Ligand-Directed Reduction-Sensitive Shell-Sheddable Biodegradable Micelles Actively Deliver Doxorubicin into the Nuclei of Target Cancer Cells

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ABSTRACT: The therapeutic performance of biodegradable micellar drugs is far from optimal due to existing challenges like poor tumor cell uptake and intracellular drug release. Here, we report on ligand-directed reduction-sensitive shell-sheddable biodegradable micelles based on poly(ethylene glycol)-poly(ε-caprolactone) (PEG-PCL) copolymer actively delivering doxorubicin (DOX) into the nuclei of target cancer cells, inducing superb in vitro antitumor effects. The micelles were constructed from PEG-SS-PCL and galactose-PEG-PCL (Gal-PEG-PCL) block copolymers, in which Gal-PEG-PCL was designed with a longer PEG than that in PEG-SS-PCL (6.0 vs 5.0 kDa) to fully expose Gal ligands onto the surface of micelles for effective targeting to hepatocellular carcinoma cells. PEG-SS-PCL combining with 10 or 20 wt % of Gal-PEG-PCL formed uniform micelles with average sizes of 56.1 and 58.2 nm (denoted as PEG-SS-PCL/Gal10 and PEG-SS-PCL/Gal20, respectively). The in vitro release studies showed that about 81.1 and 75.0% DOX was released in 12 h from PEG-SS-PCL/Gal10 and PEG-SS-PCL/Gal20 micelles under a reducing condition containing 10 mM dithiothreitol (DTT). In contrast, minimal DOX release (<12%) was observed for PEG-SS-PCL/Gal10 and PEG-SS-PCL/Gal20 micelles under nonreducing conditions as well as for reduction-insensitive Gal-PEG-PCL and PEG-PCL/Gal20 micelles in the presence of 10 mM DTT. MTT assays in HeLa and HepG2 cells showed that DOX-loaded PEG-SS-PCL/Gal20 micelles exhibited apparent targetability and significantly enhanced antitumor efficacy toward asialoglycoprotein receptor (ASGP-R)-overexpressing HepG2 cells with a particularly low half maximal inhibitory concentration (IC50) of 1.58 μg DOX equiv/mL, which was comparable to free DOX and approximately six times lower than that for nontargeting PEG-SS-PCL counterparts under otherwise the same conditions. Interestingly, confocal microscopy observations using FITC-labeled PEG-SS-PCL/Gal20 micelles showed that DOX was efficiently delivered and released into the nuclei of HepG2 cells in 8 h. Flow cytometry revealed that cellular DOX level in HepG2 cells treated with DOX-loaded PEG-SS-PCL/Gal20 micelles was much greater than that with reduction-insensitive PEG-PCL/Gal20 and nontargeting PEG-SS-PCL controls, signifying the importance of combining shell-shedding and active targeting. Ligand-directed, reduction-sensitive, shell-sheddable, and biodegradable micelles have emerged as a versatile and potent platform for targeted cancer chemotherapy.

