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Aptamer-conjugated and doxorubicin-loaded unimolecular micelles for targeted therapy of prostate cancer

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Abstract

In the absence of effective therapy for prostate cancer, there is an immense need for developing improved therapeutic options for the management of this disease. This study has demonstrated that aptamer-conjugated unimolecular micelles can improve the *in vivo* tumor biodistribution of systemically administered anti-cancer drugs in prostate cancer expressing prostate-specific membrane antigen (PSMA). The aptamer-conjugated unimolecular micelles were formed by individual hyperbranched polymer molecules consisting of a hyperbranched H40 polymer core and approximately 25 amphiphilic polylactide–poly(ethylene glycol) (PLA–PEG) block copolymer arms (H40-PLA-PEG-Apt). The unimolecular micelles with an average hydrodynamic diameter of 69 nm exhibited a pH-sensitive and controlled drug release behavior. The targeted unimolecular micelles (i.e., DOX-loaded H40-PLA-PEG-Apt) exhibited a much higher cellular uptake in PSMA positive CWR22Rv1 prostate carcinoma cells than non-targeted unimolecular micelles (i.e., DOX-loaded H40-PLA-PEG), thereby leading to a significantly higher cytotoxicity. The DOX-loaded unimolecular micelles up-regulated the cleavage of PARP and Caspase 3 proteins and increased the protein expression of Bax along with a concomitant decrease in Bcl2. These micelles also increased the protein expression of cell cycle regulation marker P21 and P27. In CWR22Rv1 tumor-bearing mice, DOX-loaded H40-PLA-PEG-Apt micelles (i.e., targeted) also exhibited a much higher level of DOX accumulation in the tumor tissue than DOX-loaded H40-PLA-PEG micelles (i.e., non-targeted). These findings suggest that aptamer-conjugated unimolecular micelles may potentially be an effective drug nanocarrier to effectively treat prostate cancer.

Keywords

Unimolecular micelles; Hyperbranched amphiphilic block polymer; Aptamer; Tumor targeted; Drug delivery

1. Introduction

Cancer of the prostate gland is one of the most frequently diagnosed cancers amongst Western men, as well as men in most developing countries. According to figures available from the American Cancer Society, prostate cancer (PCa) has surpassed heart disease as the

top killer of men over the age of 85 years [1,2]. In the United States alone, the number of projected new cases of PCa for the year 2012 was more than 240,000, with over 28,000 deaths probable from the disease [1,2]. So far, treatment of advanced PCa relies mainly on non-specific therapies, such as chemotherapies and ionizing radiation, which have low efficacy and are highly toxic to normal tissues [3,4]. Nanotechnology-based therapeutic systems have attracted tremendous attention as they have the potential to revolutionize cancer therapy and diagnosis owing to their tumor-targeting ability that allows anti-cancer drugs to be delivered more specifically to the cancer cells, thus greatly enhancing the therapeutic outcomes while minimizing any non-specific systemic toxicity [5–7]. Several recent studies have already demonstrated that prevention and therapy of PCa using nanomedicine may be a viable option for decreasing the mortality and morbidity associated with the disease [8–10].

Among the various nanoparticle systems, polymeric micelles have attracted significant attention due to their unique core–shell structures, which enable them to be excellent carriers for hydrophobic drugs [11–16]. Conventional multi-molecular polymer micelles are formed by the self-assembly of multiple linear amphiphilic block copolymers. Such multi-molecular polymer micelles may provide insufficient *in vivo* stability because their *in vivo* stability is affected by many factors including the concentration of the linear amphiphilic block copolymers, flow stress, pH values, interaction with serum proteins, etc. [17]. Drug-loaded polymer micelles that are prematurely disassembled in the blood stream lose their tumor targeting ability and cause a burst-release of the drug in the blood stream.

Various strategies have been investigated to improve the thermodynamic instability associated with self-assembled nanoparticles [18]. Among them, unimolecular micelles—which are formed by individual hyperbranched amphiphilic block copolymers—offer a promising approach. Unimolecular micelles exhibit excellent *in vivo* stability due to their covalent nature without compromising the biodegradability or drug release profile [19–23]. Unimolecular micelles can also provide a high drug loading capacity, possess a narrow nanoparticle size distribution, and offer an excellent chemical versatility that allows for further functionalization such as ligand conjugation. Various types of hyperbranched polymers or dendrimers can be used as the inner core and/or macro-initiators for the conjugation/synthesis of the amphiphilic block copolymer arms [24]. Boltorn® H40 (H40), a hyperbranched aliphatic polyester, serves as a desirable inner core/macro-initiator for unimolecular micelles due to its biodegradability, biocompatibility, globular architecture, and its large number of terminal functional groups [15,19–23].

Drug nanocarriers are desirable for targeted cancer therapy due to their passive and active tumor-targeting abilities. Their passive tumor-targeting ability can be attributed to the enhanced permeability and retention (EPR) effect exhibited by solid tumors due to their rapid growth [25]. Active tumor-targeting ability can be obtained by conjugating certain types of ligands including aptamers, antibodies, and peptides, etc., to enhance the *in vivo* tumor accumulation of the nanomedicine. Among the various targeting ligands, aptamers have gained increasing attention in recent years because they exhibit a series of desirable properties for *in vivo* tumor targeting including a high affinity and specificity for the targeted receptors/antigens, non-immunogenicity, and remarkable stability over a wide range of pHs (e.g., pH from 4 to 9), temperatures, and organic solvents without loss of activity [26–31]. Moreover, the synthesis of aptamers does not rely on biological systems and is an entirely chemical process that can minimize batch-to-batch variability [32].

