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Reduction-Responsive Disassemblable Core-Cross-Linked Micelles Based on Poly(ethylene glycol)-*b*-poly(*N*-2-hydroxypropyl methacrylamide)−Lipoic Acid Conjugates for Triggered Intracellular Anticancer Drug Release

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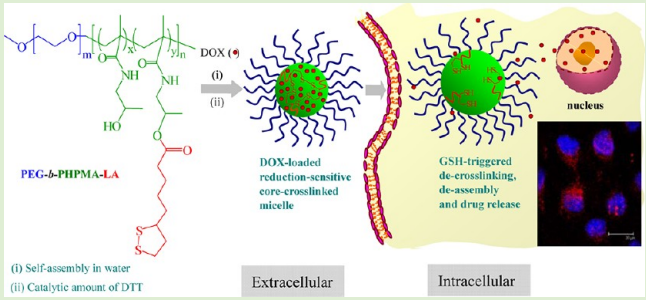
Supporting Information

ABSTRACT: Reduction-sensitive reversibly core-cross-linked micelles were developed based on poly(ethylene glycol)-*b*-poly(*N*-2-hydroxypropyl methacrylamide)−lipoic acid (PEG-*b*-PHPMA-LA) conjugates and investigated for triggered doxorubicin (DOX) release. Water-soluble PEG-*b*-PHPMA block copolymers were obtained with $M_{n,PEG}$ of 5.0 kg/mol and $M_{n,HPMA}$ varying from 1.7 and 4.1 to 7.0 kg/mol by reversible addition−fragmentation chain transfer (RAFT) polymerization. The esterification of the hydroxyl groups in the PEG-*b*-PHPMA copolymers with lipoic acid (LA) gave amphiphilic PEG-*b*-PHPMA-LA conjugates with degrees of substitution (DS) of 71−86%, which formed monodispersed micelles with average sizes ranging from 85.3 to 142.5 nm, depending on PHPMA molecular weights, in phosphate buffer (PB, 10 mM, pH 7.4). These micelles were readily cross-linked with a catalytic amount of dithiothreitol (DTT). Notably, PEG-*b*-PHPMA(7.0k)-LA micelles displayed superior DOX loading content (21.3 wt %) and loading efficiency (90%). The in vitro release studies showed that only about 23.0% of DOX was released in 12 h from cross-linked micelles at 37 °C at a low micelle concentration of 40 μg/mL, whereas about 87.0% of DOX was released in the presence of 10 mM DTT under otherwise the same conditions. MTT assays showed that DOX-loaded core-cross-linked PEG-*b*-PHPMA-LA micelles exhibited high antitumor activity in HeLa and HepG2 cells with low IC_{50} (half inhibitory concentration) of 6.7 and 12.8 μg DOX equiv/mL, respectively, following 48 h incubation, while blank micelles were practically nontoxic up to a tested concentration of 1.0 mg/mL. Confocal laser scanning microscope (CLSM) studies showed that DOX-loaded core-cross-linked micelles released DOX into the cell nuclei of HeLa cells in 12 h. These reduction-sensitive disassemblable core-cross-linked micelles with excellent biocompatibility, superior drug loading, high extracellular stability, and triggered intracellular drug release are promising for tumor-targeted anticancer drug delivery.



INTRODUCTION

Amphiphilic block copolymer micelles with a distinct hydrophobic core to accommodate sparingly water-soluble anticancer drugs like paclitaxel (PTX) and doxorubicin (DOX), and an intrinsically stealthy shell have emerged as one of the most promising platforms for tumor-targeting drug delivery.[1−4] However, one practical challenge for micellar drugs is their low in vivo stability since large volume dilution and interactions with cells and biomolecules present in the blood following i.v. injection often leads to micelle dissociation or aggregation, premature drug release, and low drug targetability.[5,6] The cross-linking of micellar core or shell could greatly improve micelle stability leading to prolonged circulation time and enhanced accumulation at the tumor sites.[7−11] The permanent cross-linking of micelles would, however, also significantly inhibit drug release at the site of action (e.g., inside the tumor cells), giving compromised therapeutic effect.[12]

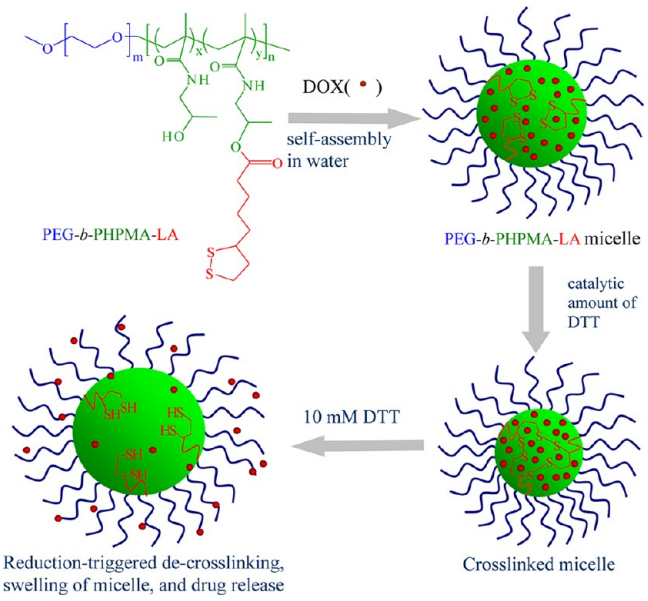
In the past years, several stimuli-sensitive de-cross-linkable micelles (also referred to as "reversibly crosslinked micelles") that are destabilized in response to a specific stimulus have been developed to meet the conflicting requirements of extracellular stability and intracellular drug release of micellar drugs. For example, Jeong et al. reported that acid-sensitive ketal-cross-linked PEG-poly(amino acid) copolymer micelles released DOX more rapidly at endosomal pH than at physiological pH.[13] Wang and Lam groups independently reported that boronate-cross-linked micelles while exhibiting good stability under physiological conditions quickly released payloads in response to low pH or competitive diol molecules.[14,15] Jing et al. prepared reduction-sensitive reversibly shell-cross-linked

Received: May 1, 2012
Revised: June 21, 2012
Published: June 29, 2012

micelles from poly(L-cysteine)-*b*-poly(L-lactide) copolymer.[16] Wang, Jerome, and our groups reported that disulfide-cross-linked micelles while displaying slow DOX release under a physiological environment rapidly released DOX under a reductive condition mimicking that of the cytoplasm and cell nucleus.[17−19] Lam and Lee groups demonstrated that PTX or docetaxel-loaded disulfide-cross-linked micelles resulted in enhanced tumor specificity and therapeutic efficacy in tumor-bearing mice as compared to the noncross-linked counterparts.[20,21] The reduction-sensitive degradable micelles have attracted great interest for "active" intracellular drug release due to the existence of a high reducing potential in the cytoplasm and cell nucleus that is about 100−1000 times higher than that in the circulation.[22−24] The work of our and several other groups has shown that reduction-sensitive deconstructible micelles are able to efficiently release anticancer drugs inside the tumor cells, resulting in markedly enhanced drug efficacy as compared to the reduction-insensitive counterparts.[25−33] These reduction-sensitive degradable micelles have demonstrated either improved extracellular stability or enhanced intracellular drug release. Nevertheless, none of them has been designed to simultaneously address the extracellular stability and the intracellular drug release dilemma.

