Synthesis and Characterization of a Multiarm Poly(acrylic acid) Star Polymer for Application in Sustained Delivery of Cisplatin and a Nitric Oxide Prodrug

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ABSTRACT: Functionalized polymeric nanocarriers have been recognized as drug delivery platforms for delivering therapeutic concentrations of chemotherapies. Of this category, star-shaped multiarm polymers are emerging candidates for targeted delivery of anticancer drugs, due to their compact structure, narrow size distribution, large surface area, and high water solubility. In this study, we synthesized a multiarm poly(acrylic acid) star polymer via macromolecular design via the interchange (MADIX)/reversible addition fragmentation chain transfer (MADIX/RAFT) polymerization and characterized it using nuclear magnetic resonance (NMR) and size exclusion chromatography. The poly(acrylic acid) star polymer demonstrated excellent water solubility and extremely low viscosity, making it highly suited for targeted drug delivery. Subsequently, we selected a hydrophilic drug, cisplatin, and a hydrophobic nitric oxide (NO)-donating prodrug, O^2-(2,4-dinitrophenyl) 1-[4-(2-hydroxy)ethyl]-3-methylpiperazin-1-yl]diazen-1-ium-1,2-diolate, as two model compounds to evaluate the feasibility of using poly(acrylic acid) star polymers for the delivery of chemotherapeutics. After synthesizing and characterizing two poly(acrylic acid) star polymer-based nanoconjugates, poly(acrylic acid)–cisplatin (acid–Pt) and poly(acrylic acid–NO (acid–NO) prodrug, the *in vitro* drug release kinetics of both the acid–Pt and the acid–NO were determined at physiological conditions. In summary, we have designed and evaluated a polymeric nanocarrier for sustained-delivery of chemotherapies, either as a single treatment or a combination therapy regimen. © 2012 Wiley Periodicals, Inc. J Polym Sci Part A: Polym Chem 000: 000–000, 2012

KEYWORDS: conjugated polymer; drug delivery systems; reversible addition fragmentation chain transfer; star polymer; water-soluble polymer

INTRODUCTION Star polymer architectures are a new generation of branched polymeric materials that consist of a single core and multiple connecting arms or chains. Star polymers usually have highly condensed and globular structures, tailorable size, and large surface areas for conjugation of drugs and imaging agents, which makes them suited as potential delivery platforms. Various star polymer carriers have been synthesized and used for the delivery of small-molecule chemotherapeutics, including doxorubicin[1] (conjugation) and paclitaxel[2] (encapsulation), proteins, such as insulin,[3] as well as genetic materials, such as DNAs[4] and siRNAs.[5] Herein, we developed a multiarm poly(acrylic acid) star polymer (Fig. 1) via MADIX/reversible addition fragmentation chain transfer (RAFT) polymerization for long sustained release of multiple chemotherapeutics simultaneously as a chemo "cocktail".

Cisplatin is a water soluble, platinum-based chemotherapeutic that has been widely used for many years in the clinic as a first-line chemotherapy for head and neck squamous cell carcinoma, ovarian cancer, and non-small cell lung cancer. The major side effect of cisplatin infusion chemotherapy is renal toxicity, which limits its use. The toxicity of cisplatin is dependent on the maximum concentration of free drug in the plasma. Slow and confined release of cisplatin into tumors can largely alleviate these toxicities. In our previous studies, we synthesized a hyaluronan–cisplatin conjugate for controlled release of subcutaneously delivered cisplatin using the biodegradable and biocompatible polymer hyaluronan.[6] The conjugates achieved superior pharmacokinetics, reduced systemic toxicity, and enhanced anticancer efficacy in rodents compared to unbound intravenous cisplatin.[7–10] However, a major drawback of this hyaluronan-based delivery platform and other linear polymers is the relatively high intrinsic viscosity of the vehicle, which makes it challenging to administer a high concentration of the chemotherapy within a small injection volume. Therefore, we sought to develop another drug carrier with desired solubility and viscosity characteristics that could sustain cisplatin over several days.

Polymer carriers can overcome problems with drug solubility and stability. We have developed a nitric oxide (NO)-donating prodrug, similar to other drugs in this class, it

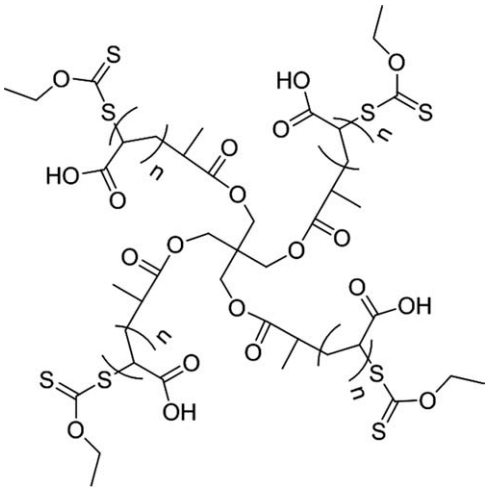

FIGURE 1 Chemical structure of multiarm poly(acrylic acid) star polymer.

releases NO *in vivo* upon activation by glutathione-s-transferase in cells. But, it is both very poorly water soluble and unstable in serum. NO has been shown to effectively inhibit the proliferation of many cancer cells, including breast cancer,[11] colon cancer,[12] non-small cell lung cancer,[13] and melanoma.[14] It is a gaseous small molecule with a half life of only seconds.[15] To deliver therapeutic concentrations of NO to tumorigenic tissues, a controlled release strategy is critical. A number of sustained release carriers have been designed for the delivery of NO, including NO-donor incorporated ethylene/vinyl acetate polymers,[16] S-nitrosothiol conjugated interpolymers,[17] as well as block copolymer of N-acryloylmorpholine, and N-acryloyl-2, 5-dimethylpiperazine.[18] To date, no combinational NO/cisplatin delivery platforms have been developed for delivering these two chemotherapeutics simultaneously. Therefore, we designed a nanocarrier-based system to generate active NO molecules and DNA-crosslinking platinum in a sustained release manner that maintains a relatively steady level of NO and platinum within the therapeutic window for a longer period of time during treatment.

