Tumor-Targeting, pH-Responsive, and Stable Unimolecular Micelles as Drug Nanocarriers for Targeted Cancer Therapy

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A new type of multifunctional unimolecular micelle drug nanocarrier based on amphiphilic hyperbranched block copolymer for targeted cancer therapy was developed. The core of the unimolecular micelle was a hyperbranched aliphatic polyester, Boltorn H40. The inner hydrophobic layer was composed of random copolymer of poly(ε-caprolactone) and poly(malic acid) (PMA-*co*-PCL) segments, while the outer hydrophilic shell was composed of poly(ethylene glycol) (PEG) segments. Active tumor-targeting ligands, i.e., folate (FA), were selectively conjugated to the distal ends of the PEG segments. An anticancer drug, i.e., doxorubicin (DOX) molecules, was conjugated onto the PMA segments with pH-sensitive drug binding linkers for pH-triggered drug release. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) analysis showed that the unimolecular micelles were uniform with a mean hydrodynamic diameter around 25 nm. The drug loading content was determined to be 14.2%. The drug release profile, cell uptake and distribution, and cytotoxicity of the unimolecular micelles were evaluated *in vitro*. The folate-conjugated micelles can be internalized by the cancer cells *via* folate-receptor-mediated endocytosis; thus, they exhibited enhanced cell uptake and cytotoxicity. At pH 7.4, the physiological condition of bloodstream, DOX conjugated onto the unimolecular micelles exhibited excellent stability; however, once the micelles were internalized by the cancer cells, the pH-sensitive hydrazone linkages were cleavable by the intracellular acidic environment, which initially caused a rapid release of DOX. These findings indicate that these unique unimolecular micelles may offer a very promising approach for targeted cancer therapy.

INTRODUCTION

Micelles formed by self-assembly of amphiphilic block copolymers have attracted significant attention in various biomedical fields, including drug and gene delivery (*1–4*). Micelle drug delivery systems can improve the solubility of hydrophobic drugs, stabilize and protect drugs that are sensitive to the surrounding environment, reduce the nonspecific uptake by the reticuloendothelial system (RES), and facilitate targeted drug delivery; however, conventional multimolecuclar micelle drug delivery systems based on the linear amphiphilic block copolymers suffer from instability *in vivo*. Multimolecular micelles are only thermodynamically stable above the critical micelle concentration (cmc) of the amphiphilic copolymers. Above the cmc, micelles are in dynamic equilibrium with the free copolymer molecules in solution, continuously breaking and reforming (*5*). When the concentration of the copolymer is below the cmc, micelles disassemble, thereby releasing the drug prematurely before reaching the tumor target. Furthermore, there are a number of other factors that may affect the *in vivo* stability of the multimolecular micelles including flow stress, interactions between the micelles and the serum components, and variations in temperature, pH, and ionic strength.

The *in vivo* instability problem associated with multimolecular micelles can be overcome by developing unimolecular micelles based on amphiphilic copolymers with a dendritic or hyperbranched structure (*6, 7*). Unimolecular micelles do not disassemble upon dilution and are stable to environmental changes due to their covalent nature, thereby providing excellent *in vivo* stability. Unimolecular micelles also offer other advantages, including high drug loading capacity and narrow nanoparticle size distribution. Furthermore, the highly branched structure of the copolymers can provide many end groups for further functionalization (*8, 9*).

To improve the efficacy of cancer therapy using drug nanocarriers such as unimolecular micelles, the unimolecular micelles must exhibit a number of desirable properties, including *in vivo* stability, high drug loading capacity, and well-controlled nanoparticle sizes, as described previously. In addition, the ability to actively target the tumor cells *via* specific targeting ligands and the ability for controlled drug release *via* stimulus-responsive drug−copolymer linkers are essential.

Drug nanocarriers are desirable for cancer therapy because they can target the tumor tissue through the enhanced permeability and retention (EPR) effect (i.e., passive targeting) (*10*). To further improve the delivery efficiency and cancer specificity, active tumor-targeting ability is also required. Active targeting can be achieved by functionalizing the nanocarriers with targeting ligands such as small molecules (e.g., folate (FA)), peptides (e.g., cRGD) and antibodies, which can recognize and bind to specific receptors/integrins that are unique to or positively expressed on cancer cells (*11*). In terms of controlled drug release, ideally, anticancer drug should not be released during circulation in the bloodstream; however, once the drug nanocarriers are internalized by the cancer cells, a sufficient

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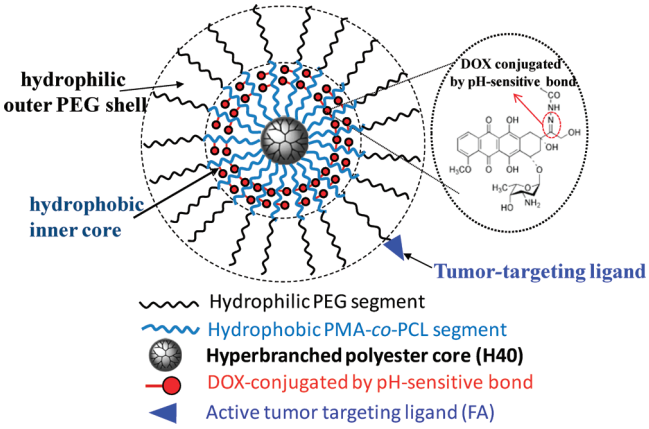
Scheme 1. Schematic Structure of Unimolecular Micelle Based on H40-(poly(β-malic acid)-hydrazone-doxorubicin-*co*-poly(ε-caprolactone))-methoxy-poly(ethylene glycol)-poly(ethylene glycol)-folate (H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA) Polymer

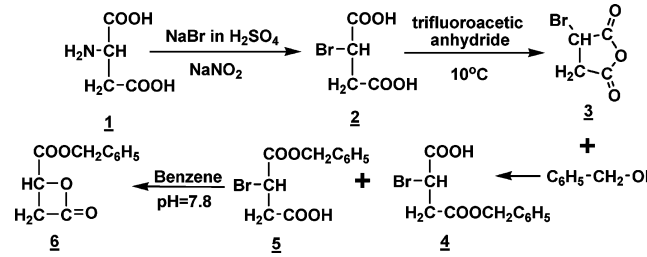


amount of anticancer drug should be released relatively quickly initially in order to effectively kill the cancer cells. The pH value of the bloodstream is approximately 7.4, while that of the endocytic compartments of the cells generally ranges from 4.5 to 6.5. This difference in pH value makes pH-triggered drug release possible (*12*).