In this paper, we report on development of robust, reduction-sensitive disassemblable and core-cross-linked micelles based on poly(ethylene glycol)-*b*-poly(*N*-2-hydroxypropyl methacrylamide)-lipoic acid (PEG-*b*-PHPMA-LA) conjugates for triggered doxorubicin release in cancer cells (Scheme 1). This novel type of "smart" micelles was designed on the basis of following considerations: (i) PEG[34,35] and PHPMA[36,37] are among the few polymers approved by the US Food and Drug Administration (FDA) for use in drug delivery; (ii) LA, a naturally occurring compound generated by human body, is currently applied for the treatment of varying diseases including Alzheimer's disease and diabetes;[38,39] (iii) The lipoylation of the hydroxyl groups in the PEG-*b*-PHPMA copolymers likely yields amphiphilic PEG-*b*-PHPMA-LA conjugates that can form core−shell structured micelles; (iv) PEG-*b*-PHPMA-LA micelles can be reversibly stabilized via disulfide cross-linking, similar to dextran-LA nanoparticles.[40] Here, synthesis and reduction-responsive disassembling of cross-linked PEG-*b*-PHPMA-LA micelles, loading and in vitro release of DOX, as well as antitumor activity of DOX-loaded core-cross-linked micelles were investigated.

Scheme 1. Illustration of Robust Reversibly Core-Cross-Linked PEG-*b*-PHPMA-LA Micelles for Efficient Loading and Reduction-Triggered Release of DOX[a]



[a]PEG-*b*-PHPMA-LA conjugates form core−shell structured micelles that can readily be cross-linked in the presence of catalytic amount of DTT. These disulfide cross-linked micelles while exhibiting excellent stability under an extracellular environment would be rapidly destabilized under a reductive condition mimicking that of the cytoplasm and cell nucleus.

EXPERIMENTAL SECTION

Materials. Poly(ethylene glycol) monomethyl ether (PEG, M_n = 5000 g/mol, Fluka) was dried by azeotropic distillation from anhydrous toluene before use. 4-Cyanopentanoic acid dithionaphthalenoate (CPADN) was synthesized according to the described procedure for 4-cyanopentanoic acid dithiobenzoate.[41] Azobisisobutyronitrile (AIBN, 98%, J&K) was recrystallized twice from methanol. Dichloromethane (DCM) and tetrahydrofuran (THF) were dried by refluxing over CaH_2 and sodium wire, respectively. Dimethyl sulfoxide (DMSO) was dried over CaH_2 and distilled prior to use. Doxorubicin hydrochloride (DOX·HCl, 99%, Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd.), *N*-2-hydroxypropyl methacrylamide (HPMA, 97.5%, Suzhou NuoHang trade Co., Ltd.), lipoic acid (98%, Acros), 4-dimethylamino pyridine (DMAP, 99%, Alfa Aesar), dicyclohexyl carbodiimide (DCC, 99%, Alfa Aesar), 1,4-dithio-D,L-threitol (DTT, 99%, Merck), and trifluoroacetic anhydride (TFAA, 99%, Alfa Aesar) were used as received.

Characterization. ^{1}H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using deuterated dimethyl sulfoxide (DMSO-d_6). The chemical shifts were calibrated against solvent signal of DMSO-d_6. For molecular weight and polydispersity determination, PEG-*b*-PHPMA copolymers were esterified with trifluoroacetic acid anhydride. The measurements were performed on a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with two linear PLgel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector. The measurements were carried out using $CHCl_3$ as the eluent at a flow rate of 0.5 mL/min at 35 °C and a series of narrow polystyrene standards for the calibration of the columns. The size of micelles was determined by a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He−Ne laser using back-scattering detection at 25 °C. 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 phosphotungstic acid (1 wt.%).

Synthesis of PEG-*b*-PHPMA Diblock Copolymers. PEG-*b*-PHPMA diblock copolymers were prepared by RAFT polymerization of HPMA using PEG-CPADN, which was obtained according to our previous report,[42] as a macro-RAFT agent. In a typical example, to a Schlenk bottle equipped with a magnetic stir bar were charged HPMA (150 mg, 1.0 mmol), PEG-CPADN (100 mg, 19 μmol), AIBN (0.46 mg, 2.8 μmol), and THF (5.0 mL). After a 30 min degassing with nitrogen flow, the reaction vessel was sealed and immersed into an oil bath thermostatted at 70 °C. The polymerization was allowed to proceed for 24 h. The resulting copolymer was isolated by precipitation in cold diethyl ether, filtration and drying in vacuo. Yield: 92%. ^{1}H NMR (400 MHz, DMSO-d_6) PEG block: δ 3.23 and 3.51; PHPMA block: δ 0.8−1.02, 2.89, 3.68, and 4.71. The M_n of PHPMA block was determined to be 7.0 kg/mol by comparing the intensities of signals at δ 3.68 and 3.51. The resulting copolymer is denoted as PEG-*b*-PHPMA(7.0k). For GPC measurements, PEG-*b*-PHPMA copolymers were esterified with trifluoroacetic acid anhydride

(TFAA). M_n (GPC) = 11.3 kg/mol; M_w/M_n = 1.19. In a similar way, we have obtained PEG-*b*-PHPMA(1.7k) and PEG-*b*-PHPMA(4.1k).

Synthesis of PEG-*b*-PHPMA-LA Conjugates. In a typical example, under a nitrogen atmosphere, to a stirred solution of lipoic acid (0.169 g, 0.82 mmol) in CH_2Cl_2 (2.0 mL) was added dropwise a solution of DCC (0.101 g, 0.49 mmol) in CH_2Cl_2 (1.0 mL). The reaction was allowed to proceed under magnetic stirring for 12 h at room temperature in the dark. The precipitate generated during the reaction was removed by filtration. The filtrate was concentrated to about 0.5 mL and added to a stirred solution of PEG-*b*-PHPMA(7.0 k) (100 mg, 8.0 μmol) in DMSO (2.0 mL), followed by addition of a solution of DMAP (50 mg, 0.41 mmol) in DMSO (2.0 mL). The flask was sealed and placed into an oil bath thermostatted at 30 °C. The reaction mixture was stirred for 48 h in the dark. The resulting PEG-*b*-PHPMA(7.0k)-LA conjugate was isolated by precipitation in cold diethyl ether, filtration, and drying in vacuo. Yield: 62%. ^{1}H NMR (400 MHz, DMSO-d_6) PEG: δ 3.23 and 3.51; PHPMA: δ 0.8−1.13, 3.14, 3.67, 4.71, and 4.83; lipoic acid moieties: δ 1.40, 1.58−1.69, 2.29, 1.89/2.43, 3.02, and 3.74. The degree of substitution (DS, defined as number of LA units per 100 hydroxyl groups of PHPMA) was determined to be 71 by comparing the integrals of signals at δ 1.40 (γ-methylene protons of LA moieties) and 3.51 (PEG methylene protons). In a similar way, PEG-*b*-PHPMA(1.7k)-LA and PEG-*b*-PHPMA(4.1k)-LA conjugates were obtained with DS of 75 and 86, respectively.

