloaddition to synthesize **(C)** poly(PAA) graft copolymers.

Table 1

Molecular weights, polydispersities, and graft densities for alkyne functional polyPAA and poly(APMA-graft-polyPAA)

Polymer	Architecture	M _{exp} (g/mol)	M _{n,theory} (g/mol)	PDI	Grafts/Polymer
1	Linear	3 600	2 500	1.38	---
2	Linear	18 500	11 500	1.53	---
3	Graft	68 300	---	1.23	16
4	Graft	142 100	---	1.22	7

^a Absolute molecular weights determined by SEC/Tosoh TSK-GEL columns (TSK- α 3000, α 3000, α 4000) (Tosoh Bioscience, Montgomeryville, PA) connected in series to a MiniDAWN TREOS multi-angle light scattering detector and Optilab rEX RI detector (Wyatt, Santa Barbara, CA). HPLC-grade DMF containing 0.1 wt% LiBr was used as the mobile phase.