Boltorn H40 has received much attention in the design of amphiphilic hyperbranched block copolymers for drug delivery application because of its biodegradability, biocompatibility, globular architecture, and high number of chain end functionalities (*13, 14*). Several excellent reports on drug nanocarriers using the hyperbranched aliphatic polyester, Boltorn H40 as the core are available in the literature. For instance, Prabaharan et al. reported a unimolecular micelle system made of a hyperbranched amphiphilic block copolymer based on Bolton H40 and copolymer of polylactide (PLA) and poly(ethylene glycol) (PEG) using FA as the active tumor-targeting ligands (*15*). Chen et al. reported a drug nanocarrier based on Bolton H40 and copolymer of polycaprolactone (PCL) and PEG, also using FA as the tumor-targeting ligand (*16*); however, in these systems, the anticancer drug, doxrubincin (DOX), was physically encapsulated only in the drug nanocarriers *via* hydrophobic interaction. As such, a significant amount of DOX may be released in the bloodstream during circulation, leading to poor targeting and reduced therapeutic effect.

In this paper, we developed a new type of multifunctional unimolecular micelle based on Boltron H40 as drug nanocarrier for targeted cancer therapy, which exhibited a pH-triggered drug release profile (Scheme 1). These unimolecular micelles were formed by amphiphilic hyperbranched copolymer, H40-((poly(β-malic acid)-hydrazone-doxorubicin)-*co*-poly(ε-caprolactone))-methoxy-poly(ethylene glycol)/poly(ethylene glycol)-folate (H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA) in aqueous solution. As shown in Scheme 1, Bolton H40 was used as the core of the amphiphilic hyperbranched copolymer. The hydrophobic segments of the amphiphilic copolymer arms were formed by random copolymerization of poly(β-malic acid) (PMA) and poly(ε-caprolactone) (PCL). The hydrophilic segments of the amphiphilic copolymer arms were formed by methoxy poly(ethylene glycol) (MPEG) or FA conjugated PEG (MPEG/PEG-FA). FA was used as the active tumor-targeting agent. FA is a vitamin with low molecular weight (M_w = 441.4) and behaves as a ligand because it has high affinity for its receptor, folate-binding protein, which is selectively overexpressed on the surface of many types of human cancer cells (*17, 18*). The PMA and PCL are important biodegradable aliphatic polyesters and of interest for biomedical applications owing to their good

Scheme 2. Synthesis Procedure of Benzyl Malolactonate Monomer



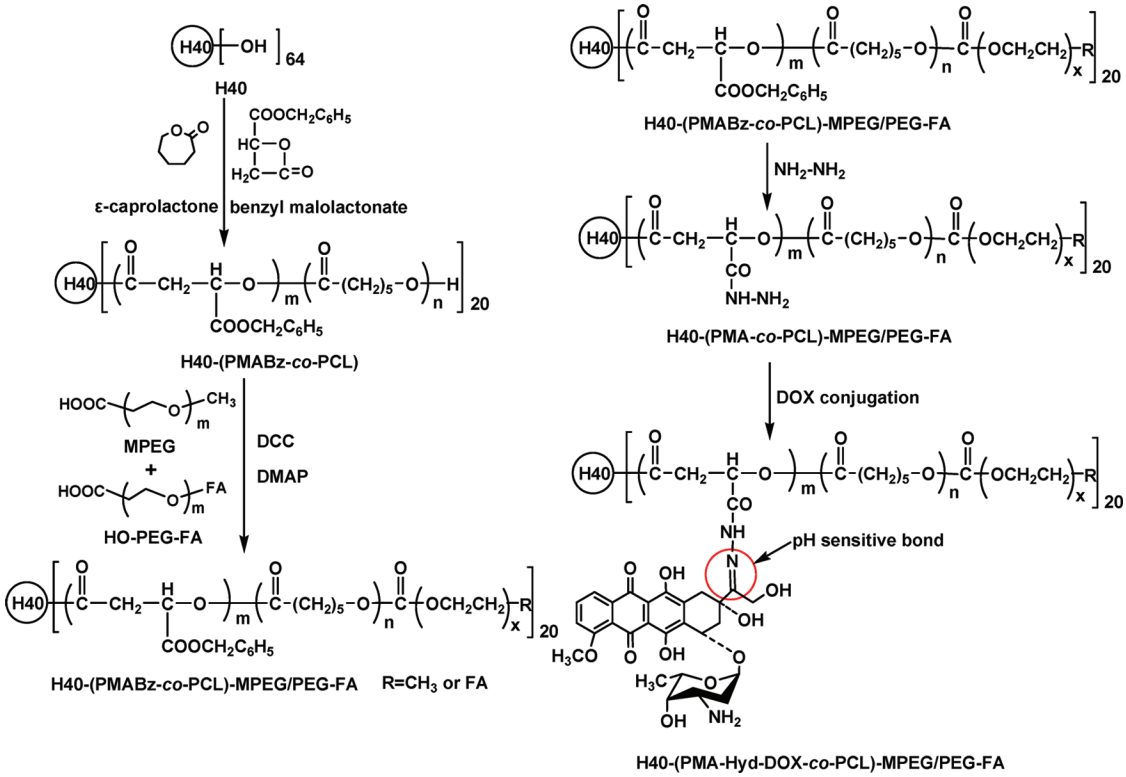
biocompatibility and low immunogenicity (*19*). The anticancer drug, doxorubicin (DOX), is widely used as an antitumor therapeutic agent and was conjugated onto the PMA-*co*-PCL segments through an acid-sensitive hydrazone bond. The random copolymer of PMA and PCL allows the DOX molecules to effectively conjugate onto the PMA segments due to the much reduced steric hindrance from the neighboring MA unit in the random copolymer. The acid-cleavable hydrazone linkages reduce the unwanted DOX release during blood circulation, yet provide quick DOX release initially once inside the tumor cells. This tumor-targeting unimolecular micelle nanosystem with a pH-triggered drug release profile and potentially excellent *in vivo* stability will open many exciting opportunities for targeted cancer therapy.