Micelle Formation and Critical Micelle Concentration (CMC). PEG-*b*-PHPMA-LA micelles were prepared by solvent exchange method. Briefly, 2.0 mL of PB buffer (10 mM, pH 7.4) was added under stirring to 1.0 mL of PEG-*b*-PHPMA-LA solution in DMSO (0.2 wt %) at 25 °C. The resulting PEG-*b*-PHPMA-LA micelle dispersion was extensively dialyzed against PB buffer (10 mM, pH 7.4) for 24 h (Spectra/Pore, MWCO 3500). The average sizes of micelles were determined at 25 °C by Zetasizer Nano ZS (Malvern Instruments).

The CMC of PEG-PHPMA-LA conjugates was determined using pyrene as a fluorescence probe. The concentration of copolymer was varied from 8.0×10^{-5} to 8.0×10^{-2} mg/mL and the concentration of pyrene was fixed at 0.6 μM. The fluorescence spectra were recorded using 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 I_{372}/I_{383} at low and high concentration regions.

Cross-Linking and Reduction-Triggered Destabilization of Micelles. The cross-linking of PEG-*b*-PHPMA-LA micelles was carried out under nitrogen atmosphere at room temperature in the presence of 10 mol % DTT relative to the lipoyl units, as reported previously.[19,40] Briefly, to 2.0 mL of PEG-*b*-PHPMA(7.0k)-LA micelle dispersion (0.4 mg/mL) was added DTT (22 μg, 10 mol % relative to the lipoyl units) solution in PB (0.1 mL). The mixture was stirred at room temperature for 1 day and dialyzed against PB (10 mM, pH 7.4) for another day (Spectra/Pore, MWCO 3500). The colloidal stability of cross-linked micelles against large volume dilution as well as against physiological and high salt concentrations were investigated using DLS.

The size change of cross-linked micelles in response to 10 mM DTT in PB (10 mM, pH 7.4) was followed by DLS measurement. Briefly, to 3.0 mL solution of PEG-*b*-PHPMA-LA micelles (40 μg/mL) in PB (10 mM, pH 7.4) previously degassed with nitrogen for 20 min was added 10 mM DTT. The solution was placed in a shaking bed at 200 rpm and 37 °C. At different time intervals, the average sizes of micelles were determined using DLS.

Loading of DOX. DOX was loaded into micelles by dropwise addition of 2.0 mL of PB (10 mM, pH 7.4) to a mixture of PEG-*b*-PHPMA-LA copolymer solution in DMSO (1.0 mL, 2.0 mg/mL) and DOX solution in DMSO (120 μL, 5.0 mg/mL) under stirring at room temperature, followed by dialysis against PB (10 mM, pH 7.4) for 24 h (Spectra/Pore, MWCO 3500). The dialysis medium was changed five times. The whole procedure was performed in the dark. The micelles were cross-linked as described above using catalytic amount of DTT.

The amount of DOX was determined using fluorescence (FLS920) measurement (excitation at 480 nm and emission at 555 nm). For determination of drug loading content (DLC), lyophilized 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) of the non-cross-linked micelles were calculated according to the following formula:

$$\text{DLC(wt \%)} = (\text{weight of loaded drug/total weight of loaded drug and polymer}) \times 100\%$$

$$\text{DLE(\%)} = (\text{weight of loaded drug/weight of drug in feed}) \times 100\%$$

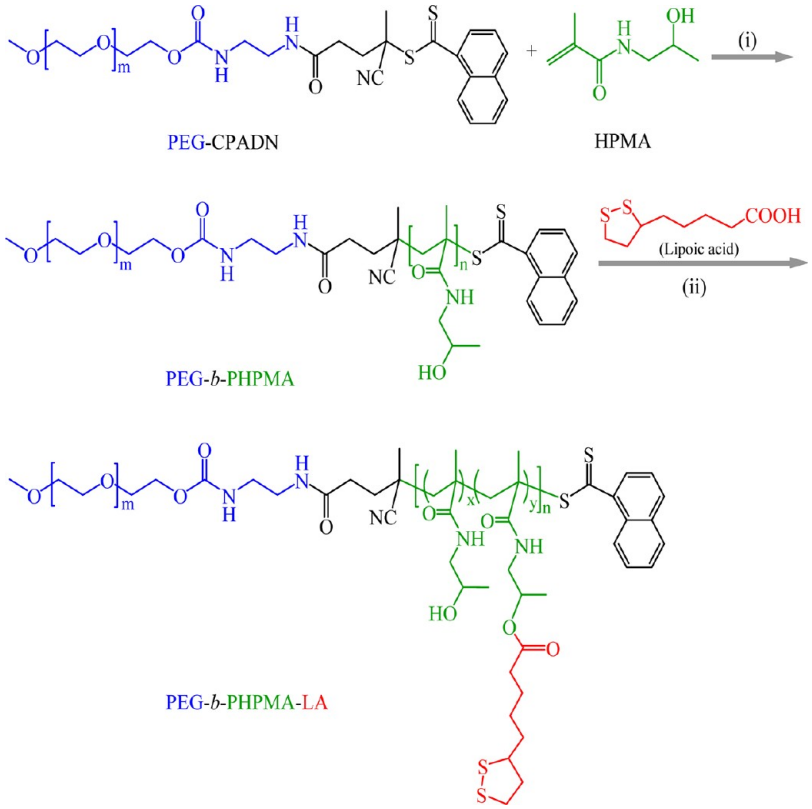
Reduction-Triggered Release of DOX. The release profiles of DOX from PEG-*b*-PHPMA(4.1k)-LA and PEG-*b*-PHPMA(7.0k)-LA micelles, either cross-linked or non-cross-linked, were studied using a dialysis tube (MWCO 12000) at 37 °C in two different media, that is, PB (10 mM, pH 7.4) with 10 mM DTT or PB (10 mM, pH 7.4) only. To acquire sink conditions, drug release studies were performed at a low micelle concentration of 40 μg/mL and with 1.0 mL of micelle solution dialysis against 20 mL of the same medium. At desired time intervals, 5.0 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 measurement (FLS920, excitation at 480 nm). The release experiments were conducted in triplicate and the results presented were the average data with standard deviations.

