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ORIGINAL PAPER

Redox-responsive micelles self-assembled from multi-block copolymer for co-delivery of siRNA and hydrophobic anticancer drug

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Abstract

Novel redox-responsive amphiphilic cationic multi-block copolymers $\mathbf{PEG_{2000}}$–$\mathbf{PLA_{3000}}$–$\mathbf{PEI_{1200}}$–$\mathbf{PLA_{3000}}$–$\mathbf{PEG_{2000}}$ and $\mathbf{PEG_{2000}}$–$\mathbf{PLA_{3000}}$–$\mathbf{PEI_{1800}}$–$\mathbf{PLA_{3000}}$–$\mathbf{PEG_{2000}}$ were synthesized and self-assembled into micelles for co-delivery of siRNA and hydrophobic doxorubicin (DOX). The chemical structure and molecular weight of the copolymers were characterized by ^{1}H nuclear magnetic resonance and gel permeation chromatography, respectively. The copolymeric micelles were examined by dynamic light scattering, and their size, zeta potential and critical micelle concentration were determined. The in vitro drug release analyses indicated that reductive environment can trigger the release of DOX and siRNA by breaking the micelles. MTT assay demonstrated that the DOX/siRNA-loaded micelles are capable of inhibiting proliferation of SGC7901 cells. The results of fluorescence microscopy and flow cytometry verify the simultaneous delivery of DOX and siRNA from the nanomicellar particles into SGC7901 cells. The reduction-responsive cationic copolymers will provide a platform for constructing drug/gene delivery system toward cancer therapy.

Keywords Amphiphilic · Multi-block copolymer · Drug carrier · Gene/drug dual delivery

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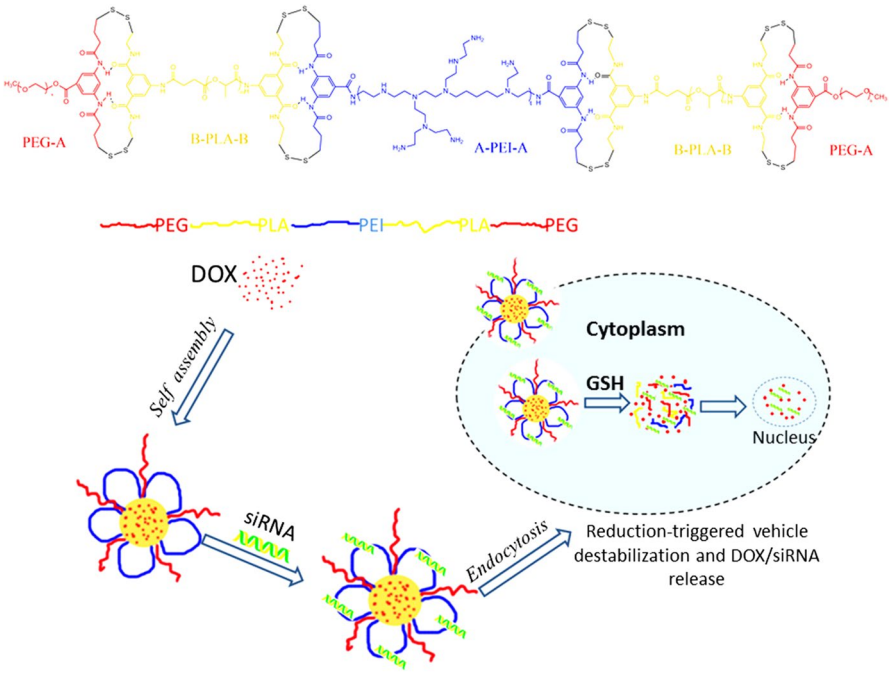
Introduction

In the past decades, polymeric micelles have attracted considerable attention due to their potential applications in nanomedicine [1]. Especially, amphiphilic copolymers have been widely used as drug delivery systems for cancer therapy. Amphiphilic copolymers can self-assemble into core–shell morphology with hydrophobic interior and hydrophilic corona in aqueous solution [2, 3]. In addition to drug delivery, cationic nanoparticles self-assembled from amphiphilic cationic copolymers have recently been employed as alternative choices and are promising for nucleic acid delivery [4]. Compared with homopolymers, copolymeric micelles used as nucleic acids delivery vectors have several unique advantages: (1) the capacity to condense and protect the nucleic acid segments [5]; (2) higher polymeric micelles stability [6]; (3) prolonged blood circulation lifetime in vivo [7]; (4) improved cell association and internalization to enhance transfection efficiency [8]. Therefore, cationic copolymers have been considered as the most prospective candidates with enormous potential in comparison with their counterparts due to their unique characteristics of forming polyelectrolyte complexes with genes and ability to protect them from various enzymes [9–12]. Besides, the structure and properties of copolymeric micelles can be sensitive to external or internal stimuli that can be utilized to control the encapsulated drug and gene release [13, 14]. The incentives include glutathione (GSH), temperature, pH, glucose and others [15].

Most cationic polymers contain amine group in their backbone [16, 17]. Polyethylenimine (PEI) has been widely applied and investigated owing to its extremely high effectivity in gene transfecting and delivering nucleic acids [18, 19]. In recent years, PEI has been used as the gold standard [20], which is low cost and easy to be derived in a wide range of molecular weight as DNA delivery vectors in vitro and in vivo [21–24]. It was found that PEI's efficacy in transfecting genetic materials increased with increasing molecular weight [25, 26]. However, a larger molecular weight of PEI led to more severe cytotoxicity. To overcome this dilemma and break the in vivo application limitation of PEI [27, 28], studies have been carried out to enhance the biocompatibility and applicability of PEI-based gene delivery systems [29]. One effective strategy is to combine the advantageous features of PEI and poly(ethylene glycol) (PEG) to reduce the toxicity of PEI [30, 31]. PEG is a nonionic hydrophilic polymer, which could shield the surface charge of the polyplexes [32]. With the polyplexes around, PEG forms a hydration shell that can reduce the intermolecular interactions and decrease the toxicity of PEI [33–35]. More remarkably, a series of studies demonstrated that PEGylation (i.e., PEG modification) could hinder the interaction of polyplexes with blood components. These valuable features of the PEGylation could enhance the serum stability of polyplexes, reduce the clearance by the reticuloendothelial system and extend their blood lifetime after intravenous administration [29].

In this paper, a ABCBA-type pentablock copolymer containing **PEI**, **PEG** and poly(L-lactic acid) **(PLA)** was designed and synthesized based on H-bonding instructed association units with double disulfide linkage (Scheme 1). The



Scheme 1 Illustration of pentablock copolymer micelles for controlled release of drug/gene

amphiphilic and cationic pentablock copolymer **PEG–PLA–PEI–PLA–PEG** could self-assemble into micelles for non-viral transfection and intracellular drug delivery [36–41]. As a cationic copolymer, the pentablock **PEG–PLA–PEI–PLA–PEG** consisted of hydrophilic **PEG** segments, hydrophobic **PLA** segments as well as a cationic **PEI** segment. The copolymeric micelles were flower-like and possessed enhanced intracellular barriers penetration, with **PEI** as the non-viral vector for siRNA delivery, **PLA** as hydrophobic segments for anticancer drug encapsulation and **PEG** as hydrophilic segments that reduce cytotoxicity, increase serum stability and blood circulation time. Compared with the micelles from the cationic triblock copolymer $\mathbf{PEG_{2000}–PLA_{5000}–PEI_{1800}}$ [14], the pentablock copolymer **PEG–PLA–PEI–PLA–PEG** micelles have more compact spherical structure, lower critical micelle concentration (CMC) and lower nitrogen to phosphorus (N/P) ratio and may be superior therapeutic agents for cancers. The results of this study show that hydrophobic DOX and FAM-siRNA can be co-delivered by the cationic copolymer with high efficiency as well as low toxicity. Therefore, the prepared polymeric micelles are expected to be a promising platform for drug/gene delivery system.