EXPERIMENTAL PROCEDURES

Materials. L-Aspartic acid, sodium bromide, sodium nitrite, trifluoroacetic anhydride, benzyl alcohol, and stannous octanoate ($Sn(Oct)_2$) were purchased from Fisher Scientific Inc., USA. Boltron H40 (64 hydroxyl groups per molecule theoretically and M_n of 2830 Da) was provided by Perstorp Polyols Inc., USA, and purified with tetrahydrofuran (THF). ε-Caprolactone (ε-CL, from Sigma-Aldrich) was purified by vacuum distillation over calcium hydride (CaH_2). Folic acid, dicyclohexylcarbodiimide (DCC), 4-dimethylamino pyridine (DMAP), and anhydrous hydrazine were purchased from Sigma-Aldrich, USA. MPEG was obtained from Fluka. α-Hydroxy-ω-amino poly(ethylene glycol) (HO-PEG-NH_2) with a M_w of 2000 was purchased from Iris Biotech GmbH and methoxy poly(ethylene glycol) (MPEG, M_w: 2000) was purchased from Fisher Scientific Inc., USA. Doxorubicin hydrochloride (DOX·HCl) was supplied by Tecoland Corporation, USA, and used as supplied. All other chemicals used were of analytical reagent grade. Phosphate and acetate buffered solutions were prepared in our laboratory. RPMI-1640 was purchased from Gibco BRL, USA. The mouse mammary carcinoma, 4T1 (ATCC), was cultured in RPMI medium supplemented with 10% fetal calf serum.

Synthesis of Benzyl Malolactonate Monomer (Scheme 2). Benzyl malolactonate monomer was prepared by a reported simple and reproducible method following Scheme 2 (*20*). First, to an ice-cold clear solution of L-aspartic acid (**1**, 50 g) and NaBr (200 g) in 2 N H_2SO_4 (300 mL), $NaNO_2$ was added progressively over a period of 2 h. The whole mixture was further stirred for 1.5 h at room temperature. After the excess reagents were decomposed by adding urea, the bromosuccinic acid was extracted with ethyl acetate twice. The extracts were combined and washed with slightly acidic water and dried over anhydrous sodium sulfate. The product **2** was recovered after evaporation of ethyl acetate. Second, the product **2** (20 g) was dissolved in anhydrous tetrahydrofuran (THF) (100 mL) containing trifluoroactetic anhydride (23.5 g) in a three-neck flask at 10 °C. The mixture was allowed to stir for 1.5 h at 10 °C and 2 h at room temperature. The THF and trifluoroacetic acid were then distillated under vacuum to yield bromosuccinic anhydride (**3**), a white crystalline compound. Then, benzyl alcohol (13 g) was immediately added to the flask containing **3**.

Scheme 3. Synthesis Procedure of H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA Polymer



The mixture was allowed to react for 4 h at room temperature and 1 h at 60 °C yielding a mixture of bromosuccinic acid monobenzyl ester isomers (**4** and **5**). Finally, the resulting mixture was dissolved in sodium hydroxide water with a pH value of 7.8. Subsequently, 200 mL of benzene was added into the mixture, which was stirred overnight at 50 °C. After cooling, the organic layer was separated from the aqueous phase and crude benzyl malolactonate was recovered after evaporation of the solvent. The product **6** was purified using column chromatography and further vacuum distilled.

Random Copolymerization of Benzyl Malolactonate and ε-Caprolactone Using H40 as Macroinitiator (H40-(PMABz-*co*-PCL)-OH). H40-(PMABz-*co*-PCL)-OH was prepared by the ring-opening polymerization of benzyl malolactonate and ε-caprolactone using H40 as a macroinitiator and $Sn(Oct)_2$ as a catalyst (Scheme 3). H40 (200 mg, 4.5 mmol of hydroxyl groups), benzyl malolactonate (0.5 g 2.43 mmol), ε-caprolactone (3.0 g 26.3 mmol), and catalyst ([catalyst]/[ε-caprolactone] = 1:1000) were added into a 250 mL flame-dried flask under N_2. The reaction was carried out under stirring at 110 °C for 24 h. After the reaction was complete, the mixture was diluted with THF, precipitated into diethyl ether, and washed with diethyl ether to produce a white H40-(PMABz-*co*-PCL)-OH powder.

Synthesis of α-Carboxyl-ω-FA Poly(ethylene glycol) (FA-PEG-COOH). FA-PEG-HO was prepared according to our previous work with a slight modification (*15*). The γ-NHS-FA was dissolved into 5 mL of a mixture of anhydrous DMSO and triethylamine (TEA) with a volume ratio of 10:1. An equimolar amount of HO-PEG-NH_2 was added to the mixture and the reaction was carried out under an anhydrous condition overnight. The product, FA-PEG-HO, was freeze−dried after dialysis against deionized water using cellulose tubing (M_w cutoff: 2000 Da). FA-PEG-COOH was prepared by reacting FA-PEG-OH (2 g, 0.57 mmol) with succinic anhydride (170 mg, 1.7 mmol) in the presence of pyridine. The reaction was carried out in anhydrous toluene under N_2 atmosphere at 70 °C for 24 h. Crude polymer obtained by precipitation into cold diethyl ether was redissolved in dichloromethane (DCM). The resulting DCM solution was washed three times with aqueous hydrochloric acid (10% in v/v) and then four times with a saturated NaCl solution. Then, the organic phase was isolated, dried over magnesium sulfate, and filtered. The final product, FA-PEG-COOH, was recovered by precipitation into cold diethyl ether and dried under vacuum.

MPEG-COOH was prepared from MPEG-OH using a procedure similar to the one described above.

Synthesis of H40-(PMABz-*co*-PCL)-MPEG/PEG-FA. H40-(PMABz-*co*-PCL)-MPEG/PEG-FA copolymer was synthesized by reacting the terminal hydroxyl groups of H40-(PMABz-*co*-PCL)-OH with the terminal carboxyl groups of anhydrous MPEG-COOH and FA-PEG-COOH. The molar ratio of MPEG-COOH and FA-PEG-COOH was 19:1 (the number of PMABz-*co*-PCL arms was 20, which was calculated according to the GPC analysis). For example, H40-(PMABz-*co*-PCL)-OH (5.52 g, 0.044 mmol) was dissolved in 30 mL anhydrous chloroform. Then, a mixture of MPEG-COOH (2.51 g, 1.26 mmol) and FA-PEG-COOH (152 mg, 0.06 mmol), DCC (272 mg, 1.32 mmol) and DMAP (161 mg, 1.32 mmol) in 20 mL anhydrous chloroform was added into the chloroform solution of H40-(PMABz-*co*-PCL)-OH. The reaction was carried out at room temperature for 24 h under stirring. The resulting solution was precipitated with cold diethyl ether twice. The precipitate was dried under vacuum. The impurities and unreacted materials of the product were removed by dialysis against deionized water using cellulose tube (M_w cutoff: 12 000 Da) for 48 h. After dialysis, the H40-(PMABz-*co*-PCL)-MPEG/PEG-FA copolymer was obtained by freeze−drying.