INTRODUCTION

In the past decades, biodegradable micelles self-assembled from amphiphilic block copolymers such as poly(ethylene glycol)-poly(ε-caprolactone) (PEG-PCL), PEG-poly(D,L-lactide) (PEG-PLA), and PEG-poly(D,L-lactide-co-glycolide) (PEG-PLGA) have been extensively studied for controlled delivery of poorly soluble anticancer drugs including doxorubicin (DOX) and paclitaxel (PTX).1–6 The preclinical and clinical studies have shown that these biodegradable micelles have advantages of prolonging drug circulation time, passive targeting to the tumor tissues via the enhanced permeability and retention (EPR) effect, decreasing drug side effects, and improving drug tolerance.7,8 It is noted, however, that current biodegradable micelles exhibit gradual drug release over a period from days to weeks via a diffusion controlled mechanism due to slow hydrolytic degradation of polyesters.9,10 This slow
drug release behavior leads to significantly reduced in vitro and in vivo antitumor efficacy. We found that reduction-sensitive shell-sheddable micelles based on poly(ethylene glycol)-SS-poly(ε-caprolactone) (PEG-SS-PCL) or dextran-SS-PCL block copolymers could rapidly release DOX under intracellular reducing conditions, resulting in greatly enhanced antitumor efficacy. A systematic study showed that intracellular drug release from DOX-loaded PEG-PCL micelles, and accordingly, their therapeutic activity could be precisely controlled by extent of shell-shedding. Wang et al. reported that DOX-loaded shell-shedding poly(ethyl ethylene phosphate)-SS-PCL micelles exhibited intracellular glutathione (GSH)-dependent DOX release behavior and could effectively overcome the multidrug resistance (MDR) in cancer cells. In recent years, different types of reduction-sensitive shell-shedding micelles, for example, based on PEG-SS-polypeptide, PEG-SS-polycarbonate, PEG-SS-polymer, star-shaped polyester-SS-hydrophilic polymer, polyester-SS-poly(ethylene glycol) monomethyl ether methacrylate) (POEMA), disulfide-linked prodrug, and disulfide-linked amphiphilic graft copolymer have been developed and explored for enhanced intracellular delivery of various lipophilic anticancer drugs including camptothecin (CPT), DOX, and PTX. The reduction-sensitive shell-shedding strategy has appeared as an effective approach to boost intracellular drug release from micelles. Nevertheless, in spite that reduction-sensitive shell-shedding micellar drugs exhibited in general much greater antitumor efficacy than their reduction-insensitive counterparts, their cytostatic activity remained low as compared to free drugs likely due to poor tumor cell uptake. The installation of targeting ligands such as RGD peptides, folate, saccharides, antibodies, and aptamers onto different biodegradable micelles has shown to enhance their specific cellular uptake. The antitumor efficacy of targeting micellar drugs based on common amphiphilic biodegradable polymers like PEG-PCL was, however, not optimal owing to slow intracellular drug release. It is interesting to note that tumor-targeting reduction-sensitive shell-shedding micelles have not been reported to date.

In this paper, we report on novel galactose-functionalized reduction-sensitive shell-shedding biodegradable micelles based on PEG-SS-PCL and Gal-PEG-PCL block copolymers for efficient and active hepatoma-targeting delivery of DOX (Scheme 1). β-D-Galactose has been widely used for hepatoma-targeting drug and gene delivery due to its high affinity to asialoglycoprotein receptor (ASGP-R) that is overexpressed in hepatocellular carcinoma cells. For example, Huang et al. reported that galactosylated chitosan nanoparticles effectively targeted to hepatoma cells. Zhou et al. reported that galactosylated FITC-labeled dextran-PCL micelles were selectively recognized by ASGP-R overexpressing HepG2 cells in vitro and preferentially accumulated in liver in vivo. We found that galactose-decorated cross-linked biodegradable PEG-PCL block copolymer micelles showed an increased accumulation in tumor tissue, resulting in significant tumor growth inhibition in nude mice bearing SMMC-7721 tumors. Gal-PEG-PCL was designed with a longer PEG than that in PEG-SS-PCL (6.0 vs 5.0 kDa) so that Gal ligands are fully exposed onto the surface of micelles. Interestingly, our results showed that incorporation of 20 wt % of Gal-PEG-PCL into PEG-SS-PCL micelles while had little adverse effect on intracellular drug release behavior significantly improved their tumor specificity and efficacy. Here, preparation of Gal-decorated shell-sheddable micelles, loading and reduction-triggered release of DOX, receptor-mediated cellular uptake and intracellular drug release, and hepatoma-specific antitumor activity were studied.