In this study, an aptamer-conjugated unimolecular micelle nanoplatfrom having a dendritic H40 core, a hydrophobic poly(L-lactide) (PLA) inner shell, and a hydrophilic poly(ethylene glycol) (PEG) outer shell was designed, characterized, and evaluated for targeted PCa

therapy (Fig. 1). The A10 aptamer can specifically recognize the extracellular domain of the prostate-specific membrane antigen (PSMA) abundantly expressed on the surface of the PCa cells [29,32]. Doxorubicin (DOX), a model anti-cancer drug that is also self-fluorescent, was physically encapsulated into the hydrophobic core of the unimolecular micelle consisting of H40 and PLA. DOX is commonly used in the treatment of a wide range of cancers; however, its cumulative cardiotoxicity and cytotoxicity to normal tissues remain major problems to be solved [33]. Both *in vitro* and *in vivo* studies, including cellular uptake, cytotoxicity, and *in vivo* tumor biodistribution, were conducted to extensively evaluate the potential of the unimolecular micelles as promising drug nanocarriers for targeted PCa therapy.

2. Materials and Methods

2.1. Materials

Boltron H40 (a hyperbranched polyester with 64 hydroxyl terminal groups per molecule; M_n : 2833 Da) was provided by Perstorp Polyols Inc., USA, and purified with acetone and tetrahydrofuran (THF). L-Lactide was purchased from Sigma-Aldrich and recrystallized from ethyl acetate before use. Succinic anhydride, 4-dimethylamino pyridine (DMAP), N-hydroxysuccinimide (NHS), 1,3-dicyclohexylcarbodiimide (DCC), and dichloromethane (DCM) were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and used without further purification. THF, triethylamine (TEA), dimethyl sulfoxide (DMSO), and dimethyl formamide (DMF) were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and were distilled before use. The heterobifunctional PEG derivatives, succinimidyl (NHS)-PEG₁₁₄-OH or methoxy (-OCH₃)-PEG₁₁₄-OH, both of which having a M_w of 5000, were acquired from JenKem Technology (Allen, TX, USA). Doxorubicin hydrochloride (DOX·HCl) (Tecoland Corporation, Irvine, CA, USA) and PSMA (A10) aptamer (Integrated DNA Technologies, Inc.) are commercially available.

2.2. Synthesis of H40-PLA

H40-PLA was prepared by the ring-opening polymerization of L-lactide using H40 as a macro-initiator and Sn(Oct)₂ as a catalyst. A 50 mL Schlenk flask was charged with H40 (400 mg, 9.04 mmol of hydroxyl groups) under an inert atmosphere and placed in an oil bath at 120 °C in order to melt it and facilitate its mixing with L-lactide. L-Lactide (4.00 g, 27.8 mmol) was slowly introduced into the flask and a catalytic amount ([catalyst]/[monomer] of 1:1000) of Sn(Oct)₂ (9 µL, 27.8 µmol) was added afterwards. The polymerization reaction mixture was stirred for 24 h. The resulting mixture was dissolved in THF and passed through a neutral alumina column. Next, the mixture was concentrated and precipitated into cold diethyl ether to yield a white H40-PLA powder. The final product was dried under vacuum for 24 h.

2.3. Synthesis of carboxyl-functionalized H40-PLA (H40-PLA-COOH)

H40-PLA-COOH was prepared by reacting H40-PLA (1.00 g, 38.1 µmol) with succinic anhydride (0.38 g, 3.8 mmol) in the presence of DMAP (0.70 g, 5.7 mmol) as a catalyst. The reaction was carried out in anhydrous DCM (10 mL) for 48 h at room temperature under stirring. Thereafter, the formed product was precipitated with cold diethyl ether and dried under vacuum. The impurities and unreacted materials of the product were removed by dialysis against deionized water using cellulose tubing (molecular weight cut-off of 3500 Da). After 48 h dialysis, the product was separated out using the freeze-drying method.

2.4. Synthesis of H40-PLA-PEG-OCH₃ (abbreviated as H40-PLA-PEG, non-targeted)

H40-PLA-COOH (30 mg, 1.04 μ mol) and MPEG-OH (methoxy (–OCH₃) PEG, 157 mg, 31.3 μ mol) were dissolved in 10 mL anhydrous DCM, which was treated with DCC (6.5 mg, 31.3 μ mol) and DMAP (0.4 mg, 3.1 μ mol) at about 0 °C. The reaction was carried out at room temperature for 48 h under stirring. After the by-product, dicyclohexylcarbodiurea, was removed by filtration, the product was precipitated with cold diethyl ether. The precipitate was then dialyzed against deionized water for 48 h using cellulose tubing (molecular weight cut-off, 12 kDa) and freeze-dried.