Synthesis of H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA. The benzyl groups of H40-(PMABz-*co*-PCL)-MPEG/PEG-FA were substituted with hydrazide groups for DOX binding by an ester-amide exchange aminolysis reaction (Scheme 3). H40-(PMABz-*co*-PCL)-MPEG/PEG-FA (2 g, 0.012 mmol) was dissolved in 100 mL of anhydrous DMF. Then, anhydrous hydrazine (5 mg, 0.156 mmol) was added to this solution. The reaction was allowed to proceed at 40 °C for 24 h, followed by dialysis against 0.25% ammonia solution using cellulose tube

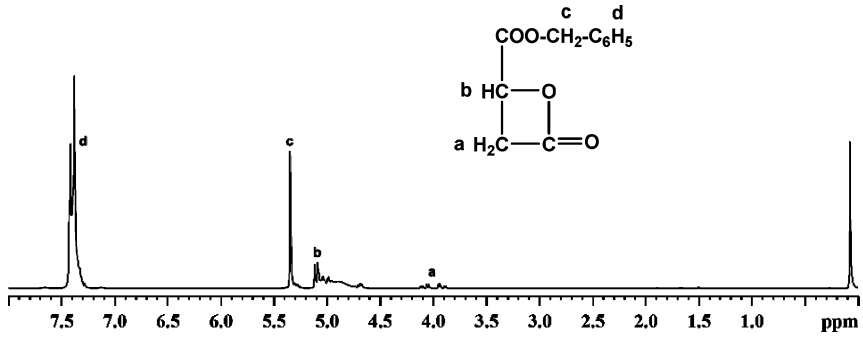


Figure 1. [1]H NMR spectrum of benzyl malolactonate monomer.

(M_w cutoff: 2000 Da) and freeze−drying. To conjugate DOX onto the amphiphilic hyperbranched copolymer, H40-(PMA-Hyd-*co*-PCL)-MPEG/PEG-FA (1.5 g, 0.009 mmol) was dissolved in 50 mL of DMF, and excess amount of DOX·HCl (150 mg, 0.26 mmol) was added. The mixture solution was stirred at room temperature for 24 h while being protected from light. The reaction solution was concentrated to a volume of 10 mL under reduced pressure and dialyzed against deionized water using cellulose tube (M_w cutoff: 2000 Da) for 48 h, followed by gel purification using Sephadex LH-20 to completely remove unbound DOX. For comparison, the H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG copolymer without FA targeting ligands was synthesized following the same procedure as described above.

Preparation of H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA Micelles. H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA micelles were prepared using the dialysis method. Briefly, 10 mg of H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA polymer was dissolved in 3 mL of DMF under stirring. Then, 5 mL of deionized water was added dropwise into the above solution. Thereafter, the mixture was dialyzed against deionized water using a dialysis tube (M_w cutoff: 2000 Da) for three days followed by freeze−drying.

Characterization. [1]H NMR spectrum was recorded on a Bruker DPX 300 spectrometer. Gel permeation chromatography (GPC) analysis was carried out at ambient temperature using the 270max Viscotek (Viscotek, USA) GPC system equipped with triple detectors, including a refractive index detector, a viscometer detector, and a light-scattering detector. THF was used as an eluent with a flow rate of 1 mL/min and polystyrene (PS) standards were used for column calibration. The amphiphilic hyperbranched block copolymer solutions in THF (5 mg/mL) were prepared. Absorbance measurements were carried out using a Varian Cary 100 Bio UV−visible spectrophotometer. The size and size distribution of the unimolecular micelles were determined by dynamic light scattering (DLS) (Beckman Coulter PCS submicrometer particle size analyzer, USA) with an angle detection at 90°. Samples for transmission electron microscopy (TEM, Hitachi H-600) analysis were prepared by drying a dispersion of the unimolecular micelles on a copper grid coated with amorphous carbon. Subsequently, a small drop of phosphotungstic acid (PTA) solution (2 wt % in water) was added to the copper grid; after 30 s the grid was blotted with filter paper for TEM observation. The DOX-loading content (DLC), defined as the weight percentage of DOX in unimolecular micelles, was quantified by UV−vis analysis using a Varian Cary model 100 Bio UV−vis spectrophotometer. The DOX was released from the unimolecular micelles in 0.1 N HCl solution to cleave the hydrazone bonds. To determine the drug content in the solution, the absorbance of DOX at 485 nm was measured using a previously established calibration. The DLC measurements were performed in triplicate for each of the samples.

Evaluation of pH Sensitivity. The unimolecular micelle's pH-responsive DOX release behavior was studied using an UV spectrophotometer. First, 50 mg freeze−dried micelle samples were dispersed in 5 mL of medium, placed in a dialysis bag with a molecular weight cutoff of 2000 Da, and then placed into various pH conditions from 7.4 to 4.0 (10 mM phosphate buffer (pH 7.4−6.0), 10 mM acetate buffer (pH 5.8−4.0)). The dialysis bag was then immersed in 100 mL of the release medium and kept in a horizontal laboratory shaker maintaining a constant temperature and stirring. At selected time intervals, the buffered solution (5 mL) outside the dialysis bag was removed for UV−vis analysis and replaced by fresh buffered solution with same volume. The amount of released DOX was analyzed with UV spectrophotometer at 485 nm. Each sample was measured three times.

Cellular Uptake and Cytotoxicity. The cellular uptake and distribution of micelles were performed using flow cytometry and confocal laser scanning microscopy (CLSM). For CLSM studies, 4T1 cells (1×10^6) were seeded onto 22 mm round glass coverslips placed in 6-well plates and grown overnight. Cells were treated with free DOX, DOX-conjugated H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA (i.e., FA-targeted), or H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG (i.e., nontargeted) micelles for 2 h (DOX concentration 20 μg/mL). Then the cells were washed and fixed with 1.5% formaldehyde. Coverslips were placed onto glass microscope slides and DOX uptake was analyzed using a Leica TCS SP2 Confocal System installed on an upright compound microscope (Leica, Wetzler, Germany). Digital monochromatic images were acquired using Leica Confocal Software (v 2.61).