MTT Assays. The cytotoxicity of cross-linked PEG-*b*-PHPMA-LA micelles was evaluated in HeLa and HepG2 cells by MTT assays. The cells were plated in a 96-well plate (5×10^3 cells/well) using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL) for 1 day. The medium was aspirated and replaced by 80 μL of fresh medium supplemented with 10% FBS. A total of 20 μL of cross-linked PEG-*b*-PHPMA-LA micelles in PB (10 mM, pH 7.4) was added to yield final micelle concentrations of 0.5 or 1.0 mg/mL. The cells were cultured at 37 °C in an atmosphere containing 5% CO_2 for 1 day. The medium was aspirated and replaced by 100 μL of fresh medium. A total of 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) solution (5 mg/mL) was added. The cells were incubated for another 4 h. The medium was aspirated, the MTT-formazan generated by live cells was dissolved in 200 μL of DMSO, and the absorbance at a wavelength of 490 nm of each well was measured using a microplate reader. The relative cell viability (%) was determined by comparing the absorbance at 490 nm with control wells containing only cell culture medium. Data are presented as average ± SD (n = 4).

The antitumor activity of DOX-loaded cross-linked PEG-*b*-PHPMA-LA micelles was studied in a similar way. Briefly, HeLa and HepG2 cells were plated in a 96-well plate (5×10^3 cells/well) using RPMI-1640 medium supplemented with 10% FBS, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL) for 1 day. The medium was aspirated and replaced by 80 μL of fresh medium supplemented with 10% FBS. A total of 20 μL of DOX-loaded cross-linked PEG-*b*-PHPMA-LA micelles in PB (10 mM, pH 7.4) was added to yield varying drug dosages from 0.1 to 40 μg/mL. The cells were cultured at 37 °C in an atmosphere containing 5% CO_2 for 2 days. The medium was aspirated and replaced by 100 μL of fresh medium and 10 μL of MTT solution (5 mg/mL). The cells were cultured for another 4 h. The cell viabilities were determined by MTT assays as described above.

Intracellular Release of DOX. The cellular uptake and intracellular release behaviors of DOX-loaded core-cross-linked PEG-*b*-PHPMA(7.0k)-LA micelles were followed with confocal laser scanning microscopy (CLSM) using HeLa cells. The cells were cultured on microscope slides in a six-well plate (5×10^5 cells/well) using RPMI-1640 medium supplemented with 10% FBS, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL).

Scheme 2. Synthesis of PEG-*b*-PHPMA-LA Conjugates by Controlled RAFT Polymerization Followed by Lipoylation[a]



[a]Reagents and conditions: (i) AIBN, THF, 70 °C, 24 h; (ii) DCC/DMAP, DMSO, 30 °C, 48 h.

The cells were incubated with DOX-loaded micelles or free DOX for 4 or 12 h at 37 °C in a humidified 5% CO_2-containing atmosphere. The culture medium was removed and the cells were rinsed three times with PBS. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The fluorescence images were obtained using confocal microscope (TCS SP2).

RESULTS AND DISCUSSION

Synthesis of PEG-*b*-PHPMA-LA Conjugates. PEG-*b*-PHPMA-LA conjugates were obtained in two steps, that is, reversible addition−fragmentation chain transfer (RAFT) polymerization of *N*-2-hydroxypropyl methacrylamide (HPMA) using PEG-CPADN ($M_{n,PEG}$ = 5.0 kg/mol; CPADN, 4-cyanopentanoic acid dithionaphthalenoate) as a macro-RAFT agent followed by lipoylation (Scheme 2). CPADN is a robust and versatile RAFT agent with which we have previously prepared a series of well-defined di- and triblock copolymers such as PEG-SS-PDEA,[42] PEG-PCL-PDEA,[43] PDMAEMA-SS-PEG-SS-PDMAEMA,[44] and PDMAEMA-PCL-PDMAEMA.[45] RAFT polymerization was performed with AIBN as the radical source in THF at 60 °C for 24 h. The results of polymerization are summarized in Table 1. ^{1}H NMR displayed clearly signals attributable to PHPMA (δ 0.8−1.03, 2.89, 3.67, and 4.71) and PEG (δ 3.23 and 3.51), respectively (Figure S1A). The M_n of PHPMA block could be estimated by comparing the intensities of signals at δ 3.67 and 3.51, which were assignable to the methine proton neighboring to the hydroxyl group of PHPMA and methylene protons of PEG, respectively. The results showed that PEG-*b*-PHPMA copolymers were obtained with $M_{n,PHPMA}$ of 1.7, 4.1, and 7.0 kg/mol at designed $M_{n,PHPMA}$ of 2.0, 4.1, and 8.0 kg/mol, respectively (Table 1). Gel permeation chromatography (GPC) of PEG-*b*-PHPMA following trifluoroacetation revealed a unimodal distribution with low polydispersities of 1.17−1.25 and molecular weights in parallel with those determined by ^{1}H NMR (Table 1). These results point out that RAFT polymerization afford PEG-*b*-PHPMA copolymers with defined structures and controlled molecular weights. In line with our expectation, PEG-*b*-PHPMA copolymers have excellent water solubility due to their double hydrophilic nature.

Table 1. Synthesis of PEG-*b*-PHPMA Copolymers

entry	copolymer	$M_{n,PHPMA}$ (kg/mol) design	$M_{n,PHPMA}$ (kg/mol) ^{1}H NMR[a]	GPC[b] M_n (kg/mol)	GPC[b] PDI[b]	yield (%)
1	PEG-*b*-PHPMA(1.7k)	2.0	1.7	8.9	1.17	86
2	PEG-*b*-PHPMA(4.1k)	4.0	4.1	9.5	1.25	88
3	PEG-*b*-PHPMA(7.0k)	8.0	7.0	11.3	1.19	92

[a]Calculated from ^{1}H NMR by comparing the intensities of signals at δ 3.67 and 3.51, which were assignable to the methine proton neighboring to the hydroxyl group of PHPMA and methylene protons of PEG, respectively. [b]PEG-*b*-PHPMA copolymers were esterified with trifluoroacetic acid anhydride (TFAA) and then analyzed by GPC measurements using $CHCl_3$ as an eluent at a flow rate of 0.5 mL/min (standards: polystyrene, 35 °C).

Lipoylated PEG-*b*-PHPMA copolymers (PEG-*b*-PHPMA-LA) were conveniently prepared by treating PEG-*b*-PHPMA copolymers with lipoic acid (LA) anhydride in DMSO at a hydroxyl/LA anhydride mole ratio of 1/1. ^{1}H NMR showed

Table 2. Characteristics of PEG-*b*-PHPMA-LA Conjugates and Cross-Linked Micelles

entry	conjugate	DS[a]	non-cross-linked micelles			cross-linked micelles	
			size (nm)[b]	PDI[b]	CMC (mg/L)[c]	size (nm)[b]	PDI[b]
1	PEG-*b*-PHPMA(1.7k)-LA	75	85.3	0.18	8.0	78.2	0.05
2	PEG-*b*-PHPMA(4.1k)-LA	86	123.6	0.10	2.0	108.7	0.07
3	PEG-*b*-PHPMA(7.0k)-LA	71	142.5	0.12	2.4	136.4	0.08

[a]Degree of substitution (DS, defined as number of substituents per 100 hydroxyl groups of PHPMA) determined by ^{1}H NMR by comparing the integrals of signals at δ 1.40 (γ-methylene protons of LA moieties) and 3.51 (PEG methylene protons). [b]Determined using Zetasizer Nano-ZS (Malvern Instruments) at 25 °C in PB (10 mM, pH 7.4). [c]Determined using pyrene as a fluorescence probe.