### EXPERIMENTAL SECTION

**Materials.** Poly(ethylene glycol)-SS-poly(ε-caprolactone) (PEG-SS-PCL, \( M_\alpha (\text{H NMR}) = 7.9 \text{ kg/mol}, M_\beta (\text{GPC}) = 11.5 \text{ kg/mol}, M_{\alpha/\beta} = 1.13 \)), poly(ethylene glycol)-f-poly(ε-caprolactone) (PEG-PCL, \( M_\alpha (\text{H NMR}) = 8.1 \text{ kg/mol}, M_\beta (\text{GPC}) = 12.6 \text{ kg/mol}, M_{\alpha/\beta} = 1.08 \)), and galactose-conjugated PEG-PCL (Gal-PEG-PCL, \( M_\alpha (\text{H NMR}) = 10.0 \text{ kg/mol}, M_\beta (\text{GPC}) = 10.8 \text{ kg/mol}, M_{\alpha/\beta} = 1.30 \)) were synthesized according to our previous reports. Tetrahydrofuran (THF) was dried by refluxing over sodium wire and distilled prior to use. N,N-Dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO) were dried by refluxing over CaH\(_2\) and distilled under reduced pressure prior to use. Dichloromethane (DCM) was dried by refluxing over CaH\(_2\) and distilled before use. Lactobionic acid (LBA, 97%, Acros), 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride (EDC, 98%, J&K), cystamine dithydrichloride (>98%, Alfa Aesar), N-hydroxysuccinimide (NHS, 98%, Alfa Aesar), dithiothreitol (DTT, 99%, Merck), triethyamine (99%, Alfa Aesar), fluorescein isothiocyanate (FITC, 98%, Sigma), and doxorubicin hydrochloride (>99%, Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd.) were used as received.

**Characterization.** \(^1\text{H NMR} \) spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using deuterated chloroform (CDCl\(_3\)) or deuterated dimethyl sulfoxide (DMSO-d\(_4\)) as a solvent. The chemical shifts were calibrated against residual solvent signals. The molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with two linear PLgel columns (Mixed-C) following a guard column and a differential refractive-index detector. The measurements were performed using THF as the eluent at a flow rate of 1.0 mL/min at 30 °C and a series of narrow polystyrene standards for the calibration of the columns. The size of micelles was determined using dynamic light scattering (DLS) at 25 °C.
°C using Zetasizer Nano-ZS (Malvern Instruments) equipped with a 633 nm He–Ne laser using backscattering detection. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 μL of 0.2 mg/mL micelle suspension on the copper grid followed by staining with 1 wt % phosphotungstic acid.

**Synthesis of FITC-Labeled PEG-SS-PCL and PEG-PCL Copolymers**. FITC-labeled PEG-SS-PCL and PEG-PCL copolymers, that is, PEG-SS-PCL-FITC and PEG-PCL-FITC, were prepared by treating PEG-SS-PCL and PEG-PCL with fluorescein isothiocyanate (FITC) respectively. In a typical experiment, 20 mg of FITC was added to a solution of PEG-SS-PCL (Mn = 7.9 kg/mol, 80 mg) in DMSO (10 mL). The mixture was stirred at 90 °C for 12 h in the dark. The reaction mixture following cooling to room temperature, was dialyzed against DMSO for 1 d and then D.I. water for 2 d. The final product was dried by lyophilization. Yield: 96%. 1H NMR (400 MHz, DMSO-d6): δ 3.51 and 3.29 (PEG methylene protons and terminal methoxy protons); δ 2.96 and 2.77 (methylene protons of the disulfide linker); δ 5.77–8.32 (protons of FITC moiety). PEG-FITC-FITC was prepared in a similar manner. Yield: 97%. 1H NMR (400 MHz, DMSO-d6): δ 3.51 and 3.29 (PEG methylene protons and terminal methoxy protons); δ 4.35, 3.98, 2.27, 1.55, and 1.29 (PCL methylene protons); δ 5.77–8.32 (protons of FITC moiety). 1H NMR showed that both PEG-SS-PCL and PEG-PCL were quantitatively functionalized with FITC.

**Preparation of Micelles and Critical Micelle Concentration (CMC)**. Typically, micelles were prepared by dropwise addition of 4.0 mL of phosphate buffer (PB, 50 mM, pH 7.4) to a DMF solution (1.0 mL) of PEG-SS-PCL or PEG-SS-PCL/Gal/PEG-PCL mixture (5.0 mg/mL) under stirring at room temperature, followed by ultrasonication for 30 min and extensive dialysis (Spectra/Pore, MWCO 3500) against PB (50 mM, pH 7.4) for 24 h.

The CMC was determined using pyrene as a fluorescence probe. The concentration of block copolymer was varied from 1.0 × 10−3 to 0.1 mg/mL and the concentration of pyrene was fixed at 1.0 μM. The fluorescence spectra were recorded using a FLs920 fluorescence spectrometer with the excitation wavelength of 330 nm. The emission fluorescence at 372 and 383 nm were monitored. The CMC was estimated as the cross-point when extrapolating the intensity ratio I373/I383, at low and high concentration regions.