To determine the DOX distribution in the nucleus and cytoplasm of the 4T1 cells, the DOX mean fluorescence intensity (MFI) in the CLSM images was measured in a 4 μm^2 area located in the nucleus or cytoplasm ($n = 10$ cells) for each sample using *ImageJ* software (http://rsb.info.nih.gov/ij). For flow cytometry, 4T1 cells (1×10^6) were seeded in 6-well culture plates and grown overnight. The cells were then treated with free DOX, DOX-conjugated H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA, or H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG micelles for 2 h (DOX concentration 20 μg/mL). Thereafter, the cells were lifted using a cell stripper (Media Tech, Inc.) and washed. The DOX uptake was analyzed using an FACSCalibur flow cytometer (BD bioscience). A minimum of 2×10^4 cells were analyzed from each sample with the fluorescence intensity shown on a four-decade log scale.

The cytotoxicity of the unimolecular micelles against 4T1 cells was studied using the MTT assay. First, 4T1 cells (1×10^4) were seeded in 96-well plates and incubated for 24 h. The media were replaced with fresh media containing free DOX, H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA, H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG micelles with different DOX concentrations or control media and incubated for 48 h. Thereafter, the wells were washed three times with warm phosphate buffer

solution and incubated again for another 3 h with RPMI containing 250 μg/mL of MTT. After discarding the culture medium, 200 μL of DMSO was added to dissolve the precipitates, and the resulting solution was measured for absorbance at 570 nm with a reference wavelength of 690 nm using a microplate reader (Molecular Devices).

RESULTS AND DISCUSSION

Synthesis and Characterization of Benzyl Malolactonate Monomer and H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA. Benzyl malolactonate is a β-substituted β-lactone monomer and was prepared by a reported simple and reproducible method according to Scheme 2 (*20*). This monomer was purified by reverse-phase silica (octadecylsilyl silica (ODS)) and then vacuum-distilled twice (bp = 90 °C, 10^{-3} mm Hg). Figure 1 shows the ^{1}H NMR spectrum of the monomer, confirming the monomer was successfully synthesized (a, 4.0 (d); b, 5.12 (t); c, 5.34 (s); d, 7.35 (s) in $CDCl_3$).

The synthesis of the H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA copolymer was carried out according to Scheme 3. First, H40-(PMABz-*co*-PCL)-OH was prepared by the random copolymerization of benzyl malolactonate and ε-caprolactone using H40 as a macroinitiator in the presence of $Sn(Oct)_2$ catalyst. This copolymer was purified by twice precipitating into diethyl ether from chloroform solution followed by filtering and was then dried under vacuum. The structure of the H40-(PMABz-*co*-PCL)-OH copolymer was confirmed by ^{1}H NMR (Figure 2a). The molar ratio of the PMABz and PCL repeat unit was approximately 1:15, which was calculated from the integral ratio of the characteristic peaks at 5.1 (MABz) and 3.9 (CL) ppm). On the basis of the absolute M_w determined by GPC (Table 1), it was estimated that each PMABz-*co*-PCL-OH arm on the hyperbranched H40-(PMABz-*co*-PCL)-OH copolymer had 2 MABz repeat units and 29 CL repeat units. To prepare the nontargeted unimolecular micelles, which can serve as a control for the study of cellular uptake and cytotoxicity of FA-targeted unimolecular micelles, the H40-(PMABz-*co*-PCL)-MPEG amphiphilic copolymer was synthesized by reacting H40-(PMABz-*co*-PCL)-OH with MPEG-COOH in the presence of DCC and DMAP. The number of arms in H40-(PMABz-*co*-PCL)-MPEG was calculated using the following equation: Number of arms = [(M_w of H40-(PMABz-*co*-PCL)-MPEG − M_w of H40-(PMABz-*co*-PCL)-OH)]/M_w of MPEG = [164 540−125 460/2000] ≈ 20. This result is consistent with our previous finding (*15*).

To achieve active tumor-targeting property, the H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA copolymer was prepared by reacting H40-(PMABz-*co*-PCL)-OH with a mixture of MPEG-COOH and FA conjugated PEG, i.e., FA-PEG-COOH, in the presence of DCC and DMAP. It is known that folate moieties with pterin heterocycles can self-assemble in an aqueous solution by hydrogen bonds and stacking interactions (*21*). As a result, introducing too many FA moieties to the amphiphilic copolymer decreases the stability of the micelles formed by the amphiphilic copolymer. In addition, the possible formation of dimers, trimers, or self-assembled tubular quarters (*21*) at a higher FA concentration is highly unfavorable for tumor cell targeting because the folate receptor can bind only one FA molecule; thus, the self-assembled FA multimers are incapable of binding to the folate receptors.

During this study, the molar ratio of the FA targeting ligand was controlled through the molar ratio between MPEG-COOH and FA-PEG-COOH, which was kept at 19:1. Thus, the estimated FA molar ratio in H40-(PMABz-*co*-PCL)-MPEG/PEG-FA was 1/20, i.e., 5%. Figure 2b shows the ^{1}H NMR spectrum of the H40-(PMABz-*co*-PCL)-MPEG/PEG-FA copolymer. The typical NMR peaks from the FA moiety appeared