2.5. Synthesis of H40-PLA-PEG-OCH₃/NHS

H40-PLA-COOH (30 mg, 1.04 μ mol), MPEG-OH (141 mg, 28.1 μ mol), and NHS-PEG-OH (15.7 mg, 3.2 μ mol) were dissolved in 10 mL anhydrous DCM, which was treated with DCC (19.2 mg, 31.3 μ mol) and DMAP (0.4 mg, 3.1 μ mol) at about 0 °C. The reaction was carried out at room temperature for 48 h under stirring. After the by-product, dicyclohexylcarbodiurea, was removed by filtration, the product was precipitated with cold diethyl ether. The precipitate was then dialyzed against deionized water for 48 h using cellulose tubing (molecular weight cut-off, 12 kDa) and freeze-dried.

2.6. Synthesis of H40-PLA-PEG-OCH₃/aptamer (abbreviated as H40-PLA-PEG-Apt, targeted)

To synthesize H40-PLA-PEG-Apt, the A10 aptamer was solubilized in a mixture of formamide/acetonitrile (vol/vol 50:50) and was then reacted with H40-PLA-PEG-OCH₃/NHS at room temperature for 24 h. The resulting H40-PLA-PEG-Apt was dialyzed in cold methanol to remove the formamide from the reaction mixture. The resulting H40-PLA-PEG-Apt hyperbranched polymer was dried under vacuum and used for the preparation of unimolecular micelles without further treatment.

2.7. Preparation of DOX-loaded H40-PLA-PEG-Apt micelles

Doxorubicin-HCl (15 mg) was first treated with 2 mol excess of triethylamine. Afterwards, they were mixed with the hyperbranched amphiphilic block copolymer (H40-PLA-PEG-Apt, 50 mg) and dissolved in 5 mL of DMF under stirring. With this mixture, 15 mL of deionized water was added dropwise. Thereafter, the mixture was dialyzed against deionized water using a dialysis tubing (molecular weight cut-off of 2 kDa) for 24 h followed by freeze-drying.

2.8. Characterization

¹H NMR spectra of all intermediate and final polymer products were recorded at 25 °C on a Bruker DPX 300 spectrometer using D₂O, CDCl₃, or DMSO as the solvent. The molecular weight of the polymers was determined by gel permeation chromatography (GPC) equipped with a refractive index detector, a viscometer detector, and a light scattering detector (Viscotek, USA). DMF (with 10 mmol/L LiBr) was used as a mobile phase with a flow rate of 1 mL/min at 35 °C. The hydrodynamic size and size distribution of the unimolecular micelles were determined by dynamic light scattering (DLS) (ZetaSizer Nano ZS90, Malvern Instrument, USA), at a polymer concentration of 0.05 mg/mL. The morphology and size of the dried unimolecular micelles were measured using a transmission electron microscopy (TEM) at 75 Kv (Hitachi H-600, Japan). To prepare the TEM sample, a drop of micelle solution (0.05 mg/mL) containing 0.8 wt.% of phosphotungstic acid was deposited onto a 200 mesh copper grid coated with carbon and dried at room temperature. The DOX loading content (DLC), defined as the weight percentage of DOX in the DOX-loaded unimolecular micelles, was quantified by a Varian Cary 300 Bio UV–visible spectrophotometer. The calibration curve of absorbance for different concentrations of DOX

was determined at 485 nm. To determine the DLC, a weighed quantity (25 mg) of DOX-loaded micelles was extracted with ethanol at room temperature for 48 h under uniform stirring. After centrifugation, the supernatant containing DOX was assayed by UV-visible spectrophotometer at a wavelength of 485 nm. All of the experiments were carried out in triplicate.

2.9. In vitro drug release study

Drug release studies were performed in a glass apparatus at 37 °C in either acetate buffer (pH 5.3) or phosphate buffer (pH 7.4) solution. 10 mg of DOX-loaded unimolecular micelles was dispersed in 5 mL of medium and placed in a dialysis bag with a molecular weight cut-off of 2 kDa. The dialysis bag was immersed in 50 mL of the release medium and kept in a horizontal laboratory shaker (100 rpm) under constant temperature. Samples of 5 mL volume were periodically removed and the same volume of fresh medium was added. The amount of released DOX was analyzed with a spectrophotometer at 485 nm. The drug release studies were performed in triplicate for each sample.

2.10. Cell culture, treatment, and western blotting

Prostate carcinoma CWR22Rv1 cells were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 and were maintained under standard cell culture conditions supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C and a 5% CO₂ environment. The cells (60–70% confluent) were treated with native drug (i.e., free DOX) or DOX-loaded H40-PLA-PEG (non-targeted) and H40-PLA-PEG-Apt (targeted) unimolecular micelles (hence forth known as non-targeted and targeted unimolecular micelles, respectively) (Equivalent DOX concentration: 1 µg/mL) in complete medium. Forty-eight hours later, the medium was aspirated and the cells were washed with cold PBS (10 mmol/L, pH 7.4) followed by incubation in an ice-cold lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na₃VO₄, 0.5% NP40, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (pH 7.4)] with a freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) over ice for 30 min. The cells were scraped and the suspension was collected in a microfuge tube and passed through a 21.5-gauge needle to break up any cell aggregates. The lysate was cleared by centrifugation at 14,000× g for 25 min at 4 °C, and the supernatant (total cell lysate) was stored at 80 °C for further analysis. For Western blotting, 25–40 µg of protein were resolved over 8%–12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer [7% nonfat dry milk/1% Tween 20; in 20 mmol/L TBS (pH 7.6)] for 1 h at room temperature, incubated with the appropriate monoclonal or polyclonal primary antibody in blocking buffer for 2 h at room temperature or overnight at 4 °C, followed by incubation with an appropriate secondary antibody HRP conjugate. The blots were exposed to enhanced chemiluminescence (Thermo Scientific Pierce, Rockford, IL) and subjected to autoradiography using a BioRad (Hercules, CA) imaging system. Densitometric measurements of the bands in the Western blot analysis were done using the digitalized scientific software program Quantity One (BioRad). The treatment protocol was performed at least three times and the individual protein expressions were analyzed three times with comparable results.