**Reduction-Triggered Destabilization of Micelles**. The change of PEG-SS-PCL/Gal20 micelle sizes in response to 10 mM DTT in PB buffer (50 mM, pH 7.4) was followed by DLS measurements. Briefly, to 3.0 mL of PEG-SS-PCL/Gal20 micelle dispersion in PB (50 mM, pH 7.4) previously degassed with nitrogen for 20 min was added DTT (final DTT concentration: 10 mM). The solution was immediately placed in a shaking bath (200 rpm) at 37 °C. At different time intervals, the micelle size was measured using DLS at 37 °C. PEG-SS-PCL micelles were used as a control.

**Loading of DOX into Micelles**. DOX-loaded micelles were prepared by dropwise addition of 4.0 mL of PB (50 mM, pH 7.4) to a solution of copolymer in DMF (1.0 mL, 5.0 mg/mL) and DOX in DMSO (50 μL, 5.0 mg/mL) under stirring at room temperature, followed by dialysis against PB (50 mM, pH 7.4) for 24 h (Spectra/Pore, MWCO 3500). The dialysis media was changed five times. The whole procedure was performed in the dark.

The amount of DOX was determined using fluorescence (FLs920) measurement (excitation at 480 nm and emission at 560 nm). For determination of drug loading content (DLC), lyopholized DOX-loaded micelles were dissolved in DMSO and analyzed with fluorescence spectroscopy, wherein calibration curve was obtained with DOX/DMSO solutions with different DOX concentrations. DLC and drug loading efficiency (DLE) were calculated according to the following formula:

\[
\text{DLC( wt\%)} = \frac{\text{weight of loaded drug}}{\text{total weight of loaded drug (drug and polymer)}} \times 100\%
\]

**Reduction-Triggered DOX Release from Micelles**. The in vitro release of DOX from micelles was studied using a dialysis tube (Spectra/Pore, MWCO 12000) at 37 °C in PB (50 mM, pH 7.4) either in the presence or absence of 10 mM DTT. To acquire sink conditions, drug release studies were performed at a micelle concentration of 1.0 mg/mL with 0.6 mL of micelle solution dialysis against 20 mL of the same media. At desired time intervals, 6 mL of release media was taken out and replenished with an equal volume of fresh media. The amount of DOX released was determined by using fluorescence measurements (FLS920). The release experiments were conducted in triplicate, and the results presented are the average data.

**MTT Assays**. The antitumor activity for DOX-loaded micelles and free DOX were determined using human hepatoblastoma cell line (HepG2 cells) and human cervical cell line (HeLa cells). The cells were plated in a 96-well plate (1 × 10^4 cells/well) using Dulbecco’s Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL). After 24 h, the cells were treated with various concentrations of DOX-loaded micelles and free DOX for 24 h. Cell viability (%) was determined by comparing the absorbance at 490 nm with control wells containing only cell culture media. The experiments were performed four times each.

The cytotoxicity of bare micelles was studied in a similar way. Briefly, HepG2 and HeLa cells were plated in a 96-well plate (1 × 10^4 cells/well) using DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL). After 24 h, the cells were treated with various concentrations of DOX-loaded micelles and free DOX for 24 h. Cell viability (%) was determined by comparing the absorbance at 490 nm with control wells containing only cell culture media. The experiments were performed four times each.

**Flow Cytometry Analysis**. HepG2 cells were seeded in a 6-well plate (1 × 10^4 cells/well) using DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL). After 24 h, the cells were treated with various concentrations of DOX-loaded micelles and free DOX solution in 400 μL of PBS were added to each well (DOX dosage: 5.0 μg/mL). After incubation at 37 °C for 4 h, the cells were digested by 0.25% (w/v) trypsin and 0.03% (w/v) EDTA. The suspensions were centrifuged at 700 × g for 3 min at 4 °C, washed twice with PBS, and then resuspended in 500 μL of PBS with 2% FBS. Fluorescence histograms were then recorded with a BD FACS-Calibur flow cytometer (Beckton Dickinson, U.S.A.) and analyzed using Cell Quest software. We analyzed 20000 gated events to generate each histogram. The gate was arbitrarily set for the detection of red fluorescence.