Materials and methods

Materials

All chemicals were purchased from Aldrich or Aladdin and were used as received unless otherwise indicated. All reactions were followed by thin-layer chromatography (TLC) (precoated 0.25-mm silica gel plates from Aldrich), and silica gel column chromatography was carried out with silica gel 60 (mesh 200–400). Dichloromethane, N,N-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were dried over calcium hydride and then purified by vacuum distillation. 3,5-Bis(4-(tritylthio) butanamido)benzoic acid (intermediate **A**), 5-amino-N^1,N^3-bis(2-(tritylthio)ethyl) isophthalamide (intermediate **B**) and **PEG_{2000}-A** were synthesized according to our reported procedure [40]. Poly(ethylene glycol) (**PEG**) was purchased from Shanghai Yare Bio. Co. Ltd, (number average molar mass (M_n) determined by gel permeation chromatography (GPC), ($M_n = 2000$, $M_w/M_n = 1.05$)) and used as received. Polylactic acid (**PLA**) was purchased from Jinan Daigang Biology Co. Ltd, (for PLA viscosity average molar (M_v) determined by viscometer $M_v = 3000$ g/mol;). Branched polyethylenimine (**PEI**, $M_w = 1800$ g/mol, $M_w = 1200$ g/mol) was purchased from Sigma-Aldrich and used as received.

SGC7901 gastric cancer cells were obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, and the cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) and antibiotics (10 units mL^{-1} penicillin and 100 units mL^{-1} streptomycin) at 37 °C in a humidified atmosphere containing 5% CO_2.

Measurements

Nuclear magnetic resonance (NMR) analyses were recorded on Bruker Avance III 400 MHz with deuterated chloroform ($CDCl_3$) or dimethyl sulfoxide-d_6 (DMSO-d_6) as solvent. The number average molar mass (M_n), weight average molar mass (M_w) and polydispersity index (PDI) were measured by gel permeation chromatography (GPC). Spectrometer dynamic light scattering (DLS) measurements were performed in aqueous solution using a Malvern Zetasizer Nano S apparatus equipped with a 4.0-mW laser operating at $\lambda = 633$ nm. All samples of 1.0 mg mL^{-1} were measured at 25 °C and at a scattering angle of 173°.

Synthesis and characterization

Synthesis of hydrophobic block B-PLA_{3000}-B

In a typical procedure, modified **PLA_{3000} (HO-PLA_{3000}-COOH)** (1.5 mmol) in 20 mL methylene chloride (DCM) was cooled in ice water and stirred for 5 min, and then, 4-dimethylaminopyridine (DMAP) (3.0 mmol) and succinic anhydride (4.0 mmol) were added. After the being stirred at room temperature for 18 h, the

reaction mixture was washed with saturated sodium bicarbonate solution and dried over anhydrous Na_2SO_4. Subsequently, the filtrate was distilled *in vacuo* to give the intermediate **HOOC-PLA$_{3000}$-COOH**. And then **HOOC-PLA$_{3000}$-COOH** (0.2 mmol), NMM (0.8 mmol), HATU (0.8 mmol) and the intermediate **B** (0.6 mmol) in anhydrous DMF (20 mL) under nitrogen atmosphere were cooled in an ice water bath, stirred by a magnetic. After stirring for 1 h, the reaction mixture was warmed up to 35 °C and continued stirring for an additional 24 h. The resulting mixture was washed with water and extracted with CH_2Cl_2, and the combined organic extracts were washed with water and brine, dried over anhydrous Na_2SO_4 and then filtered. The filtrate was evaporated *in vacuo*. The hydrophobic block **B-PLA$_{3000}$-B** was further purified by chromatography on silica gel by using CH_2Cl_2/CH_3OH (60/1) as an eluent. Yield 70%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.22–8.01 (m, 4H, ArH), 7.83 (s, 2H, ArH), 7.44–7.37 (m, 24H, ArH), 7.30–7.14 (m, 38H, ArH), 6.44 (s, 4H, –NH–), 5.43–5.02 (m, 21H, 1H per repeating unit, PLA–CH–), 4.43–4.33(m, 1H, PLA–CH–), 3.36 –3.28 (m, 8H, –NCH_2–), 2.53 (t, J = 6.3 Hz, 8H, –SCH_2–), 1.71–1.39 (m, 69H, 3H per repeating unit, PLA–CH_3). IR(KBr, cm^{-1}) 3406, 3064, 2991, 2945, 1747, 1646, 1532, 1453, 1378, 1264, 1190, 1122, 1082, 1042, 860, 746, 706, 672.

Synthesis of hydrophilic block PEG$_{2000}$-A

Common procedure: **PEG-OH** (2.0 mmol) was dissolved in 50 mL methylene chloride solution (DCM), followed by the addition of 3,5-dinitrobenzoyl chloride (4.0 mmol) and triethylamine (8.0 mmol). The reaction mixture was stirred at ice water bath for 4 h. The reaction mixture was poured into water and extracted with CH_2Cl_2, the combined organic extracts were washed with water and brine, dried over anhydrous Na_2SO_4 and filtered, and the filtrate was evaporated to give the intermediate **PEG-NO$_2$**. And then the **PEG-NO$_2$** (0.8 mmol) and 10% Pd/C (0.4 mmol) were dissolved in 50 mL methanol solution under hydrogen atmosphere. The temperature was increased to 35 °C, and the reaction mixture was stirred for 24 h. Then, the reaction mixture was cooled to room temperature, and 10% Pd/C was removed by diatomite filtration. The residue was distilled *in vacuo* to get the intermediate **PEG-NH$_2$**. After that, 4-(tritylthio)butanoic acid (2.0 mmol), 2-(7-aza-1H-benzotriazole-1-ly)-1,1,3,3-tetra-methyluronium hexafluorophosphate (HATU) (4.0 mmol) and N-methylmorpholine (NMM) (2.0 mmol) in anhydrous DMF (20 mL) under nitrogen were cooled in ice water bath with stirring for 30 min. And then **PEG-NH$_2$** was dissolved in anhydrous DMF (5 mL) and injected into the above mixture. The reaction mixture was continued stirring at room temperature for an additional 24 h. The resulting mixture was poured into water and extracted with CH_2Cl_2. The combined organic extracts were washed with water and brine, dried over anhydrous Na_2SO_4 and filtered, and the filtrate was evaporated *in vacuo*. The residue was purified by silica gel column chromatography to give the hydrophilic block **PEG$_{2000}$-A**.

