Multi-arm polymeric nanocarrier as a nitric oxide delivery platform for chemotherapy of head and neck squamous cell carcinoma

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ABSTRACT

Nitric oxide is a cell signaling molecule that can be a potent inducer of cell death in cancers at elevated concentrations. However, NO is also toxic to normal tissues and chronic exposure at low levels can induce tumor growth. We have designed a polymeric carrier system to deliver nitric oxide locoregionally to tumorigenic tissues at micromolar concentrations. A highly water solubility and biodegradable multi-arm polymer nanocarrier, sugar poly-(6-O-methacryloyl-o-galactose), was synthesized using MADIX/RAFT polymerization, and utilized to deliver high concentrations of nitric oxide to xenografts of human head and neck squamous cell carcinoma (HNSCC). The in vitro release of the newly synthesized nitric oxide donor, O2-(2,4-dinitrophenyl)-1-[4-(2-hydroxy)ethyl]-3-methylpiperazin-1-yl)diazen-1-ium-1,2-diolate and its corresponding multi-arm polymer-based nanoconjugate demonstrated a 1- and 2.3-fold increase in half-life, respectively, compared to the release half-life of the nitric oxide donor prodrug JS-K. When administered to tumor-bearing nude mice, the subcutaneously injected multi-arm polymer nitric oxide nanoparticles resulted in 50% tumor inhibition and a 7-week extension of the average survival time, compared to intravenous JS-K therapy. In summary, we have developed an effective nitric oxide anti-cancer chemotherapy that could be administered regionally to provide the local disease control, improving prognosis for head and neck cancers.

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1. Introduction

The clinical outcomes and overall survival rates for advanced HNSCC have not improved significantly over the past two decades despite advancements in surgery and treatment, so that HNSCC continues to affect over a half million new patients worldwide each year [1]. Moreover, the current therapies for head and neck cancer result in numerous adverse effects, some permanent such as possible loss the salivary gland function and muscle control in the shoulders. Thus, there is a clear need for less damaging therapies for head and neck cancer that will achieve a more favorable clinical outcome and reduce treatment morbidity [2].

Nitric oxide is a cell signaling molecule involved in many mammalian physiological processes and pathological conditions. The effects of nitric oxide on tissues are highly concentration dependent. Low levels of nitric oxide synthase (NOS) overexpression in cancer cells is frequently associated with enhanced tumor cell invasion and growth [3,4] and an increase in aerobic glycolysis capacity [5], i.e. the Warburg effect. However, high concentrations of nitric oxide can inhibit NF-kB activity, which regulates cell proliferation, and downregulating Bcl-xL expression, which modulates apoptotic pathways [6,7]. Nitric oxide has been shown to radiosensitize cancers, inhibit DNA repair mechanisms, inhibit hypoxia-induced drug resistance, and reverse the epithelia to mesenchyma phenotype transition of tumors [8–10]. Nitric oxide has a half-life of about 5 s in vivo, due to rapid reaction of the unpaired electron with hemoglobin and heme ferrous iron and subsequent decomposition into nitrate [11]. Therefore, nitric oxide donating prodrugs have been developed for anti-cancer therapy, such as JS-K [12]. JS-K is a GST-activated nitric oxide prodrug, releasing nitric oxide in vitro, inhibiting the proliferation of cancer cells, including breast cancer cells [13], non-small-cell lung cancer cells [14] and myeloma cells [15]. Because GSTs are also expressed in normal mammalian organs [16], the potential toxicity and carcinogenicity of JS-K must not be overlooked.