**Confocal Laser Scanning Microscopy (CLSM) Measurements**. HepG2 cells were plated on microscope slides in a 12-well plate (1 × 10^5 cells/well) using DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL). After 24 h, DOX-loaded FITC-labeled micelles or DOX solution in 200 μL of PBS were added to each well (DOX dosage: 5.0 μg/mL). After incubation at 37 °C and 5% CO₂ for 2 or 8 h, the culture medium was removed and the cells on microscope slides were washed three times with PBS. The cells were then fixed with 4% formaldehyde for 20 min and washed with PBS for 3 times. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue) for 20 min and washed with PBS three times.
Fluorescence images of cells were obtained using confocal microscope (TCS SP2).

**RESULTS AND DISCUSSION**

The aim of present study was to explore ligand-directed, reduction-sensitive, shell-sheddable, and biodegradable micelles as a multifunctional platform for active delivery of poorly soluble anticancer drugs into target tumor cells. Here, we chose hepatic carcinoma cells as a tumor cell model and galactose as a targeting ligand. The hepatoma-targeting shell-sheddable micelles were constructed from following two components (Table 1): (i) PEG-SS-PCL diblock copolymer \( (M_n = 5.0–2.9 \text{ kg/mol}) \) and (ii) galactose-PEG-PCL block copolymer (Gal-PEG-PCL, \( M_n = 6.0–4.0 \text{ kg/mol} \)) for active targeting to asialoglycoprotein receptor (ASGP-R) overexpressing hepatoma cells. \(^{35}\) In this study, we have also prepared PEG-PCL diblock copolymer (\( M_n = 5.0–3.1 \text{ kg/mol} \)) to be used as a reduction-insensitive control. In order to accomplish optimal exposure of Gal ligands at the surface of micelles for effective cellular targeting, Gal-PEG-PCL was equipped with a longer PEG chain than that in PEG-SS-PCL and PEG-PCL copolymers (6.0 vs 5.0 kg/mol).

**Formation of Gal-Functionalized Shell-Sheddable Micelles.** Micelles were prepared from Gal-PEG-PCL and PEG-SS-PCL block copolymers via the solvent exchange method. Notably, dynamic light scattering (DLS) showed that PEG-SS-PCL combining with 10 or 20 wt % of Gal-PEG-PCL formed uniform micelles with average sizes of 56.1 and 58.2 nm (denoted as PEG-SS-PCL/Gal10 and PEG-SS-PCL/Gal20, respectively), which were somewhat smaller than Gal-PEG-PCL micelles (69.6 nm) but larger than PEG-SS-PCL micelles (47.9 nm; Table 2). Figure 1A shows a typical size distribution profile of PEG-SS-PCL/Gal20 micelles. TEM micrograph displayed that PEG-SS-PCL/Gal20 micelles had a spherical morphology with an average particle size of about 50 nm (Figure 1B). The smaller size measured by TEM as compared to DLS was most probably due to shrinkage of the PEG shells upon drying. The critical micelle concentrations (CMC) determined by fluorescence study using pyrene as a probe were 7.1 and 6.9 mg/L for PEG-SS-PCL/Gal10 and PEG-SS-PCL/Gal20, respectively, which were comparable to those of PEG-SS-PCL and Gal-PEG-PCL micelles (Table 2).

<table>
<thead>
<tr>
<th>entry</th>
<th>copolymers</th>
<th>( M_n ) (kg/mol)</th>
<th>( ^{1} \text{H NMR}^a )</th>
<th>GPC ( ^b )</th>
<th>( M_n/M_w^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PEG-SS-PCL</td>
<td>5.0–3.0</td>
<td>5.0–2.9</td>
<td>11.5</td>
<td>1.13</td>
</tr>
<tr>
<td>2</td>
<td>Gal-PEG-PCL</td>
<td>6.0–5.0</td>
<td>6.0–4.0</td>
<td>10.8</td>
<td>1.30</td>
</tr>
<tr>
<td>3</td>
<td>PEG-PCL</td>
<td>5.0–3.0</td>
<td>5.0–3.1</td>
<td>12.6</td>
<td>1.08</td>
</tr>
</tbody>
</table>

\(^{a}\)Determined by \(^{1}\)H NMR. \(^{b}\)Determined by GPC measurements (THF as an eluent, at a flow rate of 1.0 mL/min, polystyrene standards, 30 °C).