2.11. Cellular uptake

Fluorescence microscopy was utilized to examine the cellular uptake of the free DOX and DOX-loaded unimolecular micelles. Cells were plated in a 2-chambered slide at 5000 cells/well and allowed to grow for 18 h. Post attachment, the cells were incubated for 30 min with either non-targeted or targeted unimolecular micelles or free DOX (equivalent DOX

concentration: 20 $\mu\text{g/mL}$). Unbound micelles were removed by washing three times with PBS and the cells were mounted using ProLong[®] Gold Antifade Reagent (Life Technologies, Grand Island, NY). The mountant was allowed to cure overnight in the dark at room temperature. The images were captured using a camera equipped on a Nikon Eclipse Ti inverted microscope (Nikon Instruments, Inc., Melville, NY). The experiment was repeated four times with similar results.

Flow cytometry was also used to examine the cellular uptake of the DOX-loaded targeted or non-targeted unimolecular micelles. Cells were plated in 6 well plates at 10,000 cells/well the night before treatment. The cells were then incubated for 30 min with either targeted or non-targeted unimolecular micelles, or free DOX (equivalent DOX concentration of 20 $\mu\text{g/mL}$). The cells were trypsinized and collected in the treatment media and centrifuged at 1000 rpm for 5 min. Unbound micelles and released drug were removed by aspirating the supernatant followed by washing two times with PBS with centrifugation (1000 rpm/5 min). The cells were then suspended in a flow cytometry buffer (1% BSA and 2% FBS in PBS) and the intensity of the doxorubicin fluorescence in the cells was determined on a BD FACSCalibur equipped with 488 and 633 nm lasers in the FL2 channel. The data collected from the FACS caliber was analyzed by FlowJo 10.0.5 analysis software (Tree Star, Inc., Ashland, OR). The experiments were run in triplicate and repeated three times with similar results.

2.12. Cell viability assay

Cell viability was assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay as described previously [34]. Briefly, the cells were plated at 1×10^4 per well in 200 μL of complete culture medium. The next day, cells were treated with free DOX or DOX-loaded non-targeted or targeted unimolecular micelles for 24 or 48 h. Each concentration was repeated in 10 wells. After incubation for the specified time at 37 $^{\circ}\text{C}$ in a humidified incubator, cell viability was determined. MTT (5 mg/mL in PBS) was added to each well and incubated for 2 h, after which the plate was centrifuged at $500\times g$ for 5 min at 4 $^{\circ}\text{C}$. The MTT solution was aspirated from the wells using vacuum and 0.2 mL buffered DMSO was added to each well. After a 10 min mixing, the absorbance was recorded on a microplate reader at a wavelength of 540 nm. The effect of each agent on growth inhibition was assessed as a percentage of cell viability in which vehicle-treated cells were taken as 100% viable. The experiment was repeated three times with similar outcomes.

2.13. In vivo biodistribution studies

Athymic nude mice (Charles River Laboratories, Wilmington, MA) were maintained in a temperature-regulated environment with a 12 h light/dark cycle for a week prior to the experiment. The animal protocol was approved by the Institutional Animal and Use Committee (IACUC) at the University of Wisconsin, Madison. CWR22Rv1 cells (1×10^6 cells), which are the cells that express PSMA, were suspended in a 1:1 medium mixed with Matrigel and were subcutaneously inoculated on 12 mice. About 15 days post inoculation and when the tumors reached a volume of about 200–350 mm^3 , the mice were divided into three groups such that the average tumor volume in each group was similar. Animals were then anesthetized and DOX-loaded non-targeted or targeted unimolecular micelles or native DOX were administered at 2.5 mg/kg via tail vein injection under general anesthesia (1.5% isoflurane/oxygen). Six hours post-injection, the animals were sacrificed by CO_2 asphyxia and the tissues (liver, kidney, spleen, heart, lung, and urogenital apparatus) and tumors were isolated under white light, washed in normal saline, and immediately scanned using a Xenon IVIS 200 Series (Caliper Life Sciences, Hopkinton, MA) equipped with a 150-W quartz halogen lamp and a 1 mW power scanning laser. All images were acquired by back-thinned, back-illuminated grade 1 CCD camera using a 1 s exposure time and medium binning.

Acquired images were measured and analyzed with Living Imaging software (Caliper Life Sciences). The *ex vivo* imaging experiments were repeated two times with similar observations.