EXPERIMENTAL

Materials

Unless noted otherwise, all reagents and solvents were purchased from Sigma Aldrich (St Louis, MO) or Fisher Scientific (Pittsburgh, PA) and used without further purification. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were collected on a Bruker DRX 400 spectrometer using compounds dissolved in $CDCl_3$, MeOD or D_2O. Chemical shifts were referenced to δ 7.28 and 77.0 ppm for ^{1}H NMR and ^{13}C NMR spectra, respectively. High-resolution mass spectrometry (HRMS) data were generated after flow injection analysis and manually matching peaks on an Applied Biosystems Mariner TOF spectrometer with a turbo-ionspray source. The cell culture media were purchased from MediaTech Inc (Manassas, VA).

Synthesis

Synthesis of MADIX/RAFT Agent

The MADIX/RAFT agent was synthesized based on the procedure reported previously (Fig. 2).[19] The core starting material, pentaerythritol, was esterified with 2-bromopropionyl bromide and *O*-ethylxanthic acid (potassium salt), successively, to form a four-arm sulfur compound that was used as the MADIX/RAFT initiator agent.

4-arm Core Initiator 1. Pentaerithol (1.36 g, 10 mmol) was dissolved in dry chloroform (25 mL) and pyridine (2.5 mL) and cooled to 0 °C. The 2-bromo propionyl bromide (10.01 g, 45 mmol) was added dropwise, and the reaction proceeded at ambient temperature (ca. 23 °C) for 48 h. The mixture then was neutralized with aqueous HCl (10 wt%). Then, the organic phases were washed with water, sodium bicarbonate solution (5 %), and then brine, followed by drying with sodium sulfate. The solvent was evaporated under reduced pressure, which yielded the desired compound. The molecular structure was verified by ^{1}H NMR and compared with the reported data.

A solution of the intermediate compound (2.028 g, 3.0 mmol) in chloroform (45 mL) was treated with a 10-fold excess of *O*-ethylxanthic acid, potassium salt (5.01 g, 30 mmol). The mixture was stirred at ambient temperature for 3 days, and the resulting suspension was filtered and washed with chloroform. Evaporation of the solvent followed by purification through a flash column on silica gel using 3 : 7 ethyl acetate:hexanes as eluents yielded the desired compound, and the molecular structure was verified by ^{1}H NMR and compared with the reported data.

Synthesis of 4-Arm Tert-Butyl Acrylate Star Polymer

The multiarm *tert*-butyl acrylate star polymer was synthesized based on a modification of Ting et al.'s procedure.[20] The *tert*-butyl acrylate (2.50 g, 19.5 mmol) was treated with basic alumina and dry α, α, α- trifluorotoluene (10 mL) in a

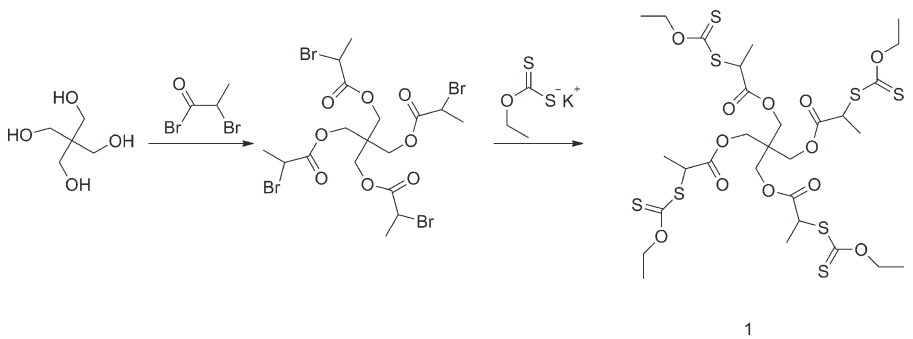

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FIGURE 2 Synthesis of MADIX/RAFT agent.

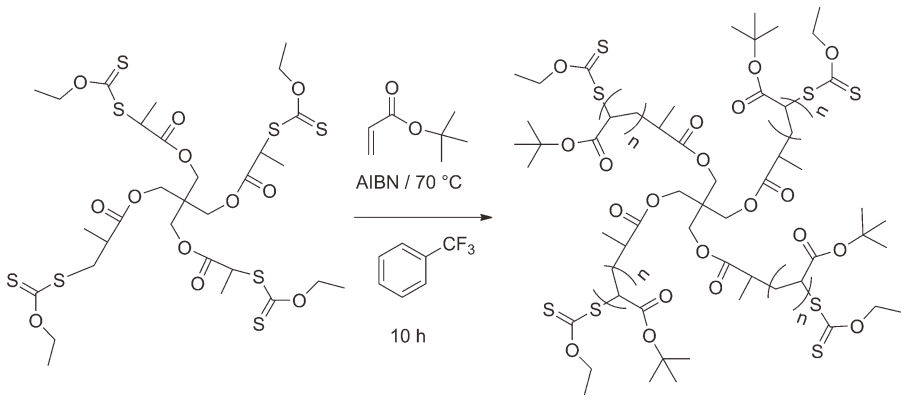

FIGURE 3 Synthesis of multiarm tert-butyl acrylate star polymers.

50-mL round bottom flask. The RAFT agent **1** (27 mg, 3.25 $\times$ 10^{-2} mmol) was added to the solution, followed by the addition of AIBN (0.53 mg, 3.25 $\times$ 10^{-3} mmol), and the reaction flask was placed on ice and purged with argon for 30 min. The flask then was transferred to a thermostatic oil bath at 70 °C for ca. 12 h. The mixture was cooled in an ice bath and poured into cold diethyl ether to obtain the precipitate followed by concentration under reduced pressure (Fig. 3). The molecular structure and molecular weight of the resulting polymer were determined by ^{1}H NMR and size exclusion chromatography (SEC), respectively.