The potential for dose-dependent carcinogenicity of nitric oxide-releasing agents has limited their development as systemically administered anti-cancer agents. The effective and safe use of these agents requires that the nitric oxide release be highly confined to tumorigenic tissues and exposure to subtherapeutic doses must be minimized. The tissue distribution of...
small molecule drugs is typically non-specific, with increased exposure in clearance organs, such as the kidneys and liver. To overcome this challenge, a drug molecule may be conjugated to a targeted delivery system so that it will be preferentially released in tumorigenic cells. Water-soluble polymers have been utilized for the preparation of drug conjugates that display significant advantages in pharmaceutical applications, including low toxicity, excellent water solubility, and tissue targeting through passive (e.g., enhanced permeation and retention effect) or receptor-based targeting [17]. Despite the targeted nature of these carriers in intravenous applications, healthy tissues will still receive substantial exposure due to long residence in the clearance organs and circulatory system. Our group and others have partially overcome this problem by delivering nanoconjugates directly to the primary tumor and the draining lymphatics [18–25]. This approach can greatly improve the response in locally advanced cancers, since the nanoconjugate and method of administration can confine the drug to only the tumor and the lymph nodes draining the tumor. An issue in the design of these drug delivery systems is the carrier must be very stable within the extravascular space, which limits the use of micelles and liposomes, and it should be under 50 nm in size for rapid clearance from the injection site. Structured polymers such as dendrimers meet these requirements [26–29], but dendrimers such as PAMAM constructs are not biodegradable and have limited capacity for water-insoluble drugs. Multi-arm or star polymers are branched nanoscale materials that have a compact structure, globular shape, and large surface area that make them highly suited for targeted drug delivery when built with non-toxic and biodegradable polymers.

Multi-arm polymers can be synthesized under a broad range of conditions with low polydispersity using reversible addition-fragmentation chain transfer polymerization (RAFT). During the RAFT process, the polymers derived keep their fragmentation chain transfer polymerization (RAFT). During the conditions with low polydispersity using reversible addition-biodegradable polymers.

suited for targeted drug delivery when built with non-toxic and biodegradable polymers. Moreover, The RAFT agent was synthesized according to the procedure reported previously (Fig. 1) [30]. Pentaerythritol was used as the starting material to react with 2-bromopropionyl bromide and 2-ethylhexanoic acid (potassium salt) successively to form a four-arm sulfur compound which was used as the MADIX/RAFT initiator agent.

2.1. Materials

Unless noted otherwise, all reagents and solvents were purchased from Sigma Aldrich and used without further purification. 1H-NMR (400 MHz) and 13C-NMR (100 MHz) spectra were collected on a Bruker DRX 400 spectrometer using compounds dissolved in CDCl3, MeOD or D2O. Chemical shifts were referenced to 57.28 and 77.0 ppm for 1H-NMR and 13C-NMR spectra, respectively. High-resolution mass spectrometry (HRMS) data were generated after flow injection analysis (FA) by manually matching peaks on an Applied Biosystems Mariner TOF spectrometer with a turbo-ion-spray source. The Griess Reagent kit was purchased from Promega Corporation (Madison, WI). Cell culture media were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Synthesis of MADIX/RAFT agent

The MADIX/RAFT agent was synthesized based on the procedure reported previously (Fig. 2) [30]. Pentaerythritol was used as the starting material to react with 2-bromopropionyl bromide and 2-ethylhexanoic acid (potassium salt) successively to form a four-arm sulfur compound which was used as the MADIX/RAFT initiator agent.

2.2.1. Synthesis of MADIX/RAFT agent

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2.2.2. Synthesis of a multi-arm sugar polymer (Fig. 1)

using MADIX/RAFT polymerization and two nitric oxide-releasing analogues based on JS-K.

![Structure of a multi-arm sugar polymer.](image_url)
succinic anhydride (20, 40, 60 and 90 mg, respectively) in 20 mL of dry dimethyl formaldehyde (Fig. 3). The mixtures were stirred at ambient temperature to form a homogeneous solution, followed by dropwise addition of 5 mL of dry pyridine. After 2 days, the solutions were dialyzed (10 kDa tubing) against 50% ethanoic water for 1 day followed by absolute ethanol for 1 day, with solvent changes every 6 h to remove the dimethyl formaldehyde, pyridine, and other small molecule impurities. Lyophilization of the resulting solution led to the desired sugar multi-arm polymers with increasing substitution degrees of carboxylic acids.