<table>
<thead>
<tr>
<th>micelles</th>
<th>before drug loading</th>
<th>after drug loading</th>
<th>DLC (^c) (wt %)</th>
<th>DLE (%)</th>
<th>CMC (^c) (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-SS-PCL/Gal10</td>
<td>56.1 ± 1.2</td>
<td>56.6 ± 1.4</td>
<td>3.8</td>
<td>75.1</td>
<td>7.1</td>
</tr>
<tr>
<td>PEG-SS-PCL/Gal20</td>
<td>58.2 ± 0.9</td>
<td>58.6 ± 2.2</td>
<td>3.9</td>
<td>77.1</td>
<td>6.9</td>
</tr>
<tr>
<td>Gal-PEG-PCL</td>
<td>69.6 ± 1.5</td>
<td>72.3 ± 1.8</td>
<td>3.8</td>
<td>75.1</td>
<td>6.2</td>
</tr>
<tr>
<td>PEG-SS-PCL</td>
<td>47.9 ± 2.1</td>
<td>48.2 ± 2.4</td>
<td>4.1</td>
<td>81.2</td>
<td>7.5</td>
</tr>
</tbody>
</table>

\(^{c}\)Determined by dynamic light scattering (DLS). \(^{c}\)Determined by fluorescence measurement. \(^{c}\)Determined using pyrene as a fluorescence probe.
The results showed that PEG-SS-PCL/Gal10 and PEG-SS-PCL/Gal20 micelles exhibited decent DOX loading efficiencies of 75.1–77.1% (Table 2). The loading of DOX had little influences on micelle size and size distribution. The in vitro drug release studies were carried out at pH 7.4 (PB, 50 mM) and 37 °C using a dialysis tube (MWCO 12000) either in the presence or absence of 10 mM DTT. As expected, minimal DOX release (<12%) was observed for all micelles in 12 h under a nonreductive condition (Figure 3). Notably, DOX release from PEG-SS-PCL, PEG-SS-PCL/Gal20 micelles were used as controls. Data are presented as the average ± standard deviation (n = 3). The inset shows zoom-in image of drug release profile in the range of lower percentage of cumulative release.

Figure 3. The in vitro drug release from DOX-loaded PEG-SS-PCL/Gal10 and PEG-SS-PCL/Gal20 micelles in PB (50 mM, pH 7.4) at 37 °C in the presence or absence of 10 mM DTT (micelle concentration: 1.0 mg/mL). PEG-SS-PCL, PEG-PCL/Gal20, and Gal-PEG-PCL micelles were used as controls. The results showed that DOX-loaded PEG-PCL/Gal20 micelles (reduction-insensitive controls) remained low under 10 mM DTT condition. These results further confirm that drug release from biodegradable micelles can be regulated by extents of shell-sheding.

Cellular Uptake and Intracellular Release of DOX. The cellular uptake and intracellular drug release behaviors of DOX-loaded PEG-SS-PCL/Gal20 micelles was markedly accelerated in the presence of 10 mM DTT under otherwise identical conditions, in which 85.8, 81.1, and 75.0% of drug was released, respectively, in 12 h. In comparison, DOX release from Gal-PEG-PCL and PEG-PCL/Gal20 micelles (reduction-insensitive controls) remained low under 10 mM DTT condition. These results further confirm that drug release from biodegradable micelles can be regulated by extents of shell-sheding.