PEG$_{2000}$-A: isolated yield: 74%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (s, 1H, ArH), 7.95 (s, 2H, –NH(C = O)–), 7.85 (s, 2H, ArH), 7.44–7.34 (m, 12H, ArH), 7.28–7.23 (m, 14H, ArH), 7.18–7.12 (m, 4H, ArH), 3.74–3.54 (m, 167H, –CH_2CH_2–), 2.35–2.24 (m, 8H, –SCH_2–, –CH_2CO–), 1.82–1.70 (m, 4H,

–$CH_2CH_2CH_2$–); IR (KBr, cm^{-1}) 2876, 1727, 1653, 1607, 1552, 1438, 1344, 1284, 1257, 1103, 948, 847, 746, 706, 619, 558.

Synthesis of cationic block A-PEI-A, intermediate **A** (2.0 mmol), NMM (2.0 mmol) and HATU (2.5 mmol) in anhydrous DMF (20 mL) under nitrogen atmosphere were cooled in an ice water bath with stirring for 1 h. PEI (1.0 mmol) was dissolved in anhydrous DMF (5 mL) and injected into the solution, while the reaction mixture was warmed up to room temperature. And then the reaction mixture was stirred for 24 h at room temperature. The resulting mixture was poured into 100 mL of ether, and the precipitate was filtered, washed thrice with ethyl ether and dried under vacuum.

A-PEI$_{1800}$-A, isolated yield: 88%. ^{1}H NMR (400 MHz, MeOD/$CDCl_3$ = 50:50) δ 8.00 (s, 2H, ArH), 7.63 (s, 4H, –NH(C = O)–), 7.52 (s, 2H, ArH), 7.42–7.33 (m, 25H, ArH), 7.23– 7.30 (m, 25H, ArH), 7.15–7.21 (m, 12H, ArH), 2.87–2.45 (m, 157H, PEI, 2H per repeating unit, –CH_2CH_2–), 2.35–2.28 (t, J = 7.5 Hz, 9H, –CH_2S), 2.23–2.17 (t, J = 7.2 Hz, 8H, –CH_2CO–), 1.78–1.66 (m, 8H, –$CH_2CH_2CH_2$–). IR (KBr, cm^{-1}) 3433, 2963, 2862, 1653, 1546, 1431, 1385, 1270, 1122, 840, 760, 695, 606, 565.

A-PEI$_{1200}$-A, isolated yield: 90%. ^{1}H NMR (400 MHz, MeOD/$CDCl_3$ = 50:50) δ 8.02 (s, 2H, ArH), 7.64 (s, 4H, –NH(C = O)–), 7.58 (s, 2H, ArH), 7.40 –7.32 (m, 24H, ArH), 7.29–7.17 (m, 23H, ArH), 7.17– 7.08 (m, 12H, ArH), 2.86–2.49 (m, 143H, PEI, 2H per repeating unit, –CH_2CH_2–), 2.34– 2.26 (t, J = 7.4 Hz, 8H, –CH_2S–), 2.26–2.14 (t, J = 7.1 Hz, 8H, –CH_2CO–), 1.80–1.68 (m, 8H, –$CH_2CH_2CH_2$–). IR (KBr, cm^{-1}) 3406, 3936, 2835, 1646, 1552, 1438, 1378, 1264, 1129, 847, 753, 693, 612, 559.

Synthesis of multi-block copolymer PEG$_{2000}$–PLA$_{3000}$–PEI–PLA$_{3000}$–PEG$_{2000}$, in a typical procedure, **B-PLA$_{3000}$-B** (20 mL), **A-PEI-A** (20 mL) and **PEG$_{2000}$-A** (20 mL) were taken from the CH_2Cl_2 stock solutions (1.0 mM) separately and mixed together in a 250-mL round bottom flask. The solvent was evaporated *in vacuo,* and the residue was dissolved with 60 mL of iodine solution (6 mM) in CH_2Cl_2. The resulting mixture was stirred at room temperature for 1 h. After that, the reaction mixture was cooled to 0 °C and a sodium thiosulfate aqueous solution (3 mM) was added until the color of iodine disappeared. The reaction mixture was then extracted with CH_2Cl_2, the organic extract was washed with brine and dried over anhydrous Na_2SO_4, filtered and evaporated, and the residue was further washed thrice with ethyl ether/acetone (35/5) and dried under vacuum to give the final product **PEG$_{2000}$–PLA$_{3000}$–PEI–PLA$_{3000}$–PEG$_{2000}$**.

PEG$_{2000}$–PLA$_{3000}$–PEI$_{1800}$–PLA$_{3000}$–PEG$_{2000}$, isolated yield: 46%, ^{1}H NMR (400 MHz, DMSO-d_6), δ 8.91 (s, 8H, ArH), 8.12 (m, 16H, ArH), 5.28–5.07 (s, 25H, 1H per repeating unit, PLA–CH–), 3.62–3.44 (m, 191H, PEG, –CH_2CH_2–), 3.06–2.66 (m, 100H, PEI, 2H per repeating unit, –CH_2CH_2–), 1.52–1.37 (m, 75H, 3H per

repeating unit, PLA–CH_3). IR (KBr, cm^{-1}) 3434, 2959, 1757, 1651, 1526, 1445, 1402, 1300, 1272, 1095, 1035, 935, 796, 706, 606, 537.

PEG_{2000}–PLA_{3000}–PEI_{1200}–PLA_{3000}–PEG_{2000}, isolated yield: 48%, ^1H NMR (400 MHz, DMSO-d_6), δ 8.76 (s, 8H), 8.04 (d, J = 78.5 Hz, 16H), 5.33–5.00 (s, 25H, 1H per repeating unit, PLA–CH–), 4.13–3.22 (m, 179H, PEG, –CH_2CH_2–), 3.10–2.58 (m, 64H, PEI, 2H per repeating unit, –CH_2CH_2–), 1.57–1.33 (s, 74H, 3H per repeating unit, PLA–CH_3). IR (KBr, cm^{-1}) 3434, 2926,1757, 1649, 1546, 1452, 1400, 1272, 1095, 1035, 1011, 955, 835, 753, 701, 605.

Preparation and characterization of self-assembled micelles

Briefly, the copolymer **PEG_{2000}–PLA_{3000}–PEI–PLA_{3000}–PEG_{2000}** (10 mg) was dissolved in 1.0 mL of DMSO and stirred at room temperature for 30 min. And then, the solution was slowly added to 8.0 mL of deionized water and stirred for another 1 h. After that, the solution was dialyzed against deionized water for 24 h (MWCO = 3500 g mol^{-1}), the deionized water was changed every 4 h for 2 days, and the dialyzate was obtained through a 0.45-μm filter membrane to give a micelle solution. The critical micelle concentration (CMC) was determined using 1,6-diphenylhexa-1,3,5-triene (DPH) as a UV probe by monitoring the absorbance at 313 nm. The concentration of the block copolymer was varied from 0.5×10^{-4} to 0.5 mg mL^{-1}, and the DPH concentration was fixed at 5×10^{-6} M. The absorbance spectra of all solution were recorded using a BioTek Synergy 2.