2.2.4. Synthesis of JS-K and JS-K analogues

The nitric oxide-donating prodrug JS-K, O₂-[2-(4-Dinitrophenyl)-1-[4-ethoxycarbonyl]piperazin-1-yl] diazen-1-ium-1, 2-diolate was synthesized. The

![Fig. 2. Synthesis of a multi-arm poly-(6-O-methacryloyl-o-galactose).](image)

![Fig. 3. Modification of multi-arm poly-(6-O-methacryloyl-o-galactose) with succinic anhydride resulted in polymers with increasing degree of acid substitution (20, 40, 60 and 90% wt/wt).](image)
The synthetic route is depicted in Fig. 4. Briefly, to a 250 mL round bottom flask were added 1.16 g of compound 1 (22.6 mmol) and 3.8 mL of 1.5 M HCl in methanol (56 mL). After stirring at ambient temperature for 1 h, the mixture was cooled to 0 °C. After cooling, 2.5 mL of 25% methanolic sodium methoxide was added. The resulting solution was stirred for 30 min and then at ambient temperature overnight under argon. The resulting solution was dialyzed against 50% v/v ethanol in water for 12 h and then absolute ethanol for 12 h using 10 kDa dialysis tubing (Fig. 5). Removal of the solvent under reduced pressure led to the desired conjugate, and the molecular structure and drug loading degree were determined by 1H-NMR. The molecular weights of the acid multi-arm polymers were determined by SEC using the ratio of the methylene protons of the acid groups to the protons of the sugar groups.

2.3.2. NMR

The molecular structures of the MADIX/RAFT agent, 4-arm polymer, acid derivatives of the 4-arm polymers, JS-K and its analogues (NO1 and NO2), and the 4-arm polymer-NO1 conjugates (sugar-NO1) were determined by 1H-NMR, 13C-NMR and HRMS. The substitution degrees of the acid groups on the sugar multi-arm polymer were determined by 1H-NMR based on the ratio of the methylene protons of the acid groups to the protons of the sugar groups. The following sugar acid polymers have been made successfully: sugar-20% acid, sugar-40% acid, sugar-60% acid, sugar-90% acid. In addition, the substitution degree of NO1 prodrug on the sugar-NO1 conjugate was also determined by 1H-NMR.

2.3.3. Solubility

The water solubilities of multi-arm poly-(6-O-methacryloyl-o-galactose) and its acid-modified derivatives were measured in 1× PBS (pH 7.4) at 37 °C. The solutions were kept under this condition and monitored for precipitation for 24 h.

2.3.4. Viscosity

The viscosity parameters were measured using a Stabinger viscometer (SV 3000, Anton Paar, USA). A series of sugar multi-arm polymer samples with or without acid modification (20%, 40%, 60%, and 90% wt/wt) were prepared by dissolving the polymers in ddH2O at three concentrations including: 1.0 mg/mL, 3.0 mg/mL, and 10.0 mg/mL. The viscosity measurements are reported in Table 1.
2.4. Cytotoxicity

Cell growth inhibition was determined in 96-well plates (3000 cells/well in 100 μL, 12 replicates/sample) using highly invasive human breast cancer cells, MDA-MB-231, non-metastatic human breast cancer cells, MDA-MB-468, and highly invasive human head and neck squamous cell cancer cells positive for epithelia growth factor receptor. MDA-1986. Drug or conjugate solutions were applied after 24 h, and 72 h post-addition, resazurin blue in 10 μL of PBS was applied to each well (final concentration 5 μM). After 4 h, well fluorescence was measured (ex/em 560/590) (SpectralMax Gemini, Molecular Devices); and the IC50 concentration determined as the midpoint between drug-free medium (positive) and cell-free (negative) controls.

2.5. Nitric oxide release

To determine the release half-life of nitric oxide from JS-K, NO1, NO2 and sugar-NO1, MDA-1986 cells were seeded in 96-well plates 24 h prior to drug treatment (100 μL/well). On the following day, cells were treated with 20 μM of either one of the nitric oxide prodrugs (NO1, NO2 or JS-K) or the polymer-based sugar-NO1. 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 (regularly) controls.

2.6. Tumor model and efficacy

The MDA-1986 human head and neck squamous cell carcinoma cells were prepared in PBS at a concentration of 2 × 106 cells/mL. Female nude mice were anesthetized with 1.5% isoflurane in 50% O2:air mixture, and 50 μL of cell solution was injected into the sub-mucosa of the mice using a 30-ga needle.