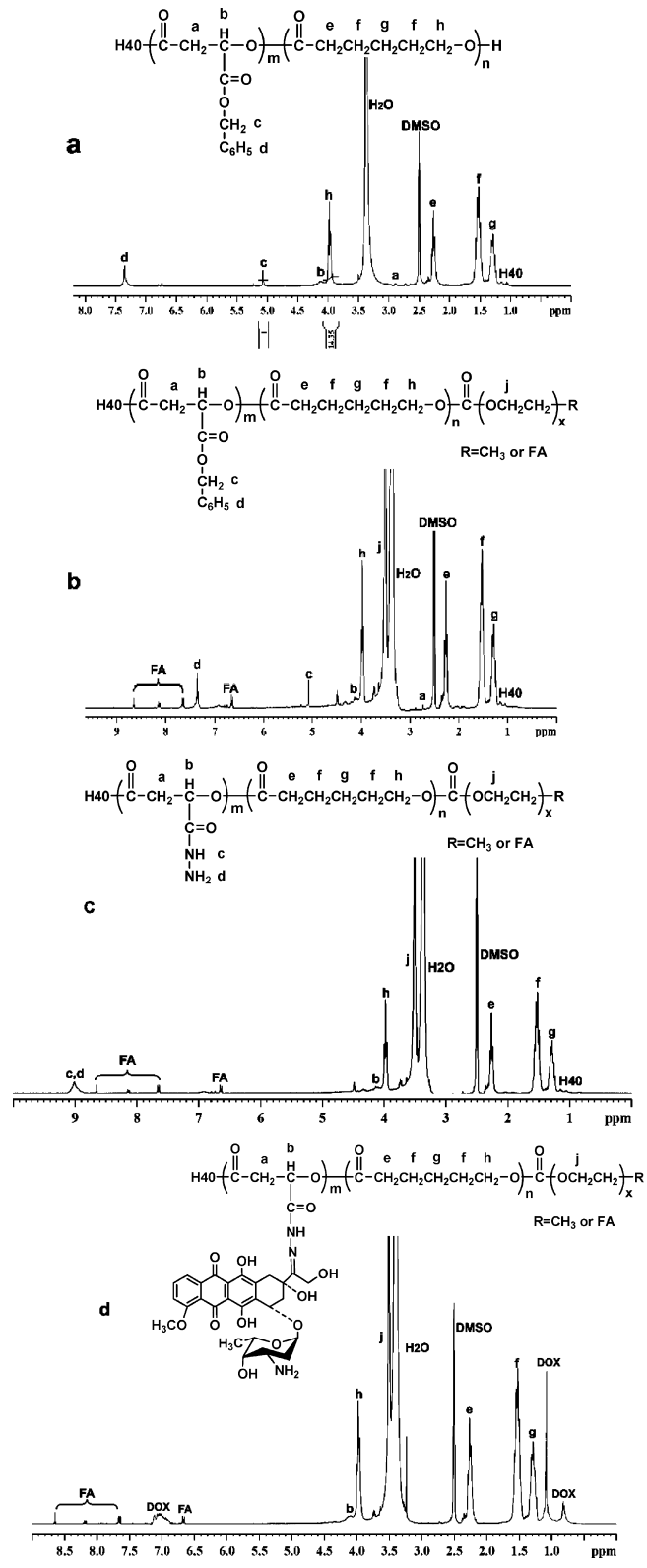


Figure 2. ^{1}H NMR spectra of (a) H40-[(poly(benzyl β-malate)-*co*-poly(ε-caprolactone)] (H40-(PMABz-*co*-PCL)-OH; (b) H40-[(poly-(benzyl β-malate)-*co*-poly(ε-caprolactone)]-methoxy-poly(ethylene glycol)-poly(ethylene glycol)-folate (H40-(PMABz-*co*-PCL)-MPEG/PEG-FA); and (c) H40-[(poly(β-malic acid-hydrazide)-*co*-poly(ε-caprolactone)]-methoxy-poly(ethylene glycol)-poly(ethylene glycol)-folate (H40-(PMA-Hyd-*co*-PCL)-MPEG/PEG-FA); (d) H40-[(poly(β-malic acid-hydrazone-DOX)-*co*-poly(ε-caprolactone)]-methoxy-poly(ethylene glycol)-poly(ethylene glycol)-folate (H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA).

Table 1. GPC Results of H40, H40-(PMABz-*co*-PCL)-OH, and H40-(PMABz-*co*-PCL)-MPEG

samples	M_n	M_w	M_w/M_n
H40	2 830	5 100	1.80
H40-(PMABz-*co*-PCL−OH)	76 500	125 460	1.64
H40-(PMABz-*co*-PCL)-MPEG	115 506	164 540	1.42

at 8.68, 8.17, 7.68, 6.98, and 6.67 ppm. The appearance of these peaks confirms the successful conjugation of FA to the hyperbranched copolymer.

The benzyl groups of the H40-(PMABz-*co*-PCL)-MPEG/PEG-FA copolymer were substituted with hydrazide groups for DOX binding through the ester-amide exchange (EAE) aminolysis reaction (*22*). The reaction was allowed to proceed at 40 °C for 24 h. The structure was confirmed by ^{1}H NMR spectrum as shown in Figure 2c. The NMR peaks originated from the benzyl ester groups (5.1 and 7.4 ppm) presented at the PMABz segments disappeared; meanwhile, the characteristic NMR peak corresponding to the hydrazine groups (9.0 ppm broadening peak) was observed. After the H40-(PMA-Hyd-*co*-PCL)-MPEG/PEG-FA copolymer was purified, it was reacted with an excess amount of DOX to form the H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA copolymer. After the reaction was complete, the polymer solution was dialyzed and further purified using Sephadex LH-20 to completely remove any unbound DOX. The structure of the H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA copolymer was confirmed by ^{1}H NMR. As shown in Figure 2d, the corresponding peaks of DOX appeared at 0.8, 1.1, and 7.0 ppm, indicating the DOX molecules were successfully conjugated onto the PMA segments by hydrazone bonds. The drug loading content determined by the UV measurement was 14.2 ± 0.3%. Theoretically, if all the hydrazide groups attached on the PMA segments are conjugated with the DOX molecules during the DOX conjugation reaction step, the DOX loading content is close to 15%. Thus, based on the DOX loading content measured by UV (14. 2%) and the theoretical maximum DOX loading content (∼15%), it can be deduced that both PMA repeat units in each arm of the 20-arm hyperbranched copolymer were conjugated with DOX molecules. Namely, there were approximately 40 DOX molecules per H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA molecule.

Micelle Properties of the H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA Copolymer. Figure 3A shows the morphology of the H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA unimolecular micelles observed by TEM. It can be seen the unimolecular micelles were spherical and most of them had a relatively uniform size, which was about 20 nm. The size and size distribution of the unimolecular micelles were further determined by DLS. The hydrodynamic size of the H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA unimolecular micelles was about 25 ± 2 nm, which was somewhat higher than the particle size determined by TEM (i.e., ∼20 nm), as expected. The size distribution, expressed by polydispersity index, was 0.145 ± 0.002. It should be noted that the resulting H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA copolymer micelles were stable over several weeks and displayed no changes in size or size distribution within experimental accuracy during this period.

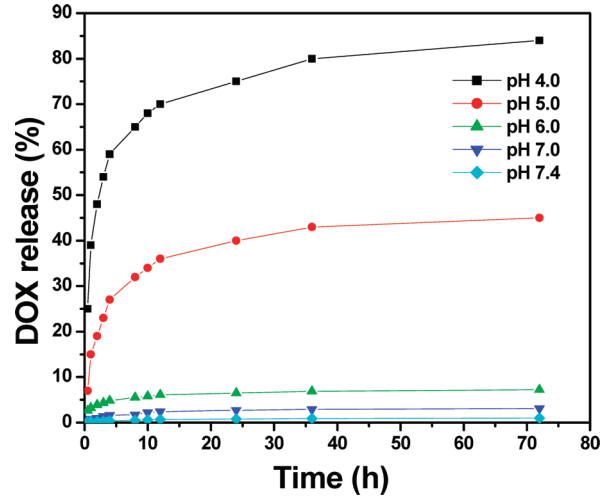


Figure 4. Release profile of DOX from the H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA micelles at different pH values.