The hepatoma-targeting antitumor activity of DOX-loaded PEG-SS-PCL/Gal10 and PEG-SS-PCL/Gal20 micelles was investigated in HepG2 cells using MTT assays. DOX-loaded PEG-PCL/Gal20, and Gal-PEG-PCL micelles were used as controls. The results showed that DOX-loaded PEG-SS-PCL/Gal10 and PEG-SS-PCL/Gal20 micelles induced pronounced antitumor effects toward HepG2 cells (Figure 6A). The half maximal inhibitory concentration (IC50) of DOX-loaded PEG-SS-PCL/Gal20 micelles was determined to be 1.58 μg DOX equiv/mL, which was comparable to that of free DOX (1.04 μg DOX/mL) and nearly 6-fold lower than that of nontargeting PEG-SS-PCL counterparts (Figure 5). In accordance with confocal observations, cells treated with DOX-loaded PEG-PCL/Gal20 micelles (reduction-insensitive) displayed about 16-fold lower intracellular free DOX level than those with DOX-loaded PEG-SS-PCL/Gal20 micelles. These results point out that Gal-decorated PEG-SS-PCL micelles are efficiently taken up by HepG2 cells via a receptor-mediated endocytosis and drug is rapidly released inside the cells. To the best of our knowledge, this represents a first report on efficient delivery and release of DOX into the nuclei of cancer cells using biodegradable micelles.

Hepatoma-Targetability and Antitumor Activity of DOX-Loaded Gal-Decorated Shell-Sheddable Micelles. The hepatoma-targeting antitumor activity of DOX-loaded PEG-SS-PCL/Gal10 and PEG-SS-PCL/Gal20 micelles was studied in HepG2 cells using confocal laser scanning microscope (CLSM) and flow cytometry. To visualize DOX release from micelles, PEG-SS-PCL and PEG-PCL block copolymers were labeled with FITC by treating them with fluorescein isothiocyanate in DMSO at 90 °C. 1H NMR of resulting PEG-SS-PCL-FITC copolymers indicated quantitative functionalization. The confocal micrographs showed that FITC fluorescence was distributed throughout the whole cells following 2 h incubation with DOX-loaded PEG-SS-PCL/Gal20 micelles (Figure 4A), indicating fast uptake of PEG-SS-PCL/Gal20 micelles by HepG2 cells. Notably, DOX fluorescence was observed in the perinuclear as well as nuclear regions of cells, suggesting that DOX has been released from PEG-SS-PCL/Gal20 micelles. We and others have shown that fluorescence of DOX encapsulated in the micelles would be self-quenched.33–45 The intracellular FITC and DOX fluorescent emissions became much stronger after a longer incubation time of 8 h (Figure 4B). It is interesting to note that DOX fluorescence was primarily detected in the nuclei of HepG2 cells, while FITC fluorescence was mainly located in the cytoplasms, confirming that DOX is released from the micelles in the cytoplasm. Moreover, the nuclear DOX fluorescence was comparable to that observed for HepG2 cells following 8 h treatment with free DOX under otherwise the same conditions (Figure 4C). These results corroborate that PEG-SS-PCL/Gal20 micelles can efficiently deliver and release DOX into the nuclei of HepG2 cells. In contrast, negligible DOX fluorescence was observed in HepG2 cells after 8 h incubation with DOX-loaded PEG-PCL/Gal20 micelles (reduction-insensitive control), though intracellular FITC fluorescence was strong (Figure 4D). Notably, our previous results showed that nontargeting shell-sheddable micelles based on PEG-SS-PCL and dextran-SS-PCL transported and released DOX primarily to the cytosol and perinuclear regions of tumor cells under similar conditions,42,43 indicating that targeting facilitates nuclear delivery of DOX. In the following, flow cytometry was employed for the quantitative determination of free DOX levels in HepG2 cells.13,46 The cells were incubated for 4 h with DOX-loaded micelles or free DOX at a drug dosage of 5.0 μg/mL. Interestingly, the results demonstrated that HepG2 cells treated with DOX-loaded PEG-SS-PCL/Gal20 micelles had a high free DOX level, which was comparable to that with free DOX and about 3.6-fold higher than that with nontargeting PEG-SS-PCL counterparts (Figure 5). In accordance with confocal observations, cells treated with DOX-loaded PEG-PCL/Gal20 micelles (reduction-insensitive) displayed about 16-fold lower intracellular free DOX level than those with DOX-loaded PEG-SS-PCL/Gal20 micelles. These results point out that Gal-decorated PEG-SS-PCL micelles are efficiently taken up by HepG2 cells via a receptor-mediated endocytosis and drug is rapidly released inside the cells. To the best of our knowledge, this represents a first report on efficient delivery and release of DOX into the nuclei of cancer cells using biodegradable micelles.
Gal-PEG-PCL ≈ PEG-PCL/Gal20, signifying the importance of combining hepatoma-targeting and active intracellular drug release. The much higher antitumor activity of DOX-loaded PEG-PCL/Gal20 and Gal-PEG-PCL counterparts suggests that slow intracellular drug release accounts more than poor cellular uptake for reduced antitumor activity of micellar drug formulations. In comparison, DOX-loaded PEG-SS-PCL/Gal20, PEG-SS-PCL/Gal10, and PEG-SS-PCL micelles revealed similar antitumor effect to HeLa cells (low expression of ASGP-R), which was about 10-fold lower than free DOX under otherwise the same conditions (Figure 6B). These results further confirm that Gal-decorated reduction-sensitive shell-sheddable micelles possess apparent targetability to ASGP-R-overexpressing hepatoma cells. MTT assays showed that PEG-SS-PCL/Gal20 and PEG-SS-PCL/Gal10 micelles, along with PEG-SS-PCL, PEG-PCL/Gal20, and Gal-PEG-PCL controls, were practically nontoxic (cell viabilities >93%) toward both HepG2 and HeLa cells up to a tested concentration of 1.5 mg/mL (Figure 7), supporting that micelles based on PEG-PCL block copolymers possess good biocompatibility. This study has made a proof-of-concept that ligand-directed, reduction-sensitive, shell-sheddable, and biodegradable PEG-PCL block copolymer micelles can actively deliver potent anticancer drugs into the nuclei of target cancer cells, inducing superb antitumor effects. In the following, we will investigate the in vivo impacts of these micelles in vivo.