This xenograft model has been used in our laboratory to evaluate a hyaluronan-based drug delivery system [19]. The tumor cells were implanted at the same time for all groups of animals. The treatments began once the primary tumors reached 50–100 mm3, which typically occurred within 2 weeks of implantation. The tumor growth was monitored 2–3 times weekly by measurement with a digital caliper in two perpendicular dimensions, and the tumor volume was calculated using the equation: tumor volume = 0.52 × (width)2 × (length). Animals were euthanized when their tumor size reached 1000 mm3 or the body score index dropped below 2 [33].

Animals bearing head and neck tumors were randomly divided into 3 groups, including Sugar-NO1 s.c. group (N = 11), JS-K i.v. group (N = 7) and untreated control group (N = 8). The treatments were administered subcutaneously next to the tumor two week after tumor cell implantation, at a dose of 10 mg/kg on the basis of NO prodrugs.

3. Results

3.1. Synthesis

The yield of the MADIX/RAFT polymerization reaction mediated by xanthates was ca. 90%. The NMR results demonstrated that the satisfactory conversion and polymerization were obtained when the reaction was allowed to proceed for 8–12 h. After deprotection, the resulting sugar multi-arm polymer is highly soluble in water and organic solvents, including MEOH, EtOH3, DMF and DMSO. The sugar multi-arm polymers were further modified using succinic anhydride to yield multi-arm polymers with increasing degrees (20, 40, 60 and 90%) of terminal carboxylic acid groups on the polymer arms.

The nitric oxide prodrug JS-K and its two analogues were successfully synthesized; the yields of the reactions are reported in NMR 3.3.2. The analogue number 1, O2-[(2,4-dinitrophenyl)-1-[4-(2-hydroxy)ethyl]-3-methylpiperaizin-1-yl]dien-1-ium-1,2-diolate, was selected to be further conjugated onto the sugar multi-arm polymer with 60% acid substitution, resulting in the formation of a multi-arm polymer-NO1 conjugate. The substitution degree of NO1 prodrug on the conjugate was determined to be approximately 12% (wt/wt) based on the ratio of the aromatic protons of the NO1 prodrug to the protons of the repeating units of the multi-arm polymer backbone.

3.2. Characterization

3.2.1. Size exclusion chromatography

The molecular weight of the multi-arm poly-(1,2:3,4-dl-O-isopropylidene-6-O-methacyryloyl-α-D-galactopyranose) synthesized was determined by SEC using polystyrene polymers as standards.
The number average molecular weight, $M_n$, and the polydispersity index, PDI, were determined to be 73,292 g/mol and 1.13, respectively (Fig. 7). After the removal of the isopropylidene protecting groups within the multi-arm polymer, the molecular weight of the resulting multi-arm poly-(6-O-methacryloyl-D-galactose) was determined by SEC under the same condition except that PEG polymers were used as standards. The $M_n$ and PDI were determined to be 45,460 g/mol and 1.20, respectively (Fig. 7). After the modification of the multi-arm poly-(6-O-methacryloyl-D-galactose) with succinic anhydride, the resulting acid derivatives of the polymer were analyzed by SEC and their molecular weights and PDIs are reported in Table 1.

3.2.2. NMR

The molecular structures of JS-K and its two analogues were determined by $^1$H-NMR, $^{13}$C-NMR and HRMS. The substitution degrees of the acid groups to the protons of the sugar side chains. In addition, the substitution degree of NO1 on the sugar multi-arm polymer backbone was also determined by $^1$H-NMR. The chemical shifts of all NMR spectra were measured as follows.

3.2.2.1. Synthesis of MADIX/RAFT agent. The desired bromo intermediate was obtained with a yield of 72% (4.89 g). $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ = 1.87 (m, 1H), 3.92 (brs, 4H), 7.98 (d, $J = 9.3$ Hz, 1H), 8.58 (dd, $J = 9.3$ Hz, 2.7 Hz, 1H), 8.89 (d, $J = 2.4$ Hz, 1H), 9.64 (brs, 2H). $^{13}$C-NMR (DMSO-$d_6$, 100 MHz): $\delta$ = 41.8, 47.4, 118.8, 122.3, 130.2, 137.4, 142.8, 153.0. HRMS (ESI) calculated for C$_{10}$H$_{13}$N$_6$O$_6$ (M$^+$): 407.0927; Found: 407.0932.