Preparation of DOX-loaded micelles

In brief, 10 mg of **PEG_{2000}–PLA_{3000}–PEI–PLA_{3000}–PEG_{2000}** was in 1.0 mL of DMSO, followed by addition of 1 mg DOX·HCl and 2 equivalents of triethylamine (TEA, purity 99%), and the solution was stirred at room temperature for 1 h. The mixture was added slowly to 8 mL of deionized water within 10 min and stirred for another 1 h. Subsequently, the solution was dialyzed against deionized water for 24 h (MWCO = 2000 g mol^{-1}), and the deionized water was exchanged every 4 h. To determine the total loading of drug, the DOX-loaded micelle solution was lyophilized and then dissolved in DMSO again. The UV absorbance of the solution at 485 nm was measured to determine the total loading of DOX. Drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following formula.

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

$$\text{DLC (wt\%)} = (\text{weight of load drug}/\text{weight in feed}) \times 100\%$$

In vitro drug release

In brief, DOX-loaded **PEG_{2000}–PLA_{3000}–PEI–PLA_{3000}–PEG_{2000} micelles** (1 mg mL^{-1}) with treatment of DTT (0 and 1 mM) were immediately measured by

fluorescence measurements (BioTek Synergy 2, EX 485 nm, EM 590 nm) at different time intervals, and 2.0 mL of the micelle solution was transferred into a membrane bag [molecular weight cutoff (MWCO = 2000 g mol^{-1})]. It was immersed into a glass bottle containing 100 mL of PBS (50 mM, pH 7.4) or PBS with 1.0 mM of DTT in a sharking water bath at 37 °C to ensure sink conditions. At predetermined time intervals, 10 mL of external buffer solution was withdrawn and replaced with 10 mL of fresh PBS or PBS with 1.0 mM of DTT. The amount of DOX released was measured by fluorescence measurement and calculated by the standard curve plotted in advance (BioTek Synergy 2, excitation wavelength at 485 nm, emission wavelength at 590 nm (DOX)). All DOX-release experiments were conducted in triplicate, and the results are the average date with standard deviations. The cumulative release E_r is calculated according to the following formula:

$$E_r = \frac{V_e \sum_1^{n-1} C_i + V_0 C_n}{m_{drug}}$$

E_r: the total cumulative release % of DOX; V_e: the replacement of PBS volume (10 mL); V_0: the total amount of phosphate buffered saline (PBS) volume (100 mL); C_i: DOX concentration of the ith replacement liquid (μg/mL) (determined by fluorescence measurement); C_n: DOX concentration of the last replacement liquid (μg/mL); m_{drug}: the total amount of DOX in micelle (μg).

Biophysical properties of the polyplexes

The capability of polymers to condense FAM-siRNA (GenePharma Company, Shanghai, China) was studied by gel retardation. FAM-siRNA (20 μM) was dissolved into DEPC water. And then the polymer solution was dropped into the DEPC water containing FAM-siRNA to form various mixtures at different N/P ratios. Polyplexes with various N/P ratios were mixed with 5 × loading buffer and loaded onto the agarose gel (1%). Gel electrophoresis was carried out at room temperature in 1 × tris-acetic/EDTA (TAE) buffer (tris acetate (40 mM), EDTA (1 mM)) at 60 V for 50 min in a Sub-Cell system. Free RNA was used as the control. The UV illuminator (ChemiDoc™ XRS+, Bio-Rad, CA, USA) could be used to visualize the gel and the bands of FAM-siRNA.

In vitro cytotoxicity assay

The SGC7901 cells were firstly floated in the solution of DMEM (Dulbecco's modified Eagle's medium) and given the supplement of 10% fetal bovine serum. And then SGC7901 cells was seeded onto 96-well plates at a density of 5000 cells per well in 100 μL of medium to preincubate in a wet circumstance with 5% CO_2 at 37 °C for 48 h. The new culture medium which contained different concentrations of polyplexes was added to continue for further culture 48 h. The surviving capability of the SGC7901 cells was performed by MTT assay. Then, 20 μL of 5 mg mL^{-1}

MTT assay stock solution was added to 100 μL 96-well plates for 4-h incubation at 37 °C, and unreacted MTT was removed. The obtained blue formazan crystals were dissolved in 100 μL per well DMSO, and the absorbance was measured at a wavelength of 490 nm using a BioTek Synergy 2.

Cell uptake

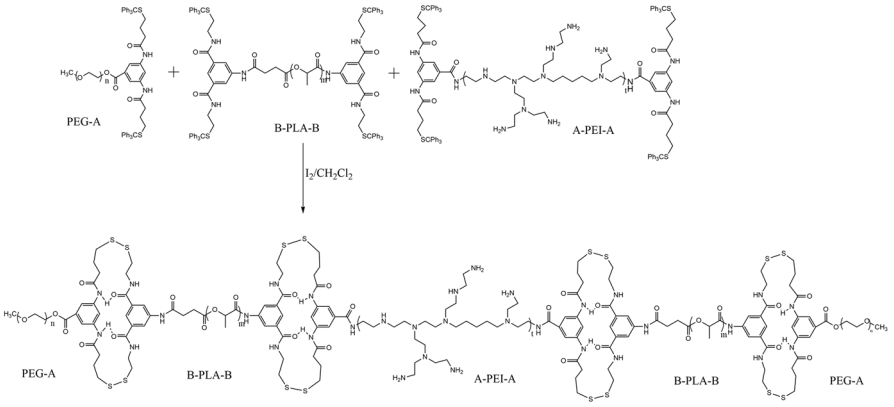
To determine the cellular uptake efficiency of the nanoparticle/siRNA (NP/FAM-siRNA) complex, the location and intensity of FAM-siRNA after cellular uptake were observed by fluorescence microscopy. SGC7901 cells (2×10^5 per well) were seeded in six-well plates and cultured for 24 h. Then, the culture medium was replaced with 500 μL DMEM without FBS containing FAM-siRNA-loaded nanomicellar particle (N/P = 15:1) with a FAM-siRNA final concentration of 200 nM, incubated for 0.5 h, 1 h, 2 h at 37 °C under 5% CO_2. Cells without polyplex treatment were considered as the control.

For flow cytometric analysis, SGC7901 cells were seeded in six-well plates at 2×10^5 per well and proliferated for 36 h before the experiment. And then the cells in each well were incubated with 200 nM FAM-siRNA formulated in DOX-loaded micelleplexes (N/P = 15:1). And after transfection for 2 h, the cells were washed with PBS and trypsinized, and re-suspended in PBS. The samples were analyzed by using a flow cytometer (Becton–Dickinson, San Jose, CA, USA). Cells without polyplex treatment were considered as the control.