Synthesis of Multiarm Acrylic Acid Star Polymers

The *tert*-butyl acrylate star polymer (2.50 g, 19.5 mmol) was dissolved in dichloromethane (200 mL), and trifluoroacetic acid (TFA, 75 mL, 975 mmol) was then added. The mixture was stirred vigorously until the generation of white precipitate ceased. The precipitate was filtered and washed with dichloromethane and diethyl ether, and then dried under reduced pressure overnight. The molecular structures and weights of the resulting polymers (Fig. 4) were determined by ^{1}H NMR and SEC, respectively.

Synthesis of Poly(acrylic acid) Star Polymer–Cisplatin Conjugates

The poly(acrylic acid) star polymer–cisplatin conjugates (acid–Pt) were synthesized according to the procedure of Cai et al.[6] Poly(acrylic acid) star polymer (50 mg) was dissolved in dd H_2O (25 mL) in a 50-mL round bottom flask. The pH of the solution was adjusted to 10 using 1-M NaOH solution and cisplatin (25 mg) was added. The solution was stirred under argon protection at ambient temperature for 36 h. The solution then was dialyzed (10-kDa MWCO tubing, Pierce, Rockford, IL) against distilled water at 4 °C for 2 days with water changes every 6 h to remove the unbound cisplatin. After dialysis the resulting polymer was concentrated by rotary evaporation to obtain the desired multiarm poly(acrylic acid) star polymer–cisplatin conjugates (Fig. 5).

Synthesis of a NO-donating Prodrug, O^2-(2,4-Dinitrophenyl) 1-{[4-(2-(2-hydroxyethoxy))ethyl] piperazin-1-yl} diazen-1-ium-1,2-diolate

The NO prodrug was synthesized according to the procedure of Chakrapani et al.[21] (Fig. 6). The 1-[2-(2-hydroxyethoxy)ethyl]piperazine (5.0 g, 27.3 mmol) was dissolved in methanol (2 mL) and treated with 25% (w/v) methanolic sodium methoxide (6.2 mL), and ether (20 mL). The resulting solution was charged with NO at 60 psi and stirred at ambient temperature for 24 h, leading to the formation of a white precipitate. The white solid was filtered, washed with ether, and dried under reduced pressure to afford the sodium 1-[2-(2-hydroxyethoxy)ethyl] homopiperazin-1-yl] diazen-1-ium-1, 2-diolate. Subsequently, the white solid was dissolved in 5%

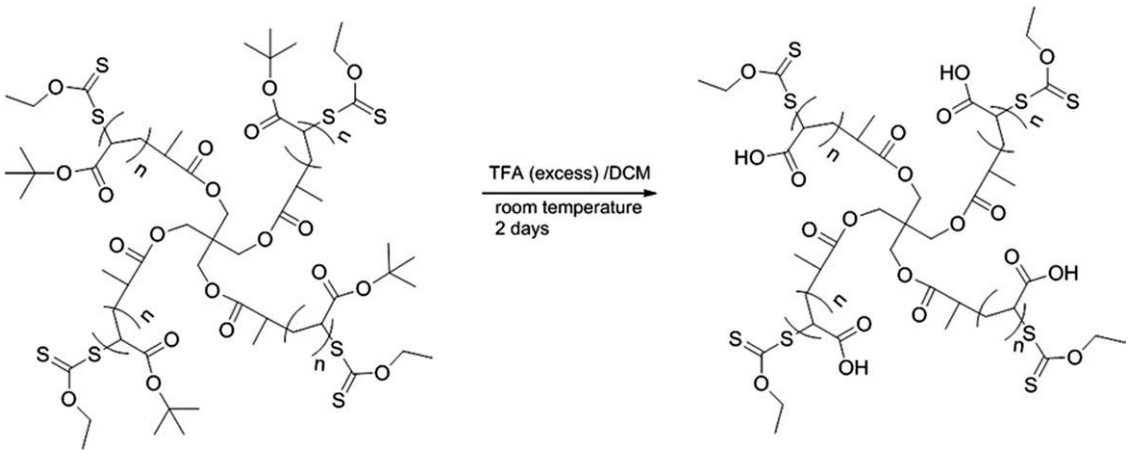

FIGURE 4 Synthesis of multiarm acrylic acid star polymer from tert-acrylate star polymer.

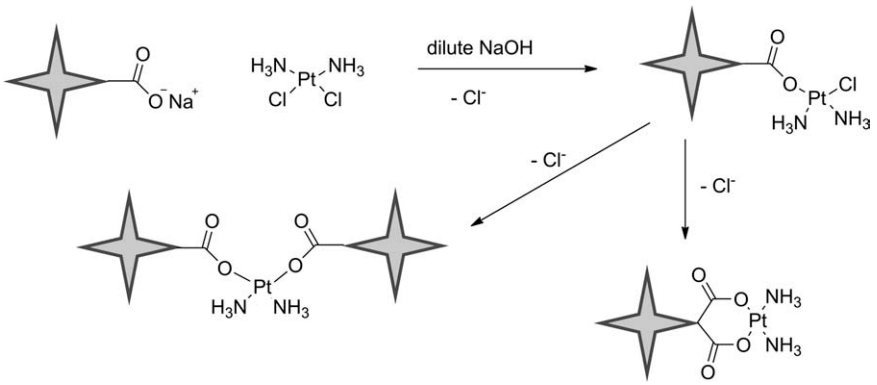

FIGURE 5 Synthesis of poly(acrylic acid) star polymer–cisplatin conjugates.

(w/v) ice cold sodium bicarbonate solution (60 mL). The solution was treated with 1-fluoro-2,4-dinitrobenzene (4.67 g, 25.1 mmol) predissolved in a mixture of *t*-BuOH (30 mL) and THF (5 mL). A yellow precipitate formed immediately and was purified by silica flash chromatography ($CHCl_3$: EtOAc = 9 : 1) to afford the desired compound. The molecular structure was verified by ^{1}H NMR and mass spectrometry.