The desired compound 2 was obtained as a yellow solid with a yield of 96%. $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ = 3.35 (t, $J = 5.4$ Hz, 3H), 3.92 (brs, 4H), 7.98 (d, $J = 9.3$ Hz, 1H), 8.58 (dd, $J = 9.3$ Hz, 2.7 Hz, 1H), 8.89 (d, $J = 2.4$ Hz, 1H), 9.64 (brs, 2H). $^{13}$C-NMR (DMSO-$d_6$, 100 MHz): $\delta$ = 41.8, 47.4, 118.8, 122.3, 130.2, 137.4, 142.8, 153.0. HRMS (ESI) calculated for C$_{10}$H$_{13}$N$_6$O$_6$ (M$^+$): 407.03897; Found: 407.0895.

The desired compound 3 was obtained as a yellow solid with a yield of 90%. $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ = 1.30 (t, $J = 5.7$ Hz, 3H), 3.29 (brs, 4H), 3.71 (t, $J = 5.2$ Hz, 4H), 4.20 (q, $J = 4.3$ Hz, 2H), 7.14 (d, $J = 9.3$ Hz, 1H), 8.31 (dd, $J = 9.2$ Hz, 2.7 Hz, 1H), 8.90 (d, $J = 2.7$ Hz, 1H). $^{13}$C-NMR (CDCl$_3$, 100 MHz): $\delta$ = 14.6, 42.2, 50.5, 62.1, 117.7, 112.2, 129.1, 137.3, 142.4, 153.7, 155.0. HRMS (ESI) calculated for C$_{12}$H$_{16}$N$_6$O$_8$ (M$^+$): 407.0927; Found: 407.0932.

The desired compound 4 was obtained as a yellow solid with a yield of 64%. $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ = 2.42 (brs, 1H), 2.66 (t, $J = 5.2$ Hz, 2H), 2.78 (t, $J = 4.9$ Hz, 4H), 3.68–3.73 (m, 6H), 7.69 (d, $J = 9.3$ Hz, 1H), 8.48 (dd, $J = 9.2$, 2.5 Hz, 1H), 8.89 (d, $J = 2.5$ Hz, 1H). $^{13}$C-NMR (CDCl$_3$, 100 MHz): $\delta$ = 50.6, 51.2, 58.1, 58.7, 117.6, 122.2, 129.1, 137.4, 142.7, 153.9. HRMS (ESI) calculated for C$_{13}$H$_{17}$N$_6$O$_{10}$ (M$^+$): 357.1159; Found: 357.1144.

The desired compound 5 was obtained as a yellow solid with a yield of 68%. $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ = 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
3.2.2.4. Synthesis of multi-arm polymer-NO conjugate. The desired multi-arm poly-(6-O-methacryloyl-D-galactose)-acid-NO1 conjugate was obtained as a yellow solid with a yield of 29%. $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ = 1.81–1.87 (m, 1H), 1.95 (brs, 1H), 2.06–2.12 (m, 2H), 2.27–2.33 (m, 1H), 2.88 (dd, $J$ = 10.2, 7.8 Hz, 1H), 3.55 (ddd, $J$ = 16.6, 10.8, 5.9 Hz, 1H), 3.68–3.73 (m, 1H), 3.85 (dt, $J$ = 8.0, 3.6 Hz, 1H), 4.23–4.25 (m, 1H), 7.16 (d, $J$ = 9.6 Hz, 1H). 8.17 (dd, $J$ = 9.6, 2.7 Hz, 1H), 8.66 (d, $J$ = 2.7 Hz, 1H).

3.2.3. Solubility

The solubilities of sugar polymer poly-(6-O-methacryloyl-D-galactose) and its acid-modified derivatives were determined in pH 7.4 PBS at 37 °C. The solubilities of sugar star polymer with 0, 20, 40, 60 and 90% acid substitution were determined to be 250, 273, 295, 314, and 309 mg/mL, respectively. The result suggested a linear increase in the solubility with increasing degrees of acid substitution from 0 to 60% wt ($R^2$ = 0.998). In addition, the intrinsic solubility of the polymer with 90% acid substitution did not further increase and it appeared to exhibit a similar solubility as the polymer with 60% acid substitution.