Results and discussion

Synthesis and characterization of pentablock copolymers PEG–PLA–PEI–PLA–PEG

According to reported procedure [10b, 22], we synthesized pentablock copolymer **PEG–PLA–PEI–PLA–PEG** with double disulfide linkage complemented by double H-bonding sequence specific units efficiently. Briefly, the hydrophilic block **PEG-A** was synthesized via reaction of carboxyl group in the intermediate **A** with amino group in **PEG-NH$_2$** in the presence of HATU/NMM [22a]. The amine group in the intermediate **B** reacted with carboxyl group in modified PLA (**COOH-PLA-COOH**) in the presence of HATU/NMM, forming hydrophobic block **B-PLA-B** [22a]. And the amine group of PEI reacted with carboxyl group of the intermediate **A** in the presence of HATU/NMM in DMF solution, forming the modified polymer **A-PEI-A** [10b]. The desired pentablock copolymer **PEG–PLA–PEI–PLA–PEG** was synthesized from **PEG-A**, **B-PLA-B** as well as **A-PEI-A** under iodine oxidation conditions (Scheme 2). The intermediates **B-PLA-B**, **PEG-A**, **A-PEI-A** and the terminal pentablock copolymer **PEG–PLA–PEI–PLA–PEG** were characterized by ^{1}H NMR and GPC. The ^{1}H NMR spectra are shown in Fig. 1, and the typical ^{1}H NMR spectrum of **PEG$_{2000}$–PLA$_{3000}$–PEI$_{1800}$–PLA$_{3000}$–PEG$_{2000}$** not only reveals the signals of the **PEG** block (3.62–3.44 ppm from the repeating -OCH$_2$CH$_2$O- unit), the **PLA** block (repeating LA residues 5.28–5.07 ppm from the -CH- groups, and



Scheme 2 Synthesis of the multi-block copolymer **PEG–PLA–PEI–PLA–PEG**

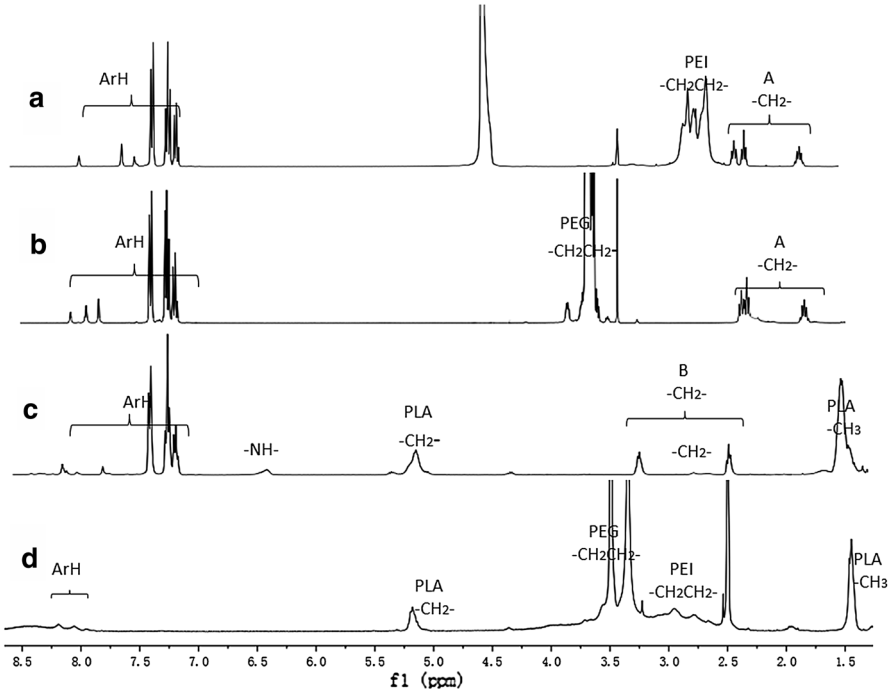


Fig. 1 ^{1}H-NMR (400 MHz) spectra of **a A-PEI$_{1800}$-A** in MeOD/$CDCl_3$ (50/50, v/v), **b PEG$_{2000}$-A** in $CDCl_3$, **c B-PLA$_{5000}$-B** in $CDCl_3$, **d PEG$_{2000}$–PLA$_{3000}$–PEI$_{1800}$–PLA$_{3000}$–PEG$_{2000}$** in DMSO

CH_3- groups of the LA units 1.52–1.37 ppm), and the **PEI** block (3.06–2.66 ppm from the repeating -NCH_2CH_2NH- unit), but also those of the intermediates **A** and **B** amide linking units (-CH_2- overlaps in the **PEI** block about 3.25–1.90 ppm). The dominant signals of the trityl groups in the spectra of **PEG$_{2000}$-A**, **B-PLA$_{3000}$-B**

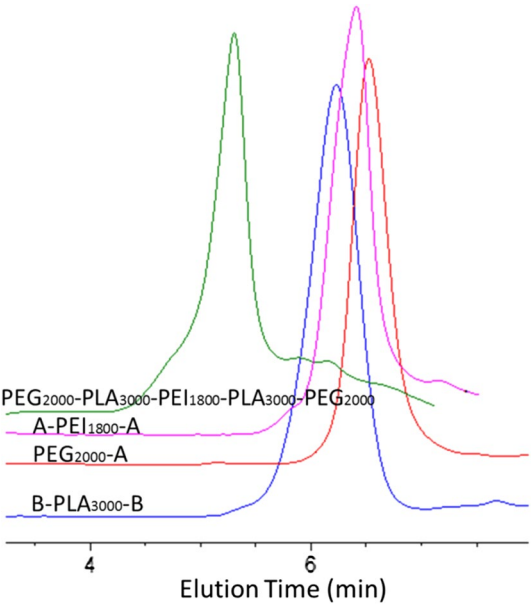


Fig. 2 GPC traces for **B-PLA$_{3000}$-B**, **PEG$_{2000}$-A**, **A-PEI$_{1800}$-A**, and **PEG$_{2000}$–PLA$_{3000}$–PEI$_{1800}$–PLA$_{3000}$–PEG$_{2000}$**

Table 1 GPC characterization of polymers

Polymers	M_n (g mol^{-1})	M_w (g mol^{-1})	PDI
PEG$_{2000}$-A	2600	2800	1.08
B-PLA$_{3000}$-B	4300	4500	1.05
A-PEI$_{1800}$-A	3200	3300	1.03
A-PEI$_{1200}$-A	2600	3000	1.15
PEG$_{2000}$–PLA$_{3000}$–PEI$_{1200}$–PLA$_{3000}$–PEG$_{2000}$	11,500	12,300	1.07
PEG$_{2000}$–PLA$_{3000}$–PEI$_{1800}$–PLA$_{3000}$–PEG$_{2000}$	13,100	13,800	1.05

Estimated by GPC (THF, 1 mL min^{-1}) using polystyrene standards

and **PEI$_{1800}$-A** disappeared completely in the spectrum of copolymer **PEG$_{2000}$–PLA$_{3000}$–PEI$_{1800}$–PLA$_{3000}$–PEG$_{2000}$**, suggesting the formation of disulfide cross-linking units indeed occurred. Figure 2 shows the GPC traces of **B-PLA$_{3000}$-B**, **A-PEI$_{1800}$-A**, **PEG$_{2000}$-A** as well as **PEG$_{2000}$–PLA$_{3000}$–PEI$_{1800}$–PLA$_{3000}$–PEG$_{2000}$**. Obviously, the M_n value of **PEG$_{2000}$–PLA$_{3000}$–PEI$_{1800}$–PLA$_{3000}$–PEG$_{2000}$** increased significantly compared with that of **B-PLA$_{3000}$-B**, **A-PEI$_{1800}$-A**, **PEG$_{2000}$-A**. The M_n and PDI of the polymers are listed in Table 1. The above results are in accordance with the structure of amphiphilic pentablock copolymer **PEG$_{2000}$–PLA$_{3000}$–PEI$_{1800}$–PLA$_{3000}$–PEG$_{2000}$** and confirmed that the double disulfide linkages indeed formed. The same cross-coupling reaction also occurred with copolymer

PEG_{2000}–PLA_{3000}–PEI_{1200}–PLA_{3000}–PEG_{2000}, in which ^{1}H NMR spectra and GPC traces are shown in Figure S1 and Figure S2 in Supporting Information.