Synthesis of Poly(acrylic acid) Star Polymer–NO Prodrug Conjugates

The poly(acrylic acid) star polymer (50 mg) was dissolved in 25 mL of ice cold DMF: dd H_2O (25 : 1, v/v) mixture in a 50-mL round bottom flask (Fig. 7), and EDCI·HCl (1.5 equiv.) and HOBt·H_2O (1.5 equiv.) were added to the solution. After 5 min, O^2-(2,4-Dinitrophenyl) 1-{[4-(2-(2-hydroxyethoxy))ethyl] piperazin-1-yl}diazen-1-ium-1,2-diolate (1.0 equiv.) was added to the solution. The reaction proceeded under argon at 0 °C for 30 min, followed by ambient temperature overnight in the dark. The resulting poly(acrylic acid)-nitric (acid–NO) conjugate was dialyzed using 10-kDa MWCO tubing against a mixture of dd H_2O: ethanol (1 : 1, v/v) at ambient temperature for 12 hours, followed by ethanol for another 12 h. The desired acid–NO conjugates were obtained after the evaporation of the solvent under reduced pressure. The molecular structure was verified by ^{1}H NMR.

Characterization

Size Exclusion Chromatography

The molecular weights and polydispersity indices (PDIs) of the multiarm *tert*-butyl acrylate star polymers were determined using EZStart 7.4 software and a Shimadzu 2010CHT system equipped with an RID-10A refractive index detector and a TSK gel multipore Hx-M 7.8 × 30 cm column using 10-mM LiCl in DMF (0.8 mL/min) as the mobile phase. The calibration curve was generated using polystyrene standards ranging from 1,180 to 339,500 g/mol. After deprotection, the resulting multiarm poly(acrylic acid) star polymer was highly soluble in methanol, water, DMF, and DMSO. The molecular weights of the multiarm poly(acrylic acid) star polymers were determined by SEC using polyethylene glycol (PEG) standards ranging from 3,070 to 66,100 g/mol (Scientific Polymer Products) (Table 1).

Determination of Drug Conjugation

The substitution degree of the NO prodrug on the acid–NO conjugate was determined by ^{1}H NMR based on the ratio of the aromatic protons of the NO prodrug to the methylene protons of the polymer core. The degree of cisplatin substitution was determined by atomic absorption spectroscopy (AAS) (Varian SpectrAA GTA-110) using platinum standards ranging from 100 to 450 ppb (Fisher Scientific).[6] The furnace program was as follows: ramp from 25 to 80 °C, hold 2 s, ramp to 120 °C, hold 10 s, ramp to 1000 °C, hold 5 s, ramp to 2700 °C, hold 2 s, cool to 25 °C over 20 s. The graphite partition tube was cleaned every 40 samples by baking at 2800 °C for 7 s. Argon was used as the injection and carrier gas.

Viscosity

The viscosity parameters were measured using a Stabinger viscometer (SVM 3000, Anton Paar) at room temperature. A series of multiarm poly (acrylic acid) star polymer samples

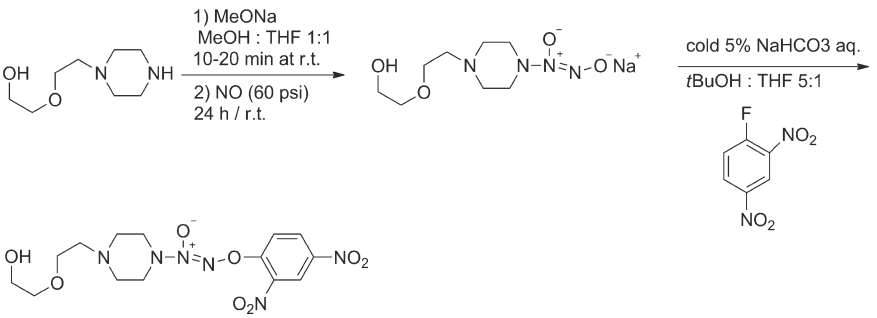

FIGURE 6 Synthesis of O^2-(2,4-Dinitrophenyl) 1-{[4-(2-(2-hydroxyethoxy))ethyl] piperazin-1-yl}diazen-1-ium-1,2-diolate.

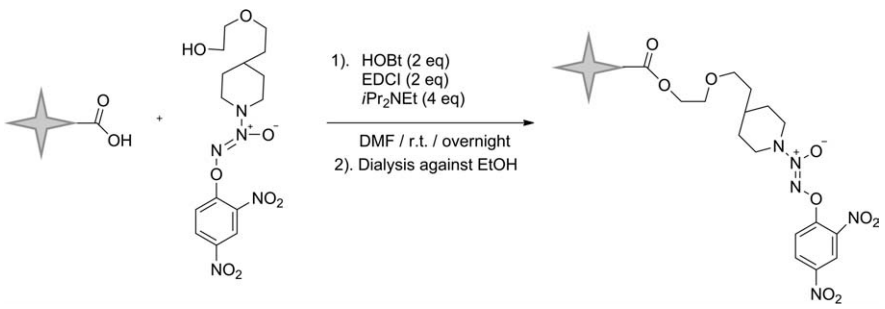

FIGURE 7 Synthesis of poly(acrylic acid) star polymer–NO prodrug conjugates.

(MWs: 64,000, 75,000, 80,300 and 110,000 g/mol) were prepared by dissolving the polymers in dd H_2O at three concentrations including: 1.0, 3.0, and 10.0 mg/mL.

In Vitro Release of Drug from Acid Star Polymers

The *in vitro* release kinetics of cisplatin was determined according to our previously published procedure.[6] The acid–Pt conjugate (10 mg) was dissolved in 10 mL of phosphate buffered saline (PBS), transferred to dialysis tubing (10 kDa MWCO), and placed in a PBS bath (pH 7.4, 37 °C, 4 L) with or without 10% bovine serum albumin and 0.2% sodium azide. Two hundred microliter aliquots were collected from the tubing at predetermined intervals and stored at −80 °C until analysis by AAS. The AAS produced a linear concentration curve from 10 to 450 ng/mL ($R^2 = 0.9998$), with a limit of detection of 5 ng/mL and a limit of quantification of 10 ng/mL (5% standard deviation).