2.14. Statistical analysis

All statistical analyzes were performed using GraphPad Prism Software (GraphPad Software, Inc., La Jolla, CA). Data are expressed as mean with 95% confidence intervals for all groups. Statistical significance of differences in all measurements between control and treated groups was determined by a 1-way ANOVA followed by a Dunnett's or Tukey's HSD test for multiple group analysis. Student's paired *t* test was used for pairwise group comparisons, as needed. All statistical tests were 2-sided, and values of *P* < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Synthesis and characterization of H40-PLA-PEG-Apt

Synthesis of H40-PLA-PEG-Apt was carried out as shown in Scheme 1. First, H40-PLA was prepared by the ring-opening polymerization of L-lactide using H40 as a macro-initiator and Sn(Oct)₂ as a catalyst under inert atmosphere at 120 °C for 24 h. The product was first purified by a neutral alumina column to remove the catalyst. Then the low molecular weight fraction was eliminated by a fractionation method of precipitation in cold diethyl ether. The formation of H40-PLA was confirmed by ¹H NMR (Fig. 2A). The peaks located at 1.54–1.56 ppm (a) and 5.10–5.16 ppm (b) were assigned to the protons of methyl and methine groups in the PLA main chains, respectively. The signal at 1.45 ppm (c) and 4.32–4.35 ppm (d) corresponded to the terminal methyl and methine protons of PLA (HOCHCH₃) of H40-PLA. The peaks at 1.18–1.22 ppm and around 4.20 ppm were assigned to the protons of the methyl groups and methylene groups of H40, respectively, confirming the dendritic structure of the H40-PLA polymer. By calculating the relative intensity of the peak at 1.45 ppm, which originated from the methyl group adjacent to the hydroxyl end group, and the peak at 1.56 ppm, which originated from the methyl groups present in the polymer chain, the molecular weight (*M_n*) and degree of polymerization (DP) of the PLA arms were found to be about 936 and 13, respectively. The average number of arms per H40-PLA molecule was estimated via comparing the molecular weights of H40 and H40-PLA determined by a GPC equipped with triple detectors, as shown in Table 1. The average number of arms per H40-PLA molecule was estimated to be 25 from the *M_n* values of H40 and H40-PLA using the following equation:

$$\text{Number of arms} = (M_n \text{ of H40-PLA} - M_n \text{ of H40}) / M_n \text{ of PLA} = [(26,208 - 2833) / 936] \approx 25$$

. This result agrees well with those reported by Kreutzer et al. and our previous studies [15,19,22,35].

In the next step, the hydroxyl terminal groups of H40-PLA were converted into carboxyl terminal groups by reacting H40-PLA with succinic anhydride in the presence of DMAP as the catalyst. The product was precipitated with diethyl ether and dialyzed against deionized water for purification. The ¹H NMR spectrum of H40-PLA-COOH (Fig. 2B) showed a new peak at 2.65 ppm (methylene groups of succinic anhydride, e, f), which confirmed the formation of H40-PLA-COOH. The third step was to conjugate hydrophilic MPEG and HO-PEG-NHS segments onto the hyperbranched hydrophobic H40-PLA-COOH molecule. The feed molar ratio of MPEG, HO-PEG-NHS, and H40-PLA-COOH was set at 22:3:1. In the ¹H NMR spectrum of the H40-PLA-PEG-OCH₃/NHS (Fig. 2C), in addition to the peaks from PLA, peaks at 3.65 ppm (g) and 3.38 ppm (h) were observed due to the methylene protons of oxyethylene units and methyl protons of MPEG, respectively. The peaks at 2.81 ppm were assigned to the ethylene group of the succinimidyl groups (NHS).

Lastly, the A10 aptamer for PSMA-targeting was conjugated onto the surface of the hyperbranched polymer H40-PLA-PEG-OCH₃/NHS. In order to achieve sufficient targeting efficiency to PSMA-expressing cells, the molar ratio of Aptamer: H40-PLA-PEG-OCH₃/NHS was set at 5% [36], which ensures that at least one A10 aptamer was conjugated onto each unimolecular micelle. The NMR spectrum of H40-PLA-PEG-Apt is shown in Fig. 2D. The appearance of a group of NMR peaks ranging from 1.8 to 2.10 ppm can be attributed to the protons of the A10 aptamer.

FTIR analyzes provide additional information about the hyper-branched amphiphilic block copolymer H40-PLA-PEG-OCH₃. The peak at 2937 cm⁻¹ was assigned to the anti-symmetric C-H stretching of CH₂. The strong peak at 1754 cm⁻¹ was attributed to the characteristic absorption of C=O stretching due to the presence of a hyperbranched polyester H40 core and PLA blocks. The absorption peak located at 1352 cm⁻¹ could be attributed to CH₂ wagging and C-C stretching in the PEG blocks. The strongest peak at 1068 cm⁻¹ was assigned to C-O-C stretching. The C-O-C stretching was also verified at 983 cm⁻¹. The FTIR results further testified to the successful formation of H40-PLA-PEG-OCH₃ [37].