Preparation and characterization of nanomicellar particle, DOX and siRNA binding to nanomicellar particle

Amphiphilic pentablock copolymer **PEG–PLA–PEI–PLA–PEG** can self-assemble into core–shell structured micelles in aqueous solution, driven by the strong hydrophobic/hydrophilic interaction between the linear chains of **PLA** and the shells of **PEI** and **PEG**. The hydrophobic **PLA** segments assembled as the inner core of the micelles, while the hydrophilic PEI and PEG segments formed the corona owning to their highly hydrophilic nature. Self-assembled blank micelles or DOX-loaded micelles were prepared by dissolving the copolymers (or DOX and the copolymers) in DMSO and dialyzed against deionized water. The CMC of the polymeric micelles was measured by UV/Vis spectroscopy, using DPH as a hydrophobic probe [42]. The absorbance of DPH as a function of the copolymer **PEG–PLA–PEI–PLA–PEG** concentration in aqueous solution at room temperature is shown in Fig. 3, and the CMC values are listed in Table 2. The CMC values were 0.046 mg mL^{-1} (Fig. 3) and 0.052 mg mL^{-1} (Figure S3 in SI) for **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** and **PEG_{2000}–PLA_{3000}–PEI_{1200}–PLA_{3000}–PEG_{2000}** micelles, respectively, confirming that the polymeric micelles were highly stable in dilute solution [43, 44]. The DOX loading of the micelles was evaluated by UV analysis, and the results showed that the DLC of **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** and **PEG_{2000}–PLA_{3000}–PEI_{1200}–PLA_{3000}–PEG_{2000}** micelles were 4.59% and 2.59%, respectively. (The theoretical DLC was set at 10%). Hence, the copolymer **PEG_{2000}–P**

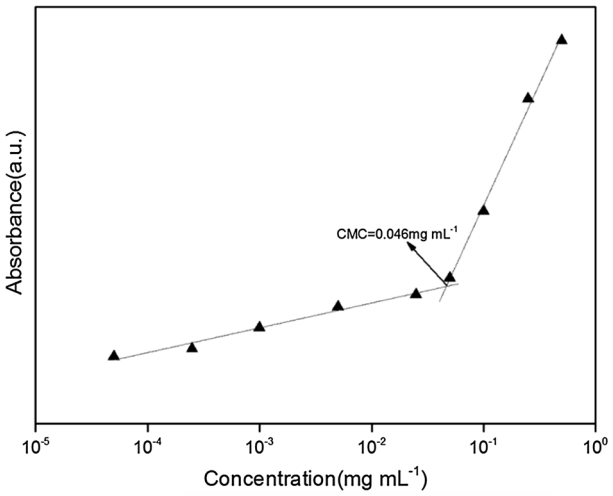


Fig. 3 Relationship of the absorbance intensity of DPH as a function of the copolymer concentration of **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** in aqueous solution at room temperature

Table 2 Properties of PEG-PLA-PEI-PLA-PEG micelles

Copolymers	CMC[a](mg mL^{-1})	Diameter[b] (nm)(*/#)	PDI[b(*/#)]	Zet[b] (mV)(*/#)	DLC[c] (%)
PEG_{2000}–PLA_{3000}–PEI_{1200}–PLA_{3000}–PEG_{2000}	0.052	32 ± 0.1/59 ± 0.5	0.234 ± 0.006/0.168 ± 0.004	46.9 ± 0.7/27.2 ± 1.1	2.59
PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}	0.046	25 ± 0.3/37 ± 0.3	0.192 ± 0.020/0.274 ± 0.011	54.7 ± 1.4/34.7 ± 1.7	4.59
PEG_{2000}-PLA_{5000}-PEI_{1800}	0.071	68 ± 0.9/66 ± 0.7	0.071 ± 0.011	39.0 ± 0.8/27.0 ± 0.6	–

Data are presented as the average ± standard deviation ($n = 3$)

[a]Determined by using a UV/Vis spectrometer

[b]Determined by DLS

[c]Drug loading content

*Blank

#DOX/siRNA-loaded micelles

LA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000} was selected as the model carrier for further evaluation. To further investigate the properties of the polymeric micelles, DLS and TEM were performed. The DLS results show that **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** micelles exhibited unimodal size distribution with a mean diameter of 25 ± 0.3 nm for blank micelles (Fig. 4a) and 37 ± 0.3 nm for DOX/siRNA-loaded micelles (Fig. 4b). Compared with that of triblock copolymer **PEG_{2000}–PLA_{5000}–PEI_{1800}** [10b], the pentablock copolymer micelles have low CMC, which indicated the micelles are relatively stable under dilute solution (Fig. 4 and Table 2). The micelles were spherical with an average size of 22 nm for blank micelles (Fig. 4e) and 37 nm for DOX/siRNA-loaded micelles (Fig. 4f) observed by TEM. High-resolution TEM images revealed the morphology of the micelles more clearly. The size of polymeric micelles is an important parameter for intracellular drug delivery, and small size (< 200 nm) of nanomicellar particle is in favor of maintaining a lower level of the reticuloendothelial system (RES) uptake and minimal renal excretion [45, 46]. Hence, the polymeric micelles of