Figure 4. CLSM images of HepG2 cells following 2 or 8 h incubation with DOX-loaded PEG-SS-PCL/Gal20 micelles (DOX dosage: 5.0 μg/mL), free DOX and DOX-loaded PEG-PCL/Gal20 micelles were used as controls. For each panel, images from left to right show cell nuclei stained by DAPI (blue), FITC fluorescence in cells (green), DOX fluorescence in cells (red), and overlays of the above images. The scale bars correspond to 25 μm in all the images: (A) DOX-loaded PEG-SS-PCL/Gal20, 2 h; (B) DOX-loaded PEG-SS-PCL/Gal20, 8 h; (C) free DOX, 8 h; (D) DOX-loaded PEG-PCL/Gal20, 8 h.

Figure 5. Flow cytometry measurements of cellular DOX level of DOX-loaded PEG-SS-PCL/Gal20 micelles in HepG2 cells following 4 h incubation (DOX dosage: 5.0 μg/mL, cell counts were 20000). Free DOX, DOX-loaded PEG-SS-PCL micelles, DOX-loaded PEG-PCL/Gal20 micelles, and HepG2 cells were used as controls.
performances of tumor-targeting shell-sheddable biodegradable micelles using different targeting ligands and tumor models.

CONCLUSIONS

We have demonstrated for the first time that ligand-directed, reduction-sensitive, shell-sheddable, and biodegradable micelles based on PEG-PCL copolymer efficiently deliver and release DOX into the nuclei of target cancer cells, resulting in superior in vitro antitumor efficacy. These multifunctional micelles offer several unique advantages: (i) They are made of biocompatible PEG and PCL that are among the few polymers widely used in the clinical settings. (ii) They can be effectively taken up by target cancer cells via the receptor-mediated endocytosis mechanism, possibly due to optimal exposure of targeting ligands at the micelle surface. It should further be noted that, by employing different targeting ligands, they can, in principle, be adapted for the effective treatment of various malignancies. (iii) They quickly release anticancer drugs into the nuclei of target cancer cells owing to a combination of efficient tumor cell uptake and fast shedding off PEG shells under the intracellular reductive condition. (iv) DOX-loaded micelles cause a markedly enhanced in vitro antitumor effect that is comparable to free DOX. To the best of our knowledge, this represents the highest in vitro antitumor activity reported for micellar doxorubicin formulations based on biodegradable PEG-polyester block copolymers. These tumor-targeting, reduction-sensitive, shell-sheddable, biodegradable micelles have appeared as a viable and potent platform for safe and efficient cancer chemotherapy.

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Notes
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