3.2. Micellar properties of hyperbranched H40-PLA-PEG-Apt copolymers

The DOX-loaded hyperbranched amphiphilic block copolymer H40-PLA-PEG-Apt formed stable unimolecular micelles in an aqueous solution due to its large number of amphiphilic arms (~25) with a proper hydrophilic to hydrophobic ratio as well as its globular architecture. The hydrophobic H40-PLA inner part formed a hydrophobic core while the hydrophilic PEG layer formed a hydrophilic shell stabilizing the resulting unimolecular micelles in an aqueous solution. It is well known that the stability and size of drug nanocarriers play an important role in the cellular internalization process as well as their *in vivo* performance. To study the stability of the H40-PLA-PEG-Apt unimolecular micelles, the size and size distribution of the freshly prepared micelles and that of the micelles stored at 4 °C in a PBS solution for one month were measured and compared. It was found that the H40-PLA-PEG-Apt micelles were relatively stable in the aqueous solution since there were no obvious changes in either the appearance (e.g., no precipitations) or the size of the unimolecular micelles over one month. The size distribution histogram of the H40-PLA-PEG-Apt micelles measured by DLS at a concentration of 0.05 mg/mL is shown in Fig. 3A. The average hydrodynamic diameter of the H40-PLA-PEG-Apt micelles was about 69 nm. Fig. 3B shows the TEM images of the unimolecular micelles that were stained by phosphotungstic acid on a carbon-coated copper grid. The unimolecular micelles possessed a spherical shape with a diameter ranging from 20 to 37 nm. The size distribution of the micelles was also relatively narrow based on both DLS and TEM analyzes. At 0.05 mg/mL, the unimolecular micelles appeared as individual nanoparticles; namely, nearly no aggregation was observed. The sizes of the unimolecular micelles measured by TEM were smaller than those measured by DLS because TEM measures the size of the dried micelles while DLS measures the hydrodynamic diameters of the micelles.

3.3. Drug loading level and in vitro drug release

The amount of DOX incorporated into the unimolecular micelles was 10.4 wt.% as measured by UV analysis using the absorption peak of DOX at 485 nm. To study the effects of pH values on the drug release rates of the DOX-loaded unimolecular micelles, *in vitro* drug release studies were performed under simulated physiological (pH 7.4) and cellular (pH 5.3) conditions at 37 °C. As shown in Fig. 4, the pH value of the medium had a strong effect on the release rate of DOX from the unimolecular micelles. Specifically, the release rate of DOX was much higher at a pH of 5.3 than at a pH of 7.4. At a pH of 7.4, the amount of DOX released after 45 h was 55%, including an initial burst release of about 10%. However, at a pH value of 5.3, the amount of DOX released was approximately 91%. As such, the pH-

sensitive DOX-loaded unimolecular micelles can reduce the amount of the drug released during circulation in the blood stream (pH 7.4), and can provide a desirable level of drug to effectively kill cancer cells once the micelles are internalized into the endocytic compartments (e.g., endosomes and lysosomes) where the pH values range from 4.5 to 6.5. A faster release of DOX from DOX-loaded polymeric micelles in acidic conditions was also reported in the literature as well as in our previous study, which is speculated to be due to the protonation of the amino group of DOX and faster degradation of the micelle core at a lower pH [23,38]. The pH-dependent drug release behavior is very desirable for targeted cancer therapy because it can greatly enhance the therapeutic efficacy of cancer treatment while minimizing the non-specific systemic spread of toxicity.

3.4. Cytotoxicity of native DOX and DOX-loaded unimolecular micelles

The effects of DOX-loaded targeted and non-targeted unimolecular micelles as well as free DOX on the proliferative ability of human prostate carcinoma CWR22Rv1 cells were studied at 24 and 48 h post-treatment. A dose responsive cytotoxicity was observed with all three treatments, with native DOX demonstrating the highest cytotoxicity at both 24 and 48 h. At 24 h post-treatment both native DOX and DOX-loaded targeted unimolecular micelles demonstrated about 50% growth inhibition at 1 $\mu\text{g/mL}$ dose (IC_{50} of 1 $\mu\text{g/mL}$), with native DOX showing a marginally superior efficacy than the targeted micelles. The observed effects were highly significant ($P < 0.001$ control vs. DOX and targeted unimolecular micelle group all doses). Non-targeted unimolecular micelles, on the other hand, only showed about a 24% inhibition at 1 $\mu\text{g/mL}$ dose of DOX (Fig. 5). However, the results were still significant as compared to the untreated control group ($P < 0.05$ control vs. non-targeted 0.25 $\mu\text{g/mL}$; $P < 0.01$ control vs. non-targeted 0.5 $\mu\text{g/mL}$ group). The low cytotoxicity exhibited by the non-targeted micelles may be attributed to their lower level of cellular uptake in comparison to that of the targeted micelles in the CWR22Rv1 cells, which was confirmed by the cellular uptake studies discussed later. Native DOX demonstrated a high level of cytotoxicity because free DOX (a DNA intercalator) can easily diffuse through the cell membrane and binds avidly to the DNA structure of the CWR22Rv1 cells; however, free DOX does not have any tumor-targeting ability *in vivo*. After 48 h treatment, there was about 76% growth inhibition ($P < 0.001$ control vs. all groups; all doses) even at the lowest tested dose of native DOX and targeted unimolecular micelles, while non-targeted micelles demonstrated an IC_{50} of 1 $\mu\text{g/mL}$ (Fig. 6). These data clearly indicate that, similar to the free DOX, DOX-loaded, targeted unimolecular micelles were effectively taken up by the CWR22Rv1 cells overexpressing PSMA due to receptor-mediated endocytosis, while non-targeted micelles failed to demonstrate a similar efficacy. Comparable effects were seen in other PSMA positive prostate carcinoma cells (data not shown) suggesting that the observed effects are general and not cell type specific. These results clearly verify our previous findings in that nanoparticle-mediated delivery of bioactive agents enhances the effectiveness of the agent [8,9] and confirms other studies in that the targeted delivery of bioactive agents results in enhanced efficacy in PCa therapy [39,40].