Effect of pH on the *in Vitro* DOX Release Rate. The pH-sensitive DOX release behavior of the unimolecular micelles was evaluated at a series of pH values. As shown in Figure 4, the DOX release rate from the unimolecular micelles increased consistently with lower pH values within the pH range of 4.0 to 7.4. A negligible amount of drug release was observed at pH 7.4, the physiological condition of the bloodstream; thus, almost no DOX will be released from the unimolecular micelles during blood circulation, which is a desirable drug delivery characteristic for drug nanocarriers. The pH values of the endocytic compartments in which the FA-conjugated unimolecular micelles are located once internalized by the tumor cells *via* FA-receptor mediated endocytosis generally ranges from 4.5 to 6.5. As shown in Figure 4, the DOX released rate increased significantly within this pH range. In acidic conditions, the acid-sensitive hydrazone bonds are cleaved releasing unmodified DOX from the unimolecular micelles, which are subsequently diffused into the cytoplasm and eventually to the nucleus. At pH 5.0, about 48% DOX was released after 72 h. Thus, once the unimolecular micelles are internalized into the tumor cells, the conjugated DOX can be released effectively to improve the therapeutic outcome of the chemotherapy.

Cellular Uptake of the Unimolecular Micelles. The behaviors of cellular uptake of the FA-conjugated H40-(PMA-Hyd-

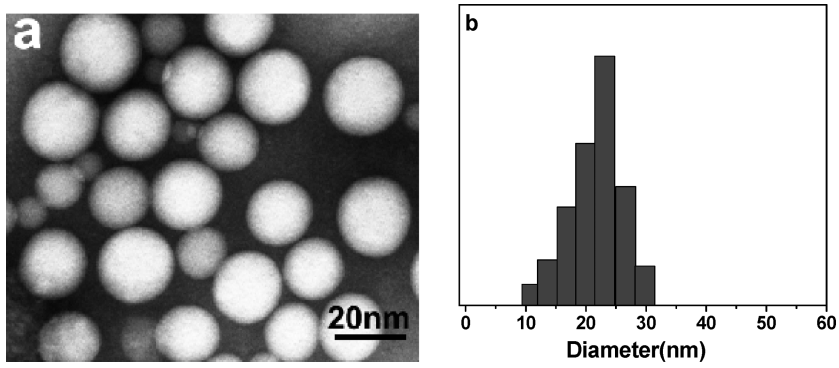


Figure 3. (a) TEM micrograph and (b) DLS histogram of unimolecular micelles based on H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA polymer.

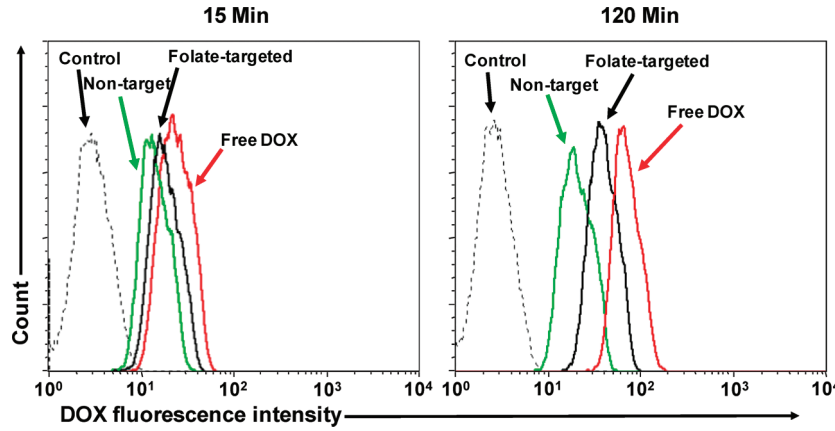


Figure 5. Flow cytometry results of 4T1 cells treated with free DOX, H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG micelles and H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA micelles at 37 °C for 15 and 120 min (DOX concentration 20 μg/mL).

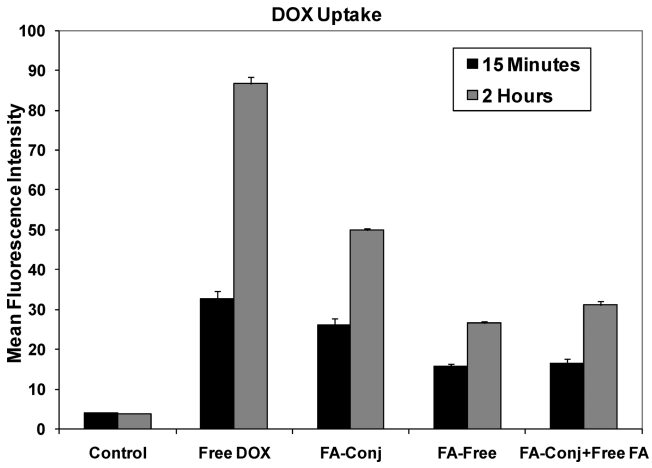


Figure 6. DOX mean fluorescence intensity (MFI) located in the 4T1 cells incubated with free DOX, nontargeted, and FA-conjugated unimolecular micelles for 15 and 120 min.