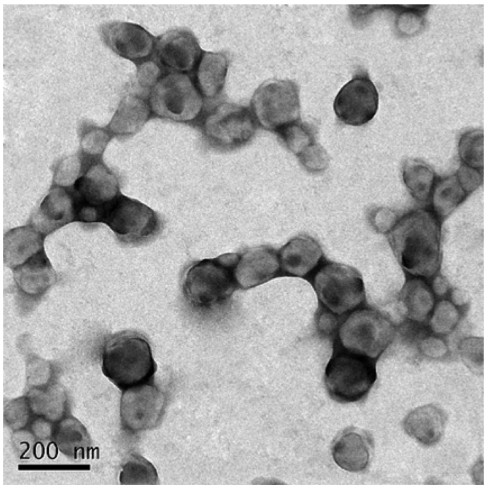


Figure 1. TEM micrograph of core-cross-linked PEG-*b*-PHPMA-(7.0k)-LA micelles.

besides signals attributable to PHPMA (δ 0.8−1.13, 3.14, 3.67, 4.71, and 4.83) and PEG (δ 3.23, 3.51) also resonances due to lipoyl ester moieties at δ 1.40, 1.58−1.69, 1.89/2.43, 2.29, 3.02, and 3.74 (Figure S1B). Furthermore, signal at δ 4.71 owing to the hydroxyl group of PHPMA was weakened following lipoylation and a new signal due to the methine proton next to the ester bond appeared at δ 4.83. The degree of substitution (DS, defined as number of LA units per 100 hydroxyl groups of PHPMA) could be determined by comparing the integrals of signals at δ 1.40 (γ-methylene protons of LA moieties) and 3.51 (PEG methylene protons). The results showed that PEG-*b*-PHPMA copolymers with $M_{n,PHPMA}$ of 1.7, 4.1, and 7.0 kg/mol had DS of 75, 86, and 71, respectively. The corresponding conjugates were in the following denoted as PEG-*b*-PHPMA-(1.7k)-LA, PEG-*b*-PHPMA(4.1k)-LA, and PEG-*b*-PHPMA-(7.0k)-LA, respectively. It should be noted that these PEG-*b*-PHPMA-LA conjugates could not readily be dissolved in water because of the hydrophobic character of lipoylated PHPMA block.

Preparation and Stability Studies of Core-Cross-Linked PEG-*b*-PHPMA-LA Micelles. PEG-*b*-PHPMA-LA micelles were readily prepared by solvent exchange method. Dynamic light scattering (DLS) measurements showed that PEG-*b*-PHPMA-LA conjugates formed monodisperse micelles with low polydispersities (PDI) of 0.10−0.18 and hydrodynamic sizes of 85.3−142.5 nm which increased with increasing the molecular weights of PHPMA-LA block (Table 2). The critical micelle concentration (CMC) determination using pyrene as a fluorescence probe showed that PEG-*b*-PHPMA(1.7k)-LA, PEG-*b*-PHPMA(4.1k)-LA and PEG-*b*-PHPMA(7.0k)-LA had low CMC of approximately 8.0, 2.0, and 2.4 mg/L, respectively (Table 2).

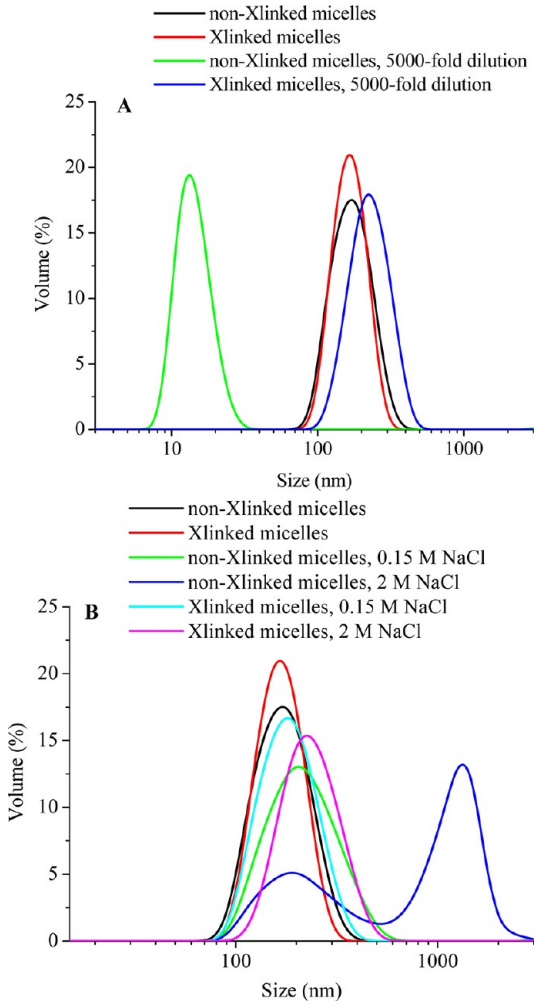


Figure 2. Stability of core-cross-linked PEG-*b*-PHPMA(7.0k)-LA micelles against 5000× dilution (A) and 150 mM or 2 M NaCl (B). The corresponding non-cross-linked micelles were used as a control.

The cross-linking of PEG-*b*-PHPMA-LA micelles were carried out in PB (pH 7.4, 10 mM) by introducing 10 mol % dithiothreitol (DTT) relative to the lipoyl units in the micelles, similar to our previous reports.[19,40] Notably, DLS revealed that micelle sizes decreased by 6−15 nm (Table 2), indicating occurrence of micelle cross-linking. These core-cross-linked micelles displayed low PDI of 0.05−0.08. The cross-linking of micelles most likely takes place via the thiol-disulfide exchange reaction wherein lipoyl rings are opened at the S−S bond, under the catalysis of DTT, to form preferentially linear disulfide polymers. TEM micrograph demonstrated that cross-linked PEG-*b*-PHPMA-LA micelles had mostly spherical

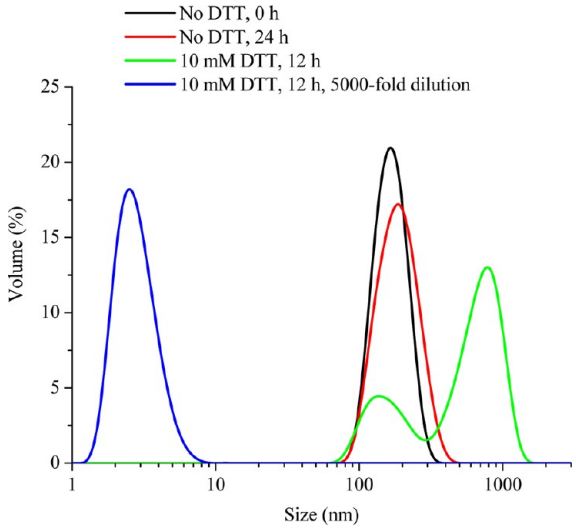


Figure 3. Change of size distribution profiles of cross-linked PEG-*b*-PHPMA(7.0k)-LA micelles in response to 10 mM DTT in PB (pH 7.4, 10 mM) at 37 °C.