3.5. Cellular uptake of non-targeted and targeted unimolecular micelles

Cellular internalization of DOX-loaded targeted and non-targeted unimolecular micelles and free DOX in the CWR22Rv1 PCa cells were studied using both confocal microscopy and flow cytometry. Fig. 7 shows the confocal images of the CWR22Rv1 cells after 30 min of treatment. The DOX concentration used for this study was 20 $\mu\text{g/mL}$. Based on the DOX fluorescence intensity (red), the level of cellular uptake decreased in the following order: free DOX, DOX-loaded targeted micelles, and DOX-loaded non-target micelles, which was consistent with the MTT assay results and the flow cytometry findings to be discussed next. Free DOX was localized in the nuclei of the cells. Similarly, DOX delivered by the targeted micelles were also largely localized in the cell nuclei. However, DOX delivered by non-

targeted micelles was mainly localized in the cytoplasm. These results again indicate that the targeted micelles were easily internalized by the PSMA positive CWR22Rv1 cells because the PSMA specific aptamers attached to the surface of the micelles while the non-targeted micelles failed to do so. Several studies previously reported similar uptake of DOX using aptamer mediated delivery [39,41]; however, no comparison between the free drug and targeted and non-targeted nanoparticles was reported in these studies.

To further verify the cellular uptake behavior of DOX-loaded targeted and non-targeted micelles and free DOX (DOX concentration: 20 $\mu\text{g/mL}$), flow cytometry analyzes were conducted. Similar to the findings observed using confocal microscopy, free DOX exhibited the highest cellular uptake followed by targeted micelles and then non-targeted micelles (Fig. 8). Native DOX resulted in an over 76 fold induction of RFI as compared to the untreated group (untreated control 3.97 vs. 285.7 in doxorubicin alone). The fluorescence intensity of native DOX was 1.6 times that of DOX-loaded targeted micelles and 3.9 times that of DOX-loaded non-targeted micelles ($P < 0.001$ control vs. treatment groups; DOX vs. targeted or non-targeted micelles).

3.6. Modulation of apoptosis and cell cycle by non-targeted and targeted micelles

The efficacy of native DOX and DOX-loaded targeted and non-targeted unimolecular micelles on the modulation of apoptosis and cell cycle in PCa cells was also studied. A significant up-regulation of Bax with a concomitant inhibition of Bcl₂ protein expression was observed suggesting a Bax/Bcl₂ ratio favoring apoptosis (Fig. 9). Importantly, the ratio was the highest with the free DOX followed by DOX-loaded targeted and non-targeted micelles. This modulation of Bax/Bcl₂ ratio is a known indicator of apoptosis in cancer cells [42–44]. In addition, the protein expression of PARP was evaluated and it was found that both native DOX and DOX-loaded targeted micelles demonstrated significant cleavage of the protein (85 kD fragment), while DOX-loaded non-targeted micelles showed little to no effect. Lastly, the protein expression of cleaved caspase-3 was assessed and it was observed that the responses of the treatments were essentially similar to what was observed in the PARP cleavage with only native DOX and DOX-loaded targeted micelles inducing cleavage of the protein.

The effects of the DOX-loaded targeted and non-targeted unimolecular micelles, as well as the native DOX on the induction of the cyclin-dependent kinase inhibitors WAF1/p21 and CIP1/p27, were also investigated. Immunoblot analysis and the densitometric quantitation of protein bands revealed that native DOX resulted in a marked induction of both p21 and p27 protein expression with DOX-loaded targeted micelles demonstrating similar effects, while DOX-loaded non-targeted micelles had little to no effect on both of these proteins. This modulation of apoptotic and cell cycle machinery proteins is well known and we and others have shown the involvement of these proteins in drug induced cell death in cancer cells [44–50].

3.7. In vivo biodistribution of the DOX-loaded targeted and non-targeted unimolecular micelles

To evaluate the effects of DOX-loaded aptamer-conjugated targeted and non-targeted micelles on the *in vivo* biodistribution of DOX, *ex vivo* imaging of all major organs 6 h post-injection were performed using a tumor xenograft assay. As described in the Materials and Methods section, PSMA positive CWR22Rv1 tumors were used for the study. Representative color coded NIR fluorescence images of the excised tumors along with the tissues (spleen, kidney, lung, liver, and heart) obtained 6 h post-treatment is shown in Fig. 10A. The fluorescence images showed that the strongest DOX fluorescence intensity was observed in tumors isolated from the targeted unimolecular micelle group followed by

tumors isolated from the non-targeted micelles treated group, while a minimal DOX fluorescence intensity was observed in the tumors isolated from the mice group treated with native DOX. The fluorescence intensity ratio obtained from all the animals in the individual groups showed a ratio of targeted micelles to native DOX as about 4 ($P < 0.05$), while the fluorescence intensity ratio between the tumors in targeted and non-targeted micelle groups was about 1.5 ($P < 0.05$) (Fig. 10B). These results established the selectivity of the drug to the tumor sites when aptamer-conjugated unimolecular micelles were used in comparison with non-targeted micelles and native DOX. *ex vivo* optical imaging was performed where there was no interference from overlaying tissues, e.g., skin and blood vessels, and thus it closely resembled intraoperative procedures in surgical oncology as opposed to whole-body imaging which might have some diagnostic value [51]. Further *in vivo* studies will help determine the antitumor effects and toxicity of these unique unimolecular micelles for targeted prostate cancer therapy.