co-PCL)-MPEG/PEG-FA unimolecular micelles and nontargeted H40-(PMA-Hyd-*co*-PCL)-MPEG unimolecular micelles against folate receptor (FR) positive 4T1 cells were studied using both flow cytometry and CLSM. Figure 5 shows the flow cytometry histograms of the 4T1 cells incubated with free DOX, FA-conjugated micelles, or nontargeted micelles for 15 min and 2 h. As the figure shows, 4T1 cells without any DOX treatment were used as a negative control and showed only the autofluorescence of the cells. The DOX fluorescence intensity (Figure 6) clearly indicated that the cellular uptake of DOX was the highest in 4T1 cells incubated with free DOX, followed by FA-conjugated micelles and then nontargeted micelles. After 15 and 120 min incubation, FA-conjugated micelles showed an increase in cellular uptake, which was approximately 1.7 and 1.9 times (Student's t test; $p < 0.005$ and $p < 0.0001$, respectively) of the cellular uptake of nontargeted micelles, respectively, while the cellular uptake of free DOX was approximately 1.3 and 1.7 times ($p < 0.05$ and $p < 0.00005$, respectively) the cellular uptake of FA-conjugated micelles, respectively. The higher cellular uptake of free DOX compared with DOX conjugated onto unimolecular micelles can be attributed to the different cellular uptake mechanism. Free DOX was transported into cells *via* diffusion (*4*), while unimolecular micelles were taken up *via* an endocytosis process (*23*). However, the higher cellular uptake by the 4T1 cells incubated with FA-conjugated micelles compared with those incubated with nontargeted micelles clearly demonstrated that FA was an effective tumor-targeting ligand for tumor cells with positive FR. Moreover, the FA competition experiment also showed that the cellular uptake level of FA-conjugated micelles in the presence of free FA was dramatically reduced and became essentially equivalent to that of nontargeted micelles indicating the cell uptake of FA-conjugated micelles was FR mediated endocytosis process (Figure 6). These findings were further confirmed by the CLSM images as shown in Figure 7. 4T1 cells incubated with free DOX (Figure 7a) showed the highest DOX fluorescence intensity in both cytoplasm and nuclear after 2 h incubation. This is because free DOX can quickly diffuse into the cytoplasm through the cell membrane and transport into the nucleus, where it can interrupt the DNA replication process (*24*). Consistent with the flow cytometry finding, 4T1 cells incubated with FA-conjugated micelles showed a much higher DOX fluorescence intensity than those incubated with nontargeted micelles due to the FA-receptor mediated endocytosis process.

Cytotoxicity of the Unimolecular Micelles. The cytotoxic effects of free DOX, H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG,

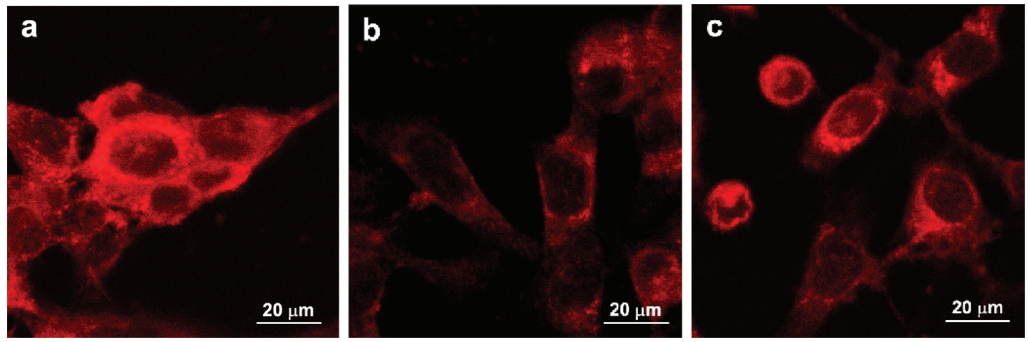


Figure 7. CLSM images of 4T1 cells treated with (a) free DOX; (b) H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG micelles; (c) H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA micelles at 37 °C for 2 h (DOX concentration 20 μg/mL).

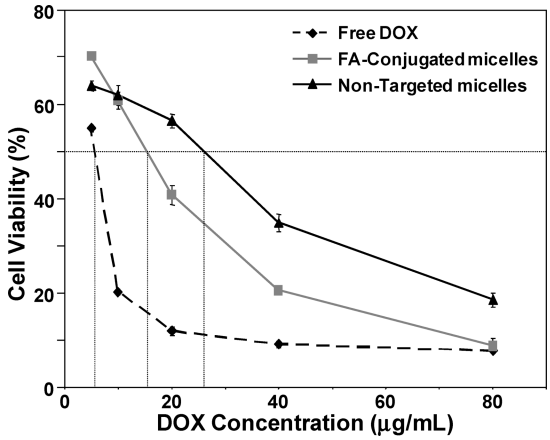


Figure 8. Cytotoxicity of free DOX, H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG micelles, and H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA micelles with different DOX concentrations against 4T1 cells (incubation time 48 h).

and H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA micelles with different DOX concentrations against 4T1 cells after 48 h incubation were studied using the MTT assay. As shown in Figure 8, at the same DOX concentration, free DOX showed the highest cytotoxicity, which is consistent with the fact that 4T1 cells incubated with free DOX had the highest cellular uptake according to the CLSM and flow cytometry analysis. In addition, free DOX can rapidly diffuse into the cell nuclei once inside the 4T1 cells and effectively inhibit the DNA replication in the cancer cells. FA-targeted micelles demonstrated a higher cytotoxicity compared with nontargeted micelles, which is also consistent with its higher cellular uptake observed by CLSM and flow cytometry. Moreover, the concentration yielding half-inhibition to cell growth (IC_{50}) was detected at 5.7, 15.5, and 26.2 μg/mL for free DOX and FA-conjugated and nontargeted micelles, respectively. The enhanced cytotoxicity exhibited by the FA-conjugated micelles again indicates that FA tumor-targeting ligands were effective in enhancing the cellular uptake of the unimolecular micelles.

CONCLUSIONS

Tumor-targeting multifunctional unimolecular micelles that exhibit a pH-triggered drug release profile and potentially excellent *in vivo* stability were developed for targeted cancer chemotherapy. The unimolecular micelles were formed by 20-arm hyperbranched amphiphilic H40-(PMA-Hyd-DOX-*co*-PCL)-MPEG/PEG-FA copolymers. Hyperbranched polyester Boltorn H40 was used as the macroinitiator for the random ring-opening copolymerization of PMABz and PCL, serving as the hydrophobic segments of the amphiphilic copolymers. The anticancer drug DOX was conjugated onto the PMA segments by acid-sensitive hydrazone bonds. Folate, the tumor-targeting ligand, was conjugated selectively (5% molar ratio) to the distal ends of the hydrophilic PEG segments. The hydrodynamic diameter of the unimolecular micelles was 25 $\pm$ 2 nm, determined by DLS. The DOX loading content was found to be 14.2%. There was very little DOX release at pH 7.4 indicating the DOX-conjugated unimolecular micelles will be very stable during blood circulation. On the other hand, DOX was released relatively quickly at lower pH values, indicating a sufficient amount of DOX may be released in the acid endocytic compartments once internalized by the tumor cells. The cellular uptake of the FA-conjugated unimolecular micelles was much higher than that of the nontargeted unimolecular micelles resulting from the FA-receptor mediated endocytosis, thereby leading to a much higher cytotoxicity. Thus, these novel tumor-targeting, pH-responsive, and potentially *in vivo* stable unimolecular micelles may provide a very promising approach for targeted cancer therapy and greatly improve the quality of cancer patient care.

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