3.2.4. Viscosity

The viscosities of sugar multi-arm polymer, poly-(6-O-methacryloyl-D-galactose), and its acid-modified derivatives (sugar polymers with 20%, 40%, 60% and 90% acid substitution) were determined in ddH$_2$O at three increasing concentrations (1.0, 3.0 and 10.0 mg/mL) (Table 1). The polymers demonstrated low viscosities, merely 5–10% higher than pure H$_2$O, regardless of the percent of the acid substitution. Furthermore, the viscosities of various polymers with different degrees of acid substitution appeared to increase with increasing polymer concentrations from 1.0 to 10.0 mg/mL.

3.3. In vitro cytotoxicity

The cytotoxicity of nitric oxide-releasing prodrugs and polymer-based conjugates was determined in a human head and neck cancer cell line, MDA-1986, and human breast cancer cell lines, MDA-MB-468 and MDA-MB-231. The IC$_{50}$ values of NO1, NO2, JS-K and sugar-NO1 are reported in Table 2. Prodrugs NO1 and NO2 had similar activity in all of the cell lines. The parent drug JS-K was more active than the prodrugs in MDA-1986 cells and less active in MDA-MB-468 cells. The sugar polymer-based conjugate was 1.6–5.3-fold less cytotoxic (mean of 2.5 fold) than the free prodrugs and JS-K in the tested cell lines.

3.4. Nitric oxide release in vitro

The release kinetics of nitric oxide prodrugs were determined in cell culture (DMEM with 10% bovine serum albumin and 1% L-glutamine) using human HNSCC cell line, MDA-1986. The concentration of nitrite, the stable breakdown product of gaseous nitric oxide, was measured using Griess assay as an indirect measurement of nitric oxide concentration in cell culture media. The assay is based on the reaction between sulfanilamide, N-1-naphthylethylenediamine dihydrochloride and nitrite, under acidic conditions to produce a fluorescent azo compound. The half-life was determined by fitting the release data to a first order decay model using GraphPad 5 ($R^2$ > 0.96 for all fits). A nitrite calibration curve ($R^2$ = 0.99) was generated under the same condition using...
a series of nitrite solutions (0, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 μM).

All three nitric oxide prodrugs, NO1, NO2 and JS-K, as well as the sugar-NO1 conjugate, produced nitric oxide in a sustained manner, with generation half-lives of 7.1, 9.0, 3.2 and 9.2 h, respectively (Table 2, Fig. 8).

### 3.5. Treatment

We have established an orthotopic rodent xenograft model of human HNSCC with rapid and sustained tumor growth in our previous study [19]. Animals in either the control group or the JS-K i.v. treatment group had tumors with a size of approximately 1000 mm³, five or six weeks after tumor cell implantation, respectively (Fig. 9A). In comparison, the animals treated with sugar-NO1 developed a tumor of an average size of approximately 200 mm³ within the same time frame, which is 5-fold smaller than the tumor sizes observed in the control and JS-K i.v. groups. Additionally, 32.7% of animals in the sugar-NO1 group survived the study (21 weeks post-tumor cell injection), and two mice had a complete response (defined as no evidence of residual disease) within 14 weeks (Fig. 9A). On the contrary, 100% of the control or JS-K treated animals were euthanized within seven weeks either due to the tumor size exceeding 1000 mm³ or necrosis of the injection site on the tail. Further, HNSCC tumor progression was delayed by nearly six weeks on average after s.c. sugar-NO1 therapy, and the survival rate was significantly extended relative to the control group (p < 0.001, Fig. 9B). The disease condition of each individual animal in the control or treated groups is reported in Table 3.

### 4. Discussion

Xanthate-mediated living radical polymerization using an MADIX/RAFT agent has been proven to successfully synthesize a number of polymeric materials, including star-shaped polymers [30] and functional diblocks [34], which could be introduced into the fields of chemical and pharmaceutical industries as additives, emulsifiers, surface coating materials as well as potential drug delivery vehicles.