4. Conclusions

The combination of targeted drug delivery and controlled drug release offered by drug nanocarriers can significantly enhance the therapeutic outcome of cancer therapy while minimizing undesirable side-effects. In this study, aptamer-conjugated unimolecular micelles made of hyperbranched amphiphilic block copolymers were synthesized and characterized for targeted PCa therapy. The unimolecular micelles possessed a uniform spherical shape with a diameter ranging from 20 to 37 nm measured by TEM. These micelles exhibited pH-sensitive and controlled drug release behavior, which can minimize the non-specific systemic spread of toxic drugs during circulation while maximizing the efficiency of tumor-targeted drug delivery. The aptamer-conjugated micelles exhibited a much higher cellular uptake in PSMA positive CWR22Rv1 cells due to PSMA-mediated endocytosis than non-targeted unimolecular micelles, thereby leading to a significantly higher cytotoxicity. In tumor-bearing mice, DOX-loaded aptamer-conjugated targeted micelles also exhibited a much higher level of DOX uptake in tumors than non-targeted micelles and free DOX according to *ex vivo* imaging. Thus, these DOX-loaded aptamer-conjugated unimolecular micelles may be promising drug nanocarriers for effective PCa therapy.

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Fig. 1. A schematic illustration of the H40-PLA-PEG-Apt nanocarriers for tumor-targeted drug delivery.

Fig. 2. ¹H NMR spectrum of (A) H40-PLA, (B) H40-PLA-COOH, (C) H40-PLA-PEG-OCH₃/NHS, and (D) H40-PLA-PEG-Apt.

Fig. 3. The (A) size distribution and (B) morphology of the H40-PLA-PEG-Apt unimolecular micelles as measured by DLS and TEM, respectively.

Fig. 4.
In vitro DOX release profiles of DOX-loaded unimolecular micelles at a pH of 5.3 and 7.4.

Fig. 5.

Effects of DOX and DOX-loaded targeted and non-targeted unimolecular micelles on cell viability. CWR22Rv1 cells were treated with free DOX, and DOX-loaded targeted and non-targeted unimolecular micelles for 24 h, and the cell growth was determined by MTT assay. The bars represent mean of three separate experiments wherein each treatment was repeated in 10 wells $\pm$ SEM. Three asterisks indicate a $P < 0.001$ compared with vehicle-treated controls, a single asterisk indicates a $P < 0.05$ compared with vehicle-treated controls, and two asterisks indicate a $P < 0.01$ compared with vehicle-treated controls.

Fig. 6. Effects of DOX and DOX-loaded targeted and non-targeted unimolecular micelles on cell viability. CWR22Rv1 cells were treated with free DOX, and DOX-loaded targeted and non-targeted unimolecular micelles for 48 h, and cell growth was determined by MTT assay. The points represent the mean of three separate experiments wherein each treatment was repeated in 10 wells; the bars represent SEM results. Three asterisks indicate a $P < 0.001$ compared with vehicle-treated controls.

Fig. 7.

Uptake of DOX in prostate cancer cells. CWR22Rv1 cells were treated with free DOX, or DOX-loaded targeted and non-targeted unimolecular micelles for 30 min, and DOX uptake by the cells was recorded on a Nikon inverted microscope. The red fluorescence represents the fluorescence intensity of DOX and its localization in the cells.

Fig. 8.

Uptake of DOX in prostate cancer cells. CWR22Rv1 cells were treated with DOX, or DOX-loaded targeted and non-targeted micelles for 30 min. DOX uptake by the cells was recorded by flow cytometry analysis on a BD FACS caliber as described under Materials and Methods. (A) The fluorescence intensity of DOX as represented by flow cytograms. (B) The relative fluorescence intensity as analyzed by FlowJo 10.0.5 analysis software, where the bars represent the SEM results. The three asterisks indicate a $P < 0.0001$ compared to vehicle-treated controls. The small case *b* indicates a $P < 0.0001$ compared to the DOX alone group.

Fig. 9. Modulation of apoptotic and cell cycle regulatory proteins. CWR22Rv1 cells were treated with DOX, and DOX-loaded targeted and non-targeted unimolecular micelles for 24 h. Protein expression was determined as described under Materials and Methods.

Fig. 10.

Ex vivo optical images of the tissues. Tissues were isolated from animals treated with DOX, and DOX-loaded targeted and non-targeted unimolecular micelles. (A) Representative images from one of the experiments for the organs. (B) Relative radiant efficiency of DOX in the excised tumors obtained from the eight animals combined together. A single asterisk indicates a $P < 0.05$ compared to the DOX only group, while the small case *a* indicates a $P < 0.05$ as compared to the targeted micelles group.

Scheme 1.

The synthesis scheme of H40-PLA-PEG-Apt nanocarriers (abbreviated as H40-PLA-PEG-Apt or targeted unimolecular micelles).

Table 1Molecular weights of H40, H40-PLA, and H40-PLA-PEG-OCH₃.

Sample	M_n	M_w	M_w/M_n
H40 ^a	2833	5100	1.80
H40-PLA	26208	36456	1.39
H40-PLA-MPEG	167130	275660	1.65

^aPerstorp data sheet.