The release half-life of NO from NO–prodrug and acid–NO conjugates was determined in cell culture. MCF-7 cells were seeded in 96-well plates 24 h before drug treatment (100 μL/well). On the following day, cells were treated with 20 μM of either the NO prodrug or the multiarm polymer based NO prodrug conjugate, acid–NO. Fifty microliters of cell culture media were collected from each plate at 10 min, 2, 7, 22, 48, and 96 h, post treatment to determine the nitrite content ($N = 5$). The Griess reaction was performed according to the manufacturer's protocol. The absorbance was measured at 535 nm using a UV microplate reader. The nitrite concentration and the corresponding nitrite oxide levels were determined using the nitrite standard curve previously generated.

Cytotoxicity

Cell growth inhibition was determined in 96-well plates. Plates were seeded with 3000 cells/well in 100 μL of media (12 replicates/sample). Drug or conjugate solutions were applied after 24 h. Resazurin blue in 10 μL of PBS was applied to each well (final concentration 5 μM) after another 72 hours. After 4 h, the well fluorescence was measured (ex/em 560/590) (SpectraMax Gemini, Molecular Devices), and the IC_{50} concentration was determined as the midpoint between drug-free medium (positive) and cell-free (negative) controls.

RESULTS AND DISCUSSION

Synthesis of Acid–NO and Acid–Pt Conjugates

The poly (*tert*-butyl acrylate) star polymers were synthesized via MADIX/RAFT polymerization mediated by xanthates in α, α, α- trifluorotoluene, using AIBN as an initiator. The MADIX/RAFT polymerization approach is of special interest to polymer and drug delivery scientists compared to other synthetic strategies involving free radical reactions, including NMP, ATRP, and RAFT, as a wide range of potential monomers could be readily used to generate well-controlled polymeric architectures. The polymerization resulted in 90% conversion of the monomers after a reaction duration of approximately 14 h. Reaction byproducts started to form if the reaction was allowed to proceed for longer than 20 h. Subsequent treatment of the poly (*tert*-butyl acrylate) star polymers of different molecular weights with TFA in dichloromethane yielded the corresponding acid star polymers. The acid star polymer and its corresponding polymer–chemotherapeutic conjugates, including acid star polymer–NO prodrug conjugate (20 wt% drug loading) and acid star polymer–cisplatin conjugates (8 wt% drug loading) were successfully synthesized. The acid star polymers were soluble in water, methanol, DMF and DMSO. Besides the multiarm poly(acrylic acid) star polymers we developed, various other polymeric systems have also been explored for locoregional delivery of anticancer drugs, including PEG-graft-α,β-poly [(N-amino acidyl)-aspartamide] polymers,[22] PLGA-4-arm-PEG branched polymeric nanoparticles,[23] and a core–shell star polymer carrier with a poly(styrene) core.[24]

Characterization – Size Exclusion Chromatography

The molecular weights of the poly (*tert*-butyl acrylate) star polymers and their corresponding acid star polymers were

TABLE 1 Molecular weights and PDIs of star polymers.

Poly(tert-butyl acrylate) Star Polymers[a]			Poly(acrylic Acid) Star Polymers[b]		
$M_{w,SEC}$	$M_{n,SEC}$	PDI	$M_{w,SEC}$	$M_{n,SEC}$	PDI
148,991	130,122	1.14	86,073	72,321	1.19
115,697	97,107	1.19	67,112	55,927	1.20
83,788	71,209	1.16	54,007	39,992	1.31

[a] Polystyrene was used as a MW standard.
[b] PEG was used as a MW standard.

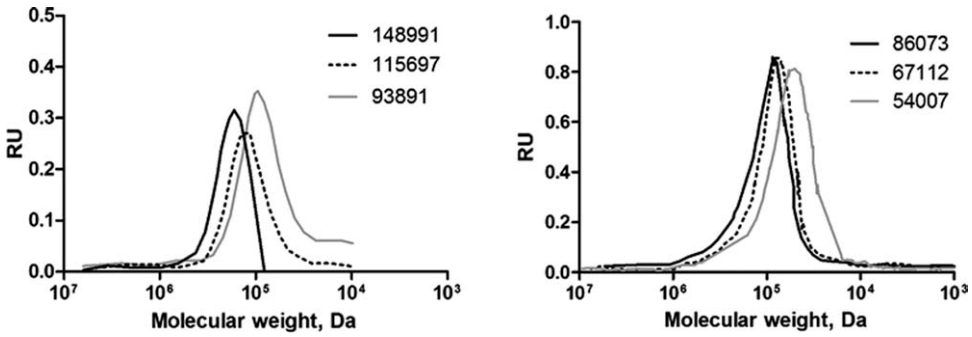

FIGURE 8 SEC traces of poly(tert-butyl acrylate) star polymers (Left panel, MWs: 148,991, 115,697, and 93,891 Da) and poly(acrylic Acid) star polymers (Right panel, MW: 86,073, 67,112, and 54,007 Da).

determined by SEC using polystyrene and PEG as standards, respectively (Fig. 8). The PDIs of poly (*tert*-butyl acrylate) star polymer and acid star polymer with increasing molecular weights were calculated and reported in Table 1. The PDIs were similar to values reported by other groups using MADIX/RAFT to form structured polymers, including four-arm star polymers reported by Stenzel et al.[19] (PDIs: 1.20–1.44). The PDI was slightly higher than ideal in part due to using linear PEGs and polystyrenes as molecular weight standards, since standards with similar architecture were not available. Also, the highly charged nature of the polymers may have led to some interactions with the column stationary phase.