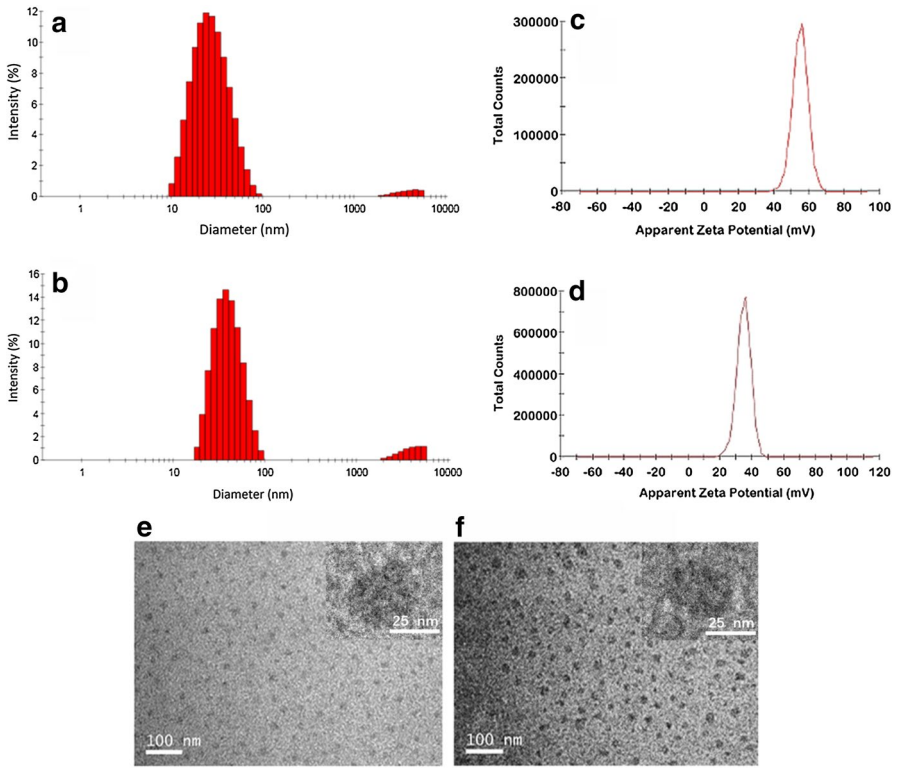


Fig. 4 Characterization of **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** micelles. Size distribution of **a** blank micelles and **b** DOX and siRNA (15:1)-loaded micelles. Zeta potential of **c** blank micelles and **d** DOX and siRNA (15:1)-loaded micelles. **e** TEM image of blank micelles; (inset) high-resolution TEM image. **f** TEM image of DOX and siRNA (15:1)-loaded micelles (N/P = 15); (inset) high-resolution TEM image

PEG–PLA–PEI–PLA–PEG can be a promising siRNA delivery complex for cancer therapy, relying on the enhanced permeation retention effect (EPR) for passive tumor targeting.

Cationic nature of the nanomicellar particles was also determined by zeta potential measurements. **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** micelles were positively charged with a zeta potential of 54.7 ± 1.4 mV (Fig. 4c) due to the presence of protonized amino groups from PEI block. When DOX and siRNA (N/P = 15:1) were bound to the nanomicellar particles, the average particle size increased to 37 ± 0.3 nm (Fig. 4b), and the zeta potential was 34.7 ± 1.7 mV (Fig. 4d).

Redox responsiveness and stimuli-triggered drug release and siRNA release

The disulfide bonds incorporated into copolymer micelles are responsive to reducing agents [47]. Here, we investigated the in vitro release of the encapsulated DOX from **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** micelles under reductive environment. The release behavior was examined by dialyzing the micelles in 0 mM and 1.0 mM DTT solution at 37 °C, respectively. The cumulative drug release from **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** micelles was 29% within 20 h in the absence of DTT. In contrast, the DOX release from the micelles increased to 63% in the presence of 1.0 mM DTT within the same period (Fig. 5). These results demonstrate that the disulfide linkage was readily cleaved by DTT, breaking the core–shell structure and accelerating the release of the encapsulated DOX.

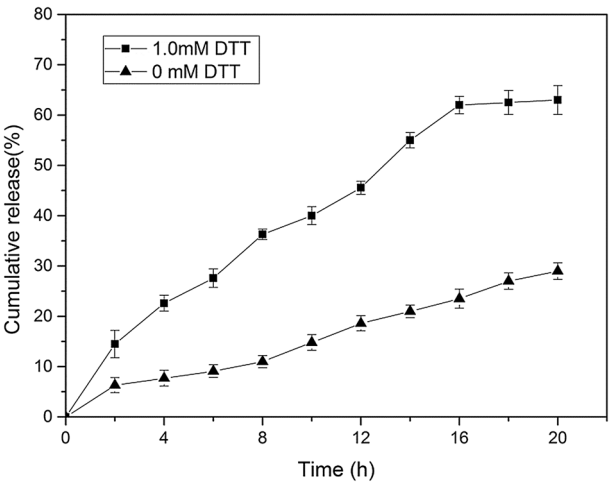


Fig. 5 *In vitro* release of encapsulated DOX from **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** micelles in PBS (pH 7.4) with or without treatment of 1 mM DTT as monitored with the fluorescence intensity of DOX (EX: 485 nm, EM: 590 nm). The data are presented as average ± standard deviation ($n = 3$)

Some researchers found that there exists fourfold higher level of GSH in tumor tissues compared with normal tissues in tumor-bearing mice [48, 49]. The disulfide bonds in **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** micelles can be reduced in the cytosol due to the reductive environment provided by intracellular glutathione. Therefore, we investigated the redox-responsive siRNA release behavior of the siRNA-loaded **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** micelles by agarose gel electrophoresis.

As shown in Fig. 6a, only a small amount of siRNA was released into the gel from **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** micelles in the absence of GSH. When the N/P ratio ≥ 15:1, most of the siRNA was retained in the wells by complexation with **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** nanomicellar particle. In Fig. 6b, most of siRNA was released from the particles in the presence of 10 mM GSH solution. However, when the N/P ratio was 20:1 and 25:1, few siRNA molecules were released into the gel. The above results show that **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** nanomicellars particle was able to inhibit siRNA migration at an N/P ratio of 15. At the same time in redox environment, disulfide bonds in the polyplexes can be destroyed and the most of siRNA can be released at the N/P ratio of 15.

In vitro cytotoxicity

As potential drug delivery and gene carrier materials, the cytotoxicity of nanomicellars particles is a key parameter for their biomedical applications [50, 51]. Cytotoxicity of **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** micelles and siRNA/

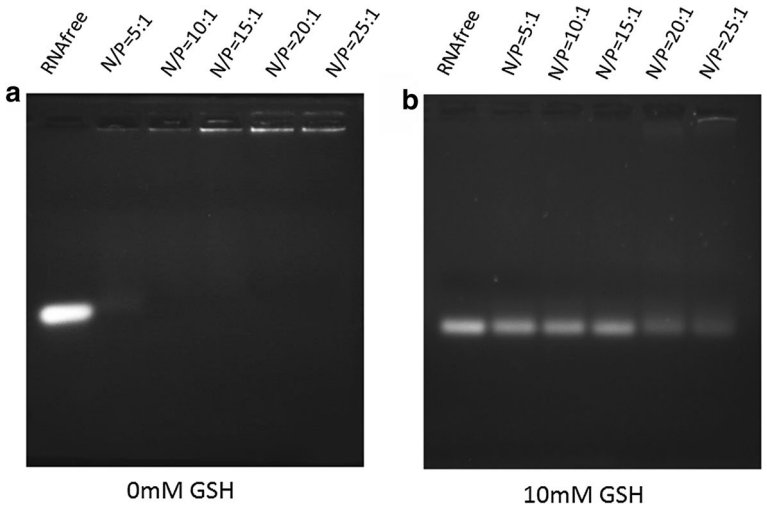


Fig. 6 Electrophoretic mobility of siRNA in the polyplexes formed by **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}**; the right image shows the influence of GSH (10 mM) on gel retardation at different N/P ratios