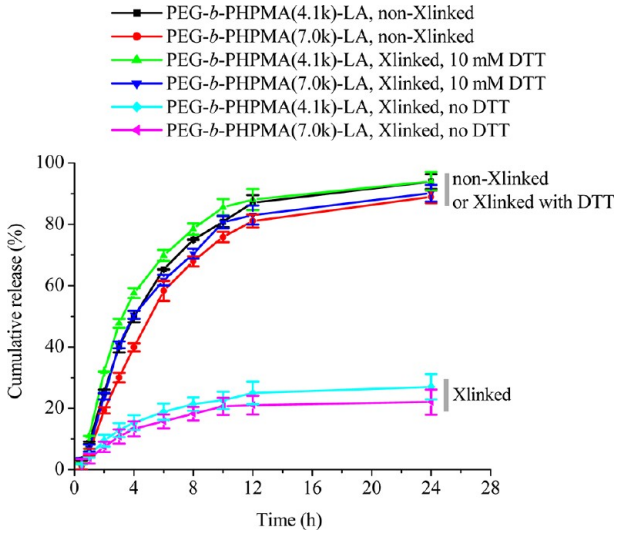


Figure 4. Reduction-triggered release of DOX from core-cross-linked PEG-*b*-PHPMA(4.1k)-LA and PEG-*b*-PHPMA(7.0k)-LA micelles at pH 7.4 and 37 °C in PB buffer. Non-cross-linked micelles were used as a control. The drug release studies were performed at a low micelle concentration of 40 μg/mL. Data are presented as mean ± SD (n = 3).

morphology and a size distribution close to that determined by DLS (Figure 1).

The stability of cross-linked PEG-*b*-PHPMA-LA micelles against extensive dilution and concentrated salt conditions was studied by DLS. Notably, cross-linked micelles following 5000-fold dilution (C < CMC) showed only slight increase in micelle sizes and maintained a low PDI (Figure 2A). In contrast, the parent non-cross-linked micelles were dissociated under otherwise the same conditions to form small aggregates (ca. 15 nm). Furthermore, cross-linked micelles exhibited also better colloidal stability under physiological and high salt conditions than their noncross-linked counterparts (Figure 2B). The non-cross-linked PEG-*b*-PHPMA-LA micelles showed increase of sizes under physiological salt and formed large aggregates under 2 M salt condition, while cross-linked micelles maintained good size distributions with little change of micelle sizes under physiological salt and slight increase of micelle sizes under 2 M NaCl condition.

The reduction-sensitivity of cross-linked PEG-*b*-PHPMA-LA micelles was investigated by monitoring change of micelle sizes in response to 10 mM DTT in PB buffer (pH 7.4, 20 mM) at 37 °C. The results showed that DTT caused significant size increase of cross-linked micelles (grow to over 800 nm) in 12 h, which upon dilution to a concentration lower than CMC of PEG-*b*-PHPMA-LA was dissociated into unimers (Figure 3), indicating occurrence of complete micelle de-cross-linking. The large size increase of cross-linked micelles following de-cross-linking is most likely due to the increased hydrophilicity of micellar core as a result of conversion of disulfide bonds into two hydrophilic thiol groups (Scheme 1). In contrast, little size change was observed for cross-linked micelles in 24 h in the absence of DTT under otherwise the same conditions. These results suggest that core-cross-linked PEG-*b*-PHPMA-LA micelles have superior colloidal stability under extracellular conditions while they are rapidly disassemblable under a reductive condition, mimicking that of the cytoplasm and cell nucleus.

Loading and In Vitro Release of DOX. Loading of DOX into micelles was performed at a theoretical drug loading content (DLC) of 23.1 wt.% and a polymer concentration of 2 mg/mL. The micelle sizes increased to varying extents following loading of DOX, in which DOX-loaded PEG-*b*-PHPMA(1.7k)-LA micelles displayed the largest average size of 213.2 nm while PEG-*b*-PHPMA(4.1k)-LA and PEG-*b*-PHPMA(7.0k)-LA showed average sizes of 136.7 and 165.6 nm, respectively (Table 3). Similar to blank PEG-*b*-PHPMA-LA micelles, DOX-loaded PEG-*b*-PHPMA-LA micelles following cross-linking with catalytic amount of DTT demonstrated shrinking of micelles by ca. 9−15 nm. The resulting DOX-loaded core-cross-linked micelles showed low PDI of 0.06−0.10. Interestingly, drug loading efficiency (DLE) of cross-linked micelles increased with increasing molecular weights of PHPMA-LA block, in which cross-linked PEG-*b*-PHPMA-(7.0k)-LA micelles gave a high DOX loading content of 21.3 wt.% and a high DOX loading efficiency of 90% (Table 3).

The in vitro drug release studies were carried out on PEG-*b*-PHPMA(4.1k)-LA and PEG-*b*-PHPMA(7.0k)-LA micelles at pH 7.4 and 37 °C at a low polymer concentration of 40 μg/mL. The results showed that release of DOX from cross-linked micelles was largely inhibited, in which only about 27.0 and

Table 3. Characteristics of DOX-Loaded Core-Cross-Linked PEG-*b*-PHPMA-LA Micelles at a Theoretical Drug Loading Content of 23.1 wt %

		non-cross-linked micelles		cross-linked micelles			
entry	conjugate	size (nm)	PDI	size (nm)	PDI	DLC (wt %)	DLE (%)
1	PEG-*b*-PHPMA(1.7k)-LA	213.2	0.17	198.2	0.10	14.2	55
2	PEG-*b*-PHPMA(4.1k)-LA	136.7	0.08	127.3	0.07	17.1	69
3	PEG-*b*-PHPMA(7.0k)-LA	165.6	0.11	153.5	0.06	21.3	90