In this study, we successfully synthesized a 4-arm branched polymer grafted with sugar moieties. The spherical multi-arm polymer has a compact structure, low polydispersity index and large surface area, making it an ideal candidate for drug delivery applications. In addition, the presence of a number of sugar molecules on the polymer chains effectively converts a hydrophobic synthetic polymer into a highly water-soluble and non-toxic polymer due to the ease of degradation of the sugar branches by the naturally-occurring enzymes in vivo. Although the polystyrene backbone is not biodegradable, the arms are connected to the core via esters subject to hydrolytic decomposition in vivo. The liberated polystyrene arms are below the renal exclusion limit and should clear from the systemic circulation. These unique properties of the polymer may be advantageous because they could be readily translated into practical benefits, including: the elimination of the presence of any organic solvents in its aqueous solution; the satisfactory injectability of even the highly concentrated polymer solution due to its low viscosity similar to water; and the low toxicity compared to other synthetic polymers, such as PAMAM dendrimers, due to its biodegradability and low cytotoxicity.

The intrinsic solubilities of the acid multi-arm polymers was highly correlated with increasing degrees of acid substitution (R² = 0.998). As the degree of acid substitution increased (0, 20, 40 and 60%), the surface charge density of the polymers as well as the average number of hydrogen bonds between the carboxylic acid groups of the polymer and the water molecules also increased, leading to favorable enthalpic interactions between the polymer and the surrounding water molecules along with the desired intra- and inter-molecular electrostatic repulsions within the multi-arm polymer itself [35,36]. The linear solubility characteristic of the acid multi-arm polymers could be further applied to other natural, semi-synthetic or synthetic polymers, promoting and possibly predicting the solubilities of their acid-modified derivatives. As the degree of acid substitution increased beyond 60%, the solubility of the resulting acid polymers plateaued and did not further increase. It is likely due to the possible crosslinking between the highly abundant carboxylic acid groups and the hydroxyl groups of the sugar molecules, reducing the flexibility of the polymer chains and restricting the overall dynamics of the polymer structure.

Low viscosity is crucial for local injectables, since injection volumes are much smaller than intravenous treatments and smaller bore needles are desired for patient comfort. High viscosity is usually associated with low injectability and great discomfort at the injection site. The sugar multi-arm polymer our laboratory synthesized demonstrated extremely low intrinsic viscosity at 1% w/v compared to other polymers for local injection, such as hyaluronan [37], resulting in a smooth delivery of high doses of therapeutics via a relatively small volume of injection.

In addition to the synthesis of this multi-arm polymer nanocarrier, the synthesis, characterization and in vivo effectiveness of two nitric-oxide releasing prodrugs, NO1 and NO2, are also reported. Since the emergence of JS-K, a glutathione s-transferase (GST)-activated nitric oxide prodrg, the underlying mechanisms regulating nitric oxide-induced cell death have been extensively investigated. To date, multiple signaling pathways have been discovered to be involved in promoting the death of cancer cells, suggesting NO is likely to mediate cell proliferation via multiple complex pathways. Sang et al. proposed that nitric oxide donating prodrugs may trigger the apoptosis of gastric cancer cells via the inactivation of AKT, a serine/threonine protein kinase, resulting in the dysregulation of cell cycle-associated proteins, and thus leading to G0/G1 arrest and the corresponding cell growth inhibition [38]. According to a study conducted by Mijatovic et al., nitric oxide also...
can induce apoptosis of melanoma cells via a TRAIL-mediated pathway, in which cells are sensitized to TRAIL and the expression of inducible nitric oxide synthase (iNOS) is altered [39]. Furthermore, Kim et al. demonstrated that nitric oxide-induced apoptosis of colon cancer cells is associated with caspases activation and it may be attributed to the downregulation of the anti-apoptotic Bcl-2 oncoprotein, along with the activation of p53 signaling pathway [40].