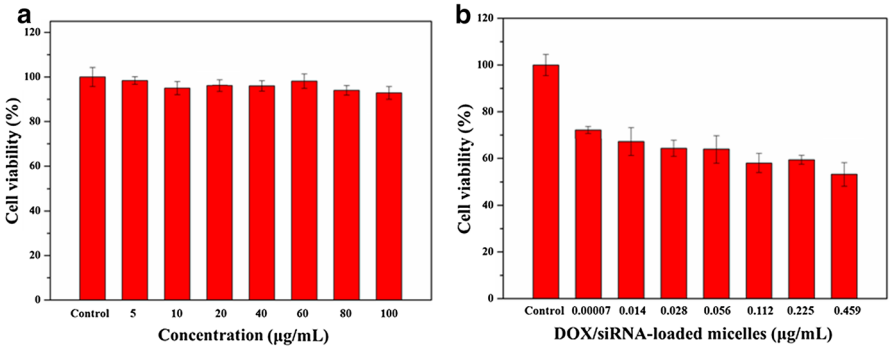


Fig. 7 Viability of SGC7901 cells incubated with **a** $\mathbf{PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}}$ blank micelles and **b** DOX/siRNA-loaded micelles for 24 h by MTT assay. Data are presented as the average ± standard deviation ($n = 5$)

DOX-loaded $\mathbf{PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}}$ in vitro was evaluated by MTT assay against SGC7901 gastric cancer cell lines. Figure 7 shows the cell viability after 24-h incubation with blank micelles or siRNA/DOX-loaded micelles at different concentrations. As shown in Fig. 7a, the cell viability remained at above 90%, indicating that the micelles have low cytotoxicity and good biocompatibility. In comparison, the viability of the cells treated with the DOX/siRNA-loaded micelles significantly decreased, indicating that the drug released from the $\mathbf{PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}}$ micelles enhances proliferation inhibition of SGC7901 cells.

$\mathbf{PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}}$ micelles co-deliver siRNA/DOX into SGC7901 gastric cancer cells

To demonstrate that the DOX/FAM-siRNA can be delivered into SGC7901 cells simultaneously by $\mathbf{PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}}$ micelles, we analyzed the cellular uptake and intracellular distribution of DOX/FAM-siRNA-loaded micelles in SGC7901 cells by fluorescence microscopy. FAM-labeled siRNA was aimed at fluorescence detection of the siRNA. Cells were incubated with DOX/FAM-siRNA-loaded micelles for 0.5 h, 1 h and 2 h, respectively. As shown in Fig. 8a, a high degree of co-localization of the red and green fluorescence signals was observed, revealing the similar distribution of DOX and siRNA in the cytoplasm.

Flow cytometry was used to investigate the cell uptake efficiency of $\mathbf{PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}}$ micelles. To observe the co-delivery of siRNA and DOX, we performed cell fluorescence for SGC7901 cells at 2 h after treatment with DOX/siRNA-loaded micelles as indicated in Fig. 8b. Fluorescence-activated cell storing analysis showed that most of the cells were located in the double-positive quadrant after 2-h incubation. This indicated that siRNA and DOX can be simultaneously delivered into the cells by the micelles.

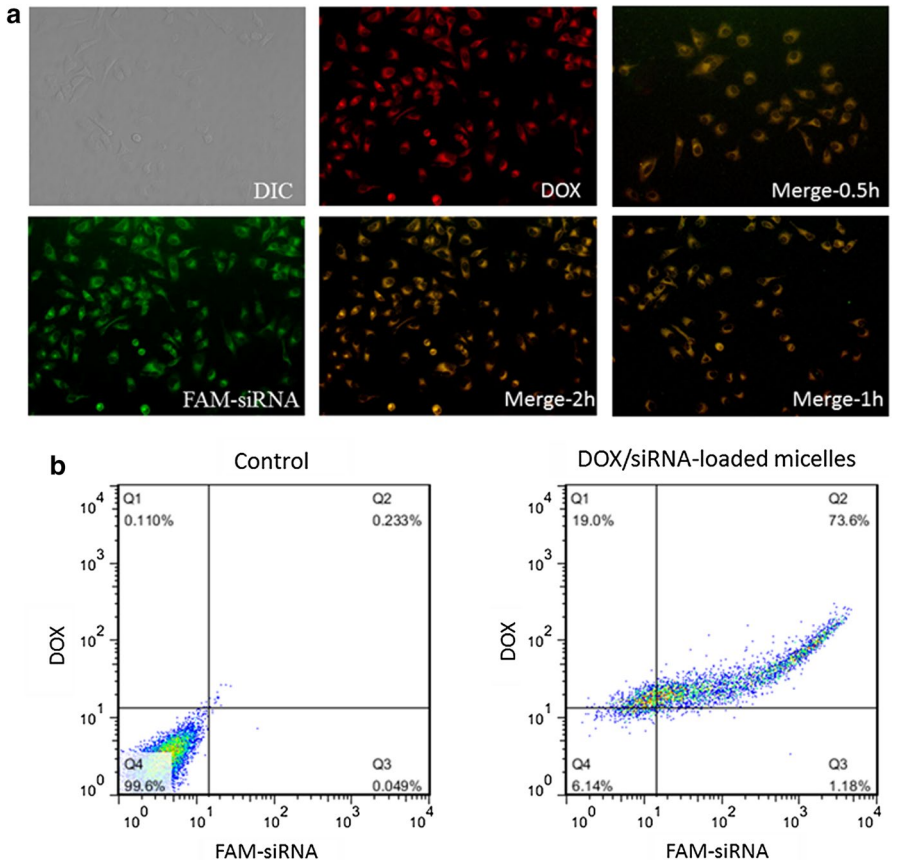


Fig. 8 Intracellular uptake and distribution of DOX/siRNA-loaded micelles in SGC7901cells. **a** SGC7901 cells were incubated with DOX/siRNA-loaded micelles for different time periods. The DOX (red) and FAM-siRNA (green) were imaged using a fluorescence microscope. **b** Fluorescence-activated cell storing analysis of SGC7901 cells incubated with DOX/siRNA-loaded micelles for 2 h. The cells were non-pretreated as control (color figure online)

Conclusion

Amphiphilic pentablock copolymer **PEG_{2000}–PLA_{3000}–PEI–PLA_{3000}–PEG_{2000}** micelles with H-bonding instructed double disulfide linkage have been synthesized successively. The redox-responsive copolymer **PEG_{2000}–PLA_{3000}–PEI–PLA_{3000}–PEG_{2000}** has low CMC. In the reductive environment provided by intracellular glutathione, the disulfide bonds were cleaved, which triggered the release of siRNA and drug. In vitro cell viability evaluation confirmed that the **PEG_{2000}–PLA_{3000}–PEI_{1800}–PLA_{3000}–PEG_{2000}** blank micelles have good cytocompatibility and the DOX/siRNA-loaded micelles are able to effectively inhibit the proliferation of gastric cancer cells. And in vitro assay study shows that nanomicellar particle could simultaneously deliver siRNA and drug into gastric cancer cells. These results

indicate that the amphiphilic pentablock copolymer **PEG_{2000}–PLA_{3000}–PEI–PLA_{3000}–PEG_{2000}** micelles provide a platform for co-delivery of hydrophobic drug and siRNA for cancer therapy.

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