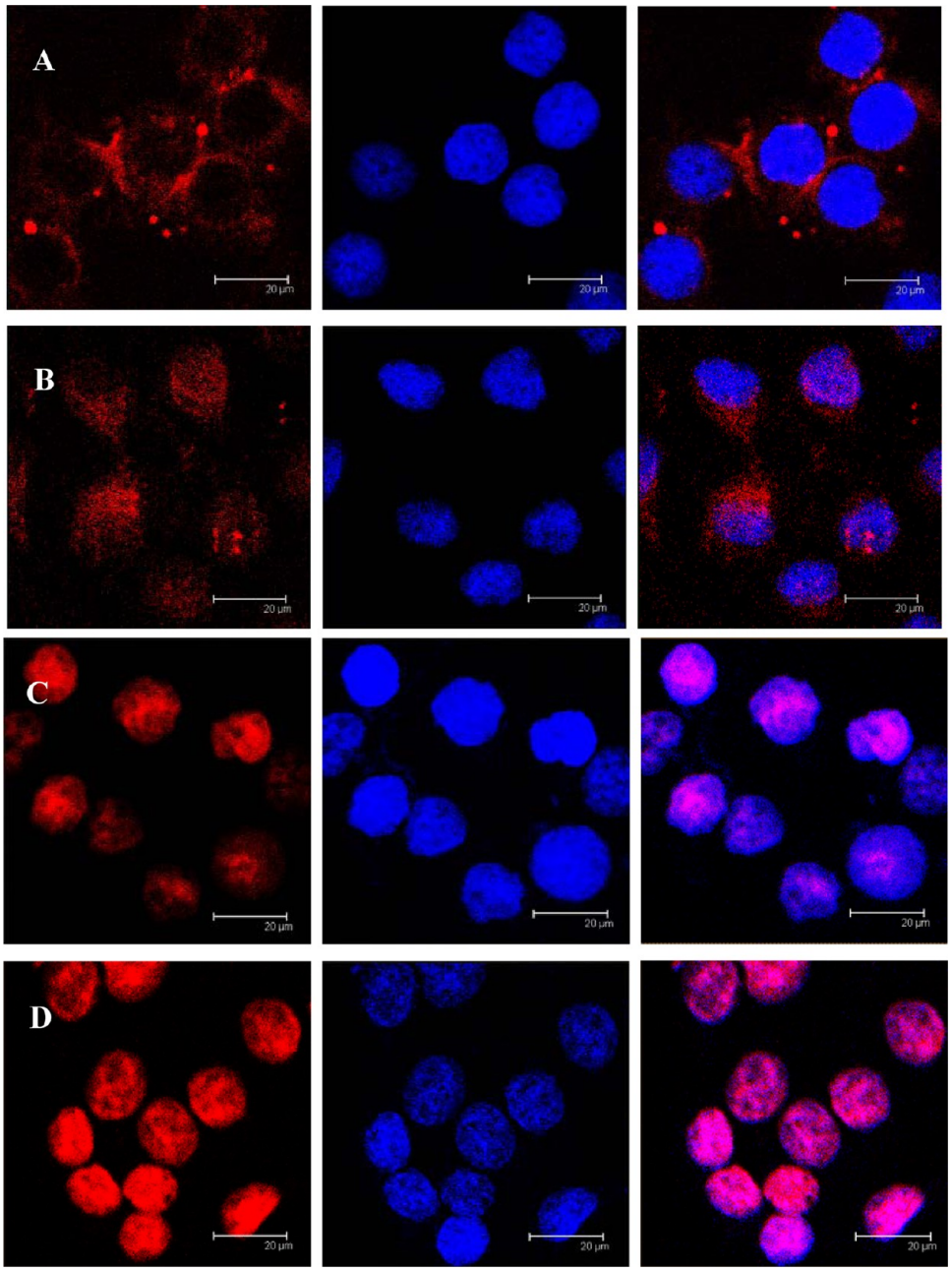


Figure 5. CLSM images of HeLa cells incubated with DOX-loaded core-cross-linked PEG-*b*-PHPMA(7.0k)-LA micelles and free DOX (10 μg/mL). For each panel, the images from left to right show DOX fluorescence in cells (red), cell nuclei stained by DAPI (blue), and overlays of the two images. The scale bars correspond to 20 μm in all the images. (A) DOX-loaded cross-linked micelles, 4 h incubation; (B) DOX-loaded cross-linked micelles, 12 h incubation; (C) free DOX, 4 h incubation; (D) free DOX, 12 h incubation.

22.0% drug was released in 24 h from cross-linked PEG-*b*-PHPMA(4.1k)-LA and PEG-*b*-PHPMA(7.0k)-LA micelles, respectively (Figure 4). However, drug release was enhanced significantly in the presence of 10 mM DTT under otherwise the same conditions, wherein about 94.0 and 90.1% drug was released from cross-linked PEG-*b*-PHPMA(4.1k)-LA and PEG-*b*-PHPMA(7.0k)-LA micelles, respectively. It is interesting to note that under a reductive condition, DOX-loaded cross-linked PEG-*b*-PHPMA-LA micelles gave virtually the same release profiles as for DOX-loaded noncross-linked counterparts (Figure 4), indicating that DTT-triggered micelle decross-linking is rapid and complete. It should further be noted that presence of 10% fetal bovine serum (FBS) had little influence on DOX release from cross-linked micelles.

Intracellular Drug Release and Antitumor Activity of DOX-Loaded Core-Cross-Linked PEG-*b*-PHPMA-LA Micelles. The cellular uptake and intracellular drug release behaviors of DOX-loaded cross-linked PEG-*b*-PHPMA-LA micelles were studied in HeLa cells using confocal laser scanning microscopy (CLSM). Interestingly, DOX fluorescence was clearly detected in HeLa cells following 4 h incubation with DOX-loaded cross-linked PEG-*b*-PHPMA(7.0k)-LA micelles (Figure 5A), indicating that DOX has been released inside cells. After a prolonged incubation time of 12 h, DOX has mostly released into the perinuclei and nuclei region of cells (Figure 5B). It should be noted that stronger DOX fluorescence was observed in HeLa cells following incubation with free DOX for 4 and 12 h (Figure 5C,D). These results confirm that cross-

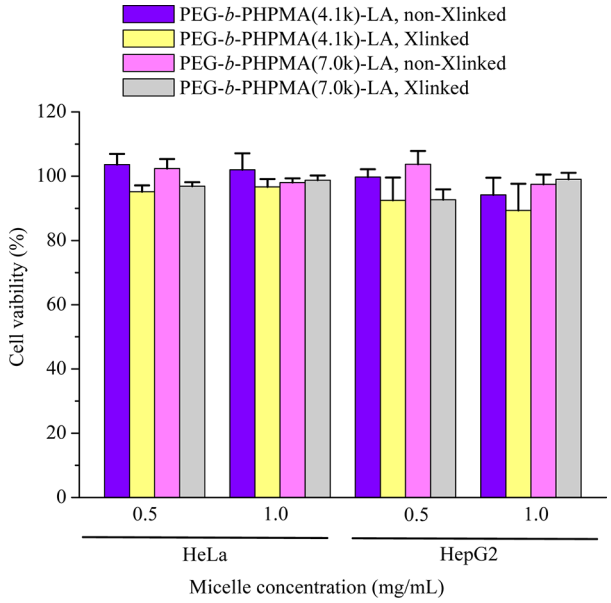


Figure 6. Cytotoxicity of PEG-*b*-PHPMA(4.1k)-LA and PEG-*b*-PHPMA(7.0k)-LA micelles determined by MTT assays in HeLa and HepG2 cells. The cells were incubated with micelles for 24 h. Data are presented as the average ± standard deviation (n = 4).