Characterization – NMR

The molecular structures of the star polymers and NO prodrug were verified using ^{1}H NMR, ^{13}C NMR and HR-MS (Fig. 9). All data were consistent with the published results (purity >99%).

O^2-(2,4-Dinitrophenyl) 1-{[4-(2-(2-hydroxyethoxy))ethyl] piperazin-1-yl}diazen-1-ium-1,2-diolate

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.69 (brs, 1H), 2.70 (t, J = 5.3 Hz, 2H), 2.80 (t, J = 5.1 Hz, 4H), 3.63–3.66 (m, 2H), 3.69–3.75 (m, 8H), 7.68 (d, J = 9.3 Hz, 1H). 8.47 (dd, J = 9.2, 2.7 Hz, 1H), 8.90 (d, J = 2.7 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 50.3, 51.5, 57.0, 61.9, 68.0, 72.3, 117.6, 122.2, 129.1, 137.2, 142.3, 153.9. HRMS (ESI) Calculated for $C_{14}H_{21}N_6O_8$ $(M+H)^+$: 401.1421; Found: 401.1413.

Poly (tert-butyl acrylate) Star Polymer

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 4.65 (q, 8H), 4.25 (q, 4H), 4.03 (q, 8H), 2.25 (brs), 1.86 (brs), 1.65–1.27 (brs, overlap).

Poly(acrylic acid) Star Polymer

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 4.66 (q, 8H), 4.30 (q, 4H), 4.14 (q, 8H), 2.25 (brs), 1.86 (brs), 1.65–1.27 (brs, overlap), 1.30 (t, 12H).

Poly(acrylic acid) Star Polymer–NO Prodrug Conjugate

^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.90 (d, J = 8.4 Hz, 1H), 8.57 (dd, J = 8.4 Hz, 1H), 7.68 (d, J = 13.2 Hz, 1H), 4.66 (q, 8H), 4.30 (q, 4H), 4.14 (q, 8H), 3.73 (m, 8H), 3.65 (m, 2H), 2.79 (m 4H), 2.70 (m, 2H), 2.73 (brs), 1.81–1.48 (brs, overlap).

Poly(acrylic acid) Star Polymer–Cisplatin Conjugate

^{1}H NMR ($CDCl_3$, 400MHz): δ = 4.68 (q, 8H), 4.38 (q, 4H), 2.55–2.37 (bs), 2.06–1.89 (bs), 1.84–1.66 (bs), 1.44 (t, 3H), 1.20 (d, 3H).

Characterization – Viscosity

The viscosity of acid star polymers slightly increased with increases in either the molecular weight (54.0–110.7 kDa) or the concentration (1–10 mg/mL) of the polymer (Fig. 10). The viscosity of the acid–NO conjugates was also evaluated and compared to the acid star polymer itself at three different concentrations. The acid star polymer selected for the drug conjugation had a molecular weight of 72.3 kD. Based on our ongoing studies, star polymers with a molecular weight close to 75 kD exhibit advantageous patterns of lymphatic drainage and retention, compared to star polymers of higher or lower molecular weights, thus, the 72.3-kD star polymer drug carrier may be a potential candidate for localized drug delivery applications. It is worth noting that none of the acid star polymers tested exhibited high viscosity compared to other polymeric injectables for localized drug delivery, including hyaluronic acid, which demonstrated a threefold increase in viscosity relative to acid star polymers with a similar molecular weight at 10 mg/mL (data not shown). The FDA recommends that injectables have a viscosity of less than 50 cP, which may be readily injected using a 25- or 27-ga needle. The viscosities of our materials were less than 2 cP, and they can be easily injected using a 31-ga needle. Thus, these polymers are highly suited for a locally administered chemotherapeutics due to their low viscosity at high concentrations, which allows the use of small-bore needles and low injection volumes.

***In Vitro* Release of Platinum from Acid–Pt**

The release kinetics of cisplatin from the acid star polymer backbone was determined in PBS (pH 7.4) at 37 °C with or without 10% serum. The half-life was determined by fitting the release data to either a zero order linear regression model (release of Pt in PBS without serum) or a first order decay model (release of Pt in serum-containing PBS) using GraphPad 5 (R^2 > 0.97 for all fits). The acid–Pt conjugates demonstrated an extended shelf-life in PBS with a platinum release half-life of approximately 120 days [Fig. 11(A)], which is significantly more stable than other sustained delivery platforms of cisplatin, including cisplatin-incorporating polymeric micelles (release half-life: ca. 4 days),[25] and dextran-based cisplatin conjugates (release half-life: ca. 2 days).[26] The presence of serum expedited the drug release from the acid polymers, which is likely due to the