Due to the complex nature of NO mediated cell death, it is less likely for cancer cells to develop a series of acquired resistance mechanisms against all governing pathways. Therefore, we sought to design a long-acting, nitric oxide-donating nanocarrier that could be given as locoregional chemotherapy for locally advanced cancers, including head and neck cancer and breast cancer, to generate cytotoxic nitric oxide molecules over time in a sustained pattern. The in vitro nitric oxide release data elucidated that the synthesized nitric oxide prodrugs, NO1 and NO2, both demonstrated longer nitric oxide release half lives in head and neck cancer cells. Additionally, the sugar-NO1 nanoconjugate exhibited the longest release half-life, which was 2.9-fold longer than the model NO-releasing drug, JS-K. The longer half-lives of the prodrugs compared to JS-K are due to the presence of electron-donating groups on the nitrogen of the 6-membered ring, which resists nucleophilic attack and generation of the Meisenheimer intermediate, whereas the carboxy in JS-K is electron-withdrawing. In addition, the sugar-NO1 conjugate may have extended the half-life by sterically hindering the prodrug from nucleophilic attack by reactive thiols. Therefore, if the in vitro drug release kinetics correctly represents the in vivo conditions, the sugar-NO1 conjugate may be a promising candidate for the controlled release of nitric oxide in tumor-bearing animals. According to a study conducted by Azizzadeh et al., NO-producing prodrugs with a longer release half-life may be more potent inhibitors of DNA synthesis and mitotic activity in the S-phase compared to short-lived NO-prodrugs [41].

Compared to JS-K, both unbound NO1 and NO2 had slightly higher IC50 values in MDA-1986 cells, whereas they both showed higher in vitro toxicity in MDA-MB-468 cells. Even though the carrier-based sugar-NO1 conjugate had higher IC50 values than the standard JS-K, according to our previous studies using hyaluronan-cisplatin and hyaluronan-doxorubicin conjugates, the subcutaneously administered, sustained nitric oxide-releasing platform may provide additional benefits including: modified drug pharmacokinetics by avoiding the potentially harmful Cmax; reduced systemic toxicities due to limited drug exposure within the circulatory system and highly perfused, toxin-susceptible organs (kidneys, liver and heart); and improved local disease control via localized delivery of highly concentrated NO therapy [18–22].

In the following in vivo evaluation of the anti-cancer efficacy of the sugar-NO1 therapy, we demonstrated that the subcutaneously injected conjugate (10 mg/kg) significantly improved the tumor inhibition in HNSCC xenografts, extending the average life span of the animals by six weeks, which was approximately one third the length of the time frame of the study. In contrast, the intravenous therapy of JS-K at an equivalent dose only resulted in a slight regression of tumor growth and a very limited increase of animals average survival rate. In addition, the localized injection also provided benefit in terms of tumor response, compared to the i.v. therapy, resulting in 25% of complete response and 25% of partial response. Unfortunately the entire group of animals received i.v. therapy developed progressive disease and none of them responded to the JS-K treatment. Furthermore, one third of the animals in the JS-K i.v. group experienced necrosis and inflammation of the injection site on their tails, which may be attributed to the toxicity of JS-K.

Besides HNSCC, this localized drug delivery platform could be adapted to other types of cancers, including breast cancer and prostate cancer, which were shown to be responsive to JS-K therapy [12,13,42,43]. Furthermore, recent investigations have discovered that nitric oxide may be involved in the reversal of cisplatin resistance in head and neck cancer [44], ovarian cancer [45,46] and lung cancer [47], via mechanisms of survivin modulation, depletion of cellular thiols, and inhibition of Bcl-2 ubiquitination, respectively. Similar re-sensitizing effects of nitric oxide were also reported in the reversal of doxorubicin chemo-resistance in human colon cancer [48]. Herein, this NO-eluting nanocarrier could either be utilized as a single treatment for JS-K responsive cancers, or incorporated into a combination therapy regimen to revert drug-induced chemo-resistance. Our ongoing studies will focus on optimizing the in vitro and in vivo properties of this type of nitric oxide-releasing nanocarriers, laying the foundation for future translational study, to develop a safe and effective nitric oxide formulation for treating cisplatin and doxorubicin resistant cancers.
5. Conclusions

We have developed a locoregional nitric oxide delivery platform for treating locally advanced head and neck cancer using multi-arm polymer nanoparticles. The polymeric architectures were success-
fully synthesized and characterized, which released nitric oxide chemotherapy in a sustained fashion, and significantly inhibited the growth of HNSCC in vivo.

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