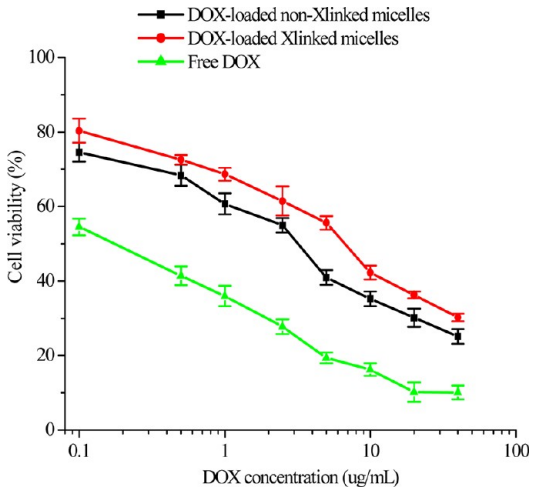


Figure 7. Antitumor activity of DOX-loaded core-cross-linked PEG-*b*-PHPMA(7.0k)-LA micelles in HeLa cells. DOX-loaded non-cross-linked PEG-*b*-PHPMA(7.0k)-LA micelles and free DOX were used as controls. The cells were treated with DOX-loaded micelles or free drug for 48 h. Data are presented as the average ± standard deviation (n = 4).

linked PEG-*b*-PHPMA-LA micelles are able to deliver and release DOX into the nuclei of cancer cells. The lower DOX fluorescence observed for cells treated with DOX-loaded cross-linked micelles than those with free DOX is most likely due to poor cellular uptake of micelles that are stealthed by a dense layer of PEG shells. The cellular uptake of micelles can be enhanced by installing a specific ligand such as folic acid and antibody fragment at the micelle surfaces.[46]

The cytotoxicity of PEG-*b*-PHPMA-LA micelles was investigated in HeLa and HepG2 cells by MTT assays. The results showed that both non-cross-linked and cross-linked PEG-*b*-PHPMA-LA micelles were practically nontoxic to HeLa and HepG2 cells (cell viabilities: 89.4−103.7%) at concentrations of 0.5 and 1.0 mg/mL (Figure 6), indicating that PEG-*b*-PHPMA-LA micelles possess excellent biocompatibility. As a matter of fact, PEG and PHPMA are among the few synthetic water-soluble polymers approved for use in drug carriers by the U.S. Food and Drug Administration (FDA) while LA is a natural antioxidant agent produced by human body.[34−39] PEG-*b*-PHPMA-LA copolymer are, therefore, solely made of biocompatible components.

The antitumor activity of DOX-loaded cross-linked PEG-*b*-PHPMA-LA micelles was studied in HeLa and HepG2 cells. In the following, PEG-*b*-PHPMA(7.0)-LA micelles were selected because of their high drug loading levels. The results showed that DOX-loaded cross-linked PEG-*b*-PHPMA(7.0)-LA micelles induced pronounced antitumor effect with a cytotoxicity profile close to that for DOX-loaded non-cross-linked counterparts (Figure 7). For example, at a drug dosage of 20 μg/mL, cell viabilities of 36.3 and 30.1% were observed for HeLa cells following 48 h incubation with DOX-loaded cross-linked and noncross-linked PEG-*b*-PHPMA-LA micelles, respectively. The IC_{50} (i.e., inhibitory concentration to produce 50% cell death) of DOX-loaded cross-linked PEG-*b*-PHPMA(7.0)-LA micelles was determined to be 6.7 and 12.8 μg/mL for HeLa and HepG2 cells, respectively, which were not much higher than those observed for the non-cross-linked counterparts (3.2 and 5.0 μg/mL for HeLa and HepG2 cells, respectively). It should further be noted that the antitumor activity of DOX-loaded cross-linked PEG-*b*-PHPMA-LA micelles is comparable to or higher than that reported for DOX-loaded nontargeted block copolymer micelles[47] as well as endosomal pH-activatable PEG-*g*-DOX prodrugs.[48] These results support that core-cross-linking has no significant adverse effect on the intracellular drug release of PEG-*b*-PHPMA(7.0)-LA micelles, likely due to fast de-cross-linking of micelles triggered by the intracellular level of glutathione.[23] However, DOX-loaded cross-linked PEG-*b*-PHPMA(7.0)-LA micelles revealed obviously lower antitumor activity as compared to free DOX (IC_{50} of 0.17 and 0.41 μg/mL for HeLa and HepG2 cells, respectively; Figure 7), which agrees well with the intracellular DOX release observations (Figure 5) and is most probably because of poor cellular uptake of micelles. It should be noted, nevertheless, that for in vivo applications, tumor cells are not likely be treated with free DOX at such a high concentration for such a long time. In addition, the antitumor activity of DOX-loaded core-cross-linked PEG-*b*-PHPMA-LA micelles may be boosted by decorating micelle surface with a targeting ligand such as folic acid and antibody fragment that facilitates specific cellular uptake of micelles.[12,49] These reduction-responsive disassemblable core-cross-linked micelles have uniquely integrated several indispensable features such as excellent biocompatibility, superior stability, high drug loading, reduction-triggered de-cross-linking, and rapid intracellular drug release, which provides an advanced platform for tumor-targeting drug delivery.

■ CONCLUSIONS

We have demonstrated that reduction-responsive disassemblable core-cross-linked micelles based on PEG-*b*-PHPMA-LA conjugates while possessing superior stability under extracellular environments undergo rapid de-cross-linking and disassembly under a reductive condition mimicking that of the intracellular compartments, resulting in efficient intracellular drug release and high antitumor activity. These "smart" micelles have integrated several features: (i) they consist solely

of biocompatible components (i.e., PEG, PHPMA and lipoic acid), thereby offering excellent biocompatibility; (ii) they show superior stability with inhibited (premature) drug release under extracellular environments; (iii) they exhibit high drug loading levels (up to 21 wt % loading content and 90% loading efficiency for doxorubicin); and (iv) they rapidly release loaded drugs in response to the intracellular level of reductive condition as well as inside tumor cells, retaining great antitumor effects. It should further be noted that core-cross-linked PEG-*b*-PHPMA-LA micelles can readily be obtained. These reduction-responsive disassemblable core-cross-linked micelles, elegantly addressing the extracellular stability and intracellular drug release dilemma of the current nanocarrier systems, are highly promising for targeted anticancer drug delivery.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H NMR spectra of PEG-*b*-PHPMA(7.0k) diblock copolymer and PEG-b-PHPMA(7.0k)-LA conjugate, release of DOX from core-cross-linked PEG-*b*-PHPMA(7.0k)-LA micelles at a micelle concentration of 0.4 mg/mL at pH 7.4 and 37 °C in PB buffer, and DOX fluorescence calibration curve. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (50973078, 20974073, 51003070, 51103093, and 51173126) and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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