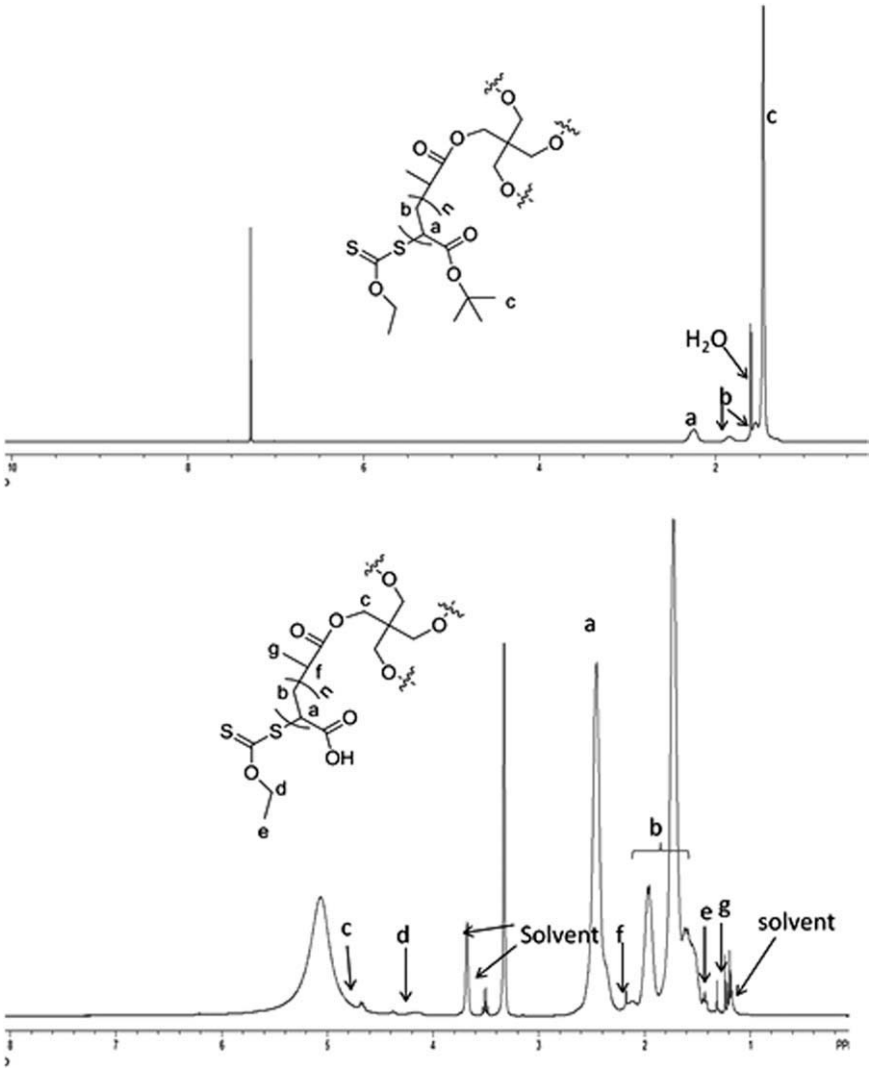

FIGURE 9 ^{1}H NMR spectra of poly (tert-butyl acrylate) star polymer (top panel, solvent: $CDCl_3$), and poly(acrylic acid) star polymer (bottom panel, solvent: MeOD).

competitive binding between the platinum and proteins present in the serum. The conjugates were able to sustain the release of cisplatin over 9 days (95% complete) with a release half-life of approximately 36.7 h in serum-containing PBS [Fig. 11(B)], suggesting satisfactory stability in plasma *in vivo*. If this delivery platform could be translated into the clinic, it may be used as an adjuvant or maintenance chemotherapy post-surgery, releasing a steady concentration of platinum for an extended period of time, which may eradicate the residual disease and potential nanometastases or

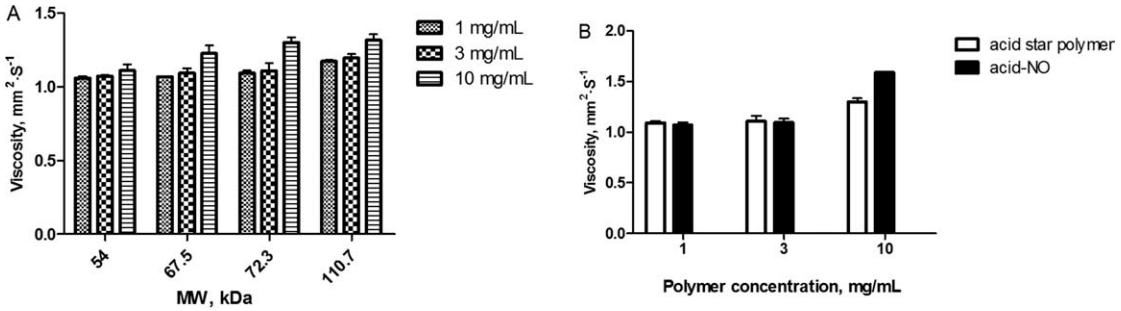

FIGURE 10 Viscosity measurements of (A) a poly(acrylic acid) star polymer at concentrations of 1, 3, and 10 mg/mL, and (B) a star polymer NO prodrug conjugate (acid–NO, 72.3 kDa).

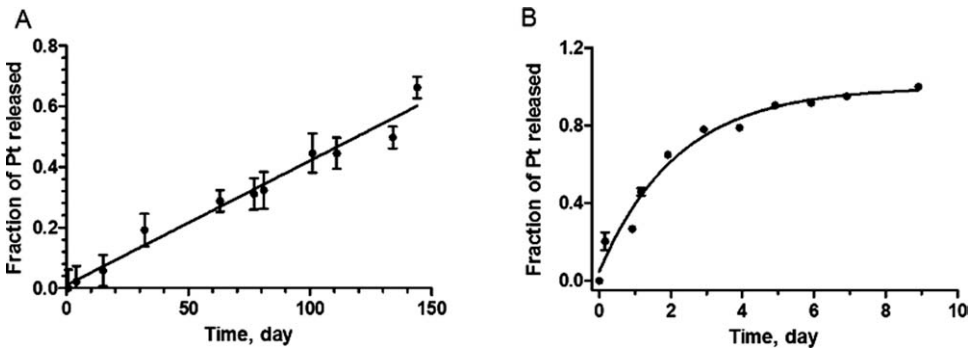

FIGURE 11 Release of platinum from acid–Pt conjugates in (A) PBS, and (B) PBS with 10% serum at 37 °C ($N = 3$). The release half-lives were determined to be 120 days and 36.7 h, using a linear regression or a first order decay model in GrapPad 5, respectively.

micrometastases in the surrounding tissues and draining lymph nodes.

In Vitro Release of NO from Acid–NO

The release kinetics of NO from both the prodrug (NO–prodrug) and the carrier–NO prodrug conjugate (acid–NO) were determined in cell culture media. The half-lives were determined be 7.4 and 5.3 h for NO–prodrug and acid–NO, respectively, by fitting the release data to a first order decay model using GraphPad 5 ($R^2 > 0.97$ for all fits, Table 2). The acid–NO exhibited a shorter release half-life compared to the NO–prodrug, which is largely due to the initial burst release of NO (Fig. 12). The poly(acrylic acid) star polymer backbone created a more acidic microenvironment surrounding the conjugates once they were solubilized in cell culture media, which triggered the liberation of NO immediately. Without the burst release, the acid–NO demonstrated a similar $t_{1/2}$ as the NO–prodrug. Another NO-donating prodrug in preclinical studies, JS-K,[11,27,28] has a NO release half-life of approximately 3.2 h (data not shown), which is shorter than the release $t_{1/2}$ of either the NO–prodrug or the acid–NO. All of the aforementioned NO–prodrugs tested exhibited significantly longer half-lives of NO release compared to the gaseous NO, which has a $t_{1/2}$ of 0.05–0.18 ms in blood.[29] According to Fetz et al.,[30] pretreatment of head and neck cancer cells using NO-donors, including S-nitroso-N-acetyl-penicillamine, reverted the cells' cisplatin resistance via the modulation of survivin. Thus, a drug delivery platform that generates fast-releasing NO, along with slow-releasing platinum, may be a potential candidate for the delivery of synergistic cisplatin and NO combination chemotherapy.

TABLE 2 IC_{50}s and release half-lives of acid-Pt and acid-NO conjugates.

Drugs/Conjugates	IC_{50} in MCF-7 Cells (μM)	Release Half-Life (h)
Cisplatin	20 ± 4	–
Acid–Pt	>100	36.7[a]
NO–prodrug	48 ± 28	7.4[b]
Acid–NO	64 ± 10	5.3[b]

[a] Release half-life was determined in PBS with 10% serum at 37 °C.
[b] Release half-lives were determined in cell culture media with MCF-7 cells.

Cytotoxicity of Acid–NO and Acid–Pt Conjugates

The *in vitro* antiproliferative activities of cisplatin, NO-donating prodrug, and their star polymer-based conjugates, acid–Pt and acid–NO, were evaluated in cell culture using a human breast cancer cell line, MCF-7 (Table 2). Acid–NO conjugates demonstrated a similar IC_{50} to the free NO–prodrug. Release of NO from the polymeric matrix inhibited the growth of cancer cells. The acid–Pt appeared to be less cytotoxic than cisplatin in the cells. This is likely due to the slow release of the active drug, which was not unexpected for a sustained-delivery platform. One of the obstacles that has not yet been overcome in infusion chemotherapy is the poor tumor penetration and accumulation of small-molecule anticancer agents. Usually, the majority of the chemotherapeutics have been cleared from systemic circulation, via the kidneys, before a significant concentration of the anticancer agent is achieved in the tumorigenic tissues. However, the acid–Pt and acid–NO nanoconjugates may greatly enhance the intratumoral drug concentration compared to the normal tissues due to the enhanced permeability and retention (EPR) effect,

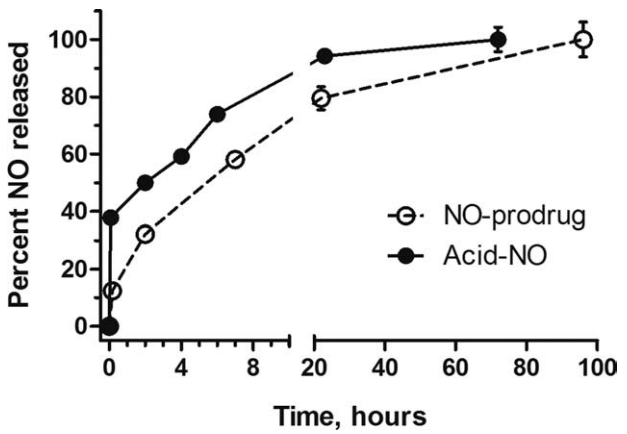

FIGURE 12 Release of NO from NO–prodrug and acid–Pt conjugates in cell culture media at 37 °C ($N = 3$). The half-lives were determined be 7.4 and 5.3 h for NO–prodrug and acid–NO, respectively, by fitting the release data to a first order decay model using GraphPad 5 ($R^2 > 0.97$ for all fits).

in which nanoformulations tend to penetrate tumors more effectively via leaky, fenestrated tumor blood vessels. In addition, the acid–Pt and acid–NO formulations could be given subcutaneously as a local injection adjacent to the tumor to eradicate potential metastasis in the tumor-draining lymph nodes; whereas, it is clinically infeasible to inject free cisplatin or NO subcutaneously due to the severe tissue damage that the anticancer agents would create. After local injection of the acid–Pt, or the acid–NO, the nanocarriers diffuse into the surrounding cutaneous tissues and enter the lymphatic vessels filled with lymph fluid; subsequently, they follow the lymph and reach the sentinel lymph node, the first tumor draining lymph node, and deliver the chemotherapeutics to the metastases. Furthermore, both of the star polymer-based conjugates could be given simultaneously as a localized combination therapy. NO has been shown to overcome chemoresistance in a cisplatin-insensitive human head and neck squamous cell carcinoma cell line.[30] The concurrent administration of both the cisplatin- and NO-releasing conjugates may minimize the incidence of acquired chemoresistance during therapy and maximize the efficacy of the combination treatment. Similar to cisplatin, NO has also been found to sensitize cancer cells to ionizing radiation,[31] which may be used as an adjuvant combination regimen post radiation therapy. In addition, NO was recently found to increase the antitumor activity of other chemotherapeutics, for example, doxorubicin.[32] This discovery can be further explored using a combinational polymer-based, sustained-release doxorubicin (hyaluronan-doxorubicin)[33] and NO (acid–NO) delivery platform that our laboratory developed for treating cancers that are responsive to doxorubicin therapy, including certain types of breast, lung and ovarian cancers.

CONCLUSION

In summary, we have successfully synthesized a multiarm poly(acrylic acid) star polymer architecture suited for the multimodal delivery of both hydrophilic and hydrophobic chemotherapeutics, as either a single-drug chemotherapy or a combinational regimen. This strategy has laid the foundation for future investigations of the delivery of chemo-cocktails using multiple anticancer agents that possess a synergism *in vivo*.

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