



## Co-delivery of siRNA and paclitaxel into cancer cells by biodegradable cationic micelles based on PDMAEMA–PCL–PDMAEMA triblock copolymers

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### ABSTRACT

Biodegradable cationic micelles were prepared from PDMAEMA–PCL–PDMAEMA triblock copolymers and applied for the delivery of siRNA and paclitaxel into cancer cells. PDMAEMA–PCL–PDMAEMA copolymers were readily obtained by reversible addition-fragmentation chain transfer (RAFT) polymerization of dimethylaminoethyl methacrylate (DMAEMA) using CPADN–PCL–CPADN (CPADN: 4-cyanopentanoic acid dithionaphthalenoate; PCL: 3600 Da) as a macro-RAFT agent. The molecular weights of PDMAEMA blocks, controlled by monomer/CPADN–PCL–CPADN mole ratios, varied from 2700, 4800 to 9100 (denoted as polymer **1**, **2** and **3**, respectively). These triblock copolymers formed nano-sized micelles in water with positive surface charges ranging from +29.3 to +35.5 mV. Both micelles **1** and **2** revealed a low cytotoxicity. Gel retardation assay showed that micelles **1** and **2** could effectively complex with siRNA at and above N/P ratios of 4/1 and 2/1, respectively. Notably, GFP siRNA complexed with micelle **1** exhibited significantly enhanced gene silencing efficiency as compared to that formulated with 20 kDa PDMAEMA or 25 kDa branched PEI in GFP-expressed MDA-MB-435-GFP cells. Moreover, micelle **1** loaded with paclitaxel displayed higher drug efficacy than free paclitaxel in PC3 cells, due to most likely improved cellular uptake. The combinatorial delivery of VEGF siRNA and paclitaxel showed an efficient knockdown of VEGF expression. Confocal laser scanning microscope studies on GFP siRNA complexed with Nile red-loaded micelle revealed that Nile red was delivered into GFP-expressed MDA-MB-435-GFP cells and that GFP expression was significantly inhibited. These results demonstrated that cationic biodegradable micelles are highly promising for the combinatorial delivery of siRNA and lipophilic anti-cancer drugs.

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### 1. Introduction

RNA interference (RNAi) mediated by small interfering RNA (siRNA) has emerged as one of the most advanced and versatile tools for biological research as well as a one of the most promising therapeutic strategy for various human diseases such as viral infections, genetic diseases, cardiovascular disorders, and cancers [1,2]. Unlike chemotherapeutics, siRNA exhibits in general a high specificity and a low non-specific toxicity. However, siRNA can not be applied directly in that it will be rapidly degraded in culture and in vivo, its cellular internalization is poor due to its negative surface charge, and it may induce type I interferon responses and stimulate the production of pro-inflammatory cytokines [3]. A variety of

delivery systems, either viral or non-viral, have been investigated for siRNA delivery in vitro and in vivo. Despite their high transfection efficiency, viral vectors are confronted with production, cost, and safety issues. Recently, non-viral vectors in particular cationic polymers have received growing interests due to many advantages they offer over their viral counterparts including improved safety, low immune responses, enabling repeated uses, and ease of production [4–6]. However, in comparison with DNA, siRNA is short and most synthetic carriers including polyethylenimine (bPEI) and chitosan exhibit insufficient condensation and colloidal stability, resulting in low level of gene knockdown [7]. In the past several years, different strategies to improve siRNA complexation have been developed [8,9], which include development of siRNA bio-conjugates [10–12], hydrophobic modification of polycations [13], and increase of polycation charge density [14].

The carrier-induced toxicity has been another challenge for cationic polymers. Usually, in order to condense siRNA, polycations

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are required to have high molecular weights and high charge densities, which are often associated with acute and/or long-term toxicity. Recently, different types of biodegradable polycations such as poly( $\beta$ -amino esters) [15], poly(amino ester glycol urethane) [16], bioreducible poly(amido amine)s [17,18], and bioreducible polyspermines [19] have been explored as low cytotoxic carriers to deliver siRNA into cells. Notably, Wang and coworkers reported that cationic degradable micelles self-assembled from amphiphilic triblock copolymer PEG-*b*-poly( $\epsilon$ -caprolactone)-*b*-poly(2-aminoethyl ethylene phosphate) mediated efficient siRNA delivery [20]. Lavasanifar and coworkers reported that MDR-1-targeted siRNA formulated with micelles based on spermine or tetraethylene-pentamine-grafted PEG-*b*-poly( $\epsilon$ -caprolactone) exhibited efficient gene silencing for P-glycoprotein expression [21]. The cationic micelles are particularly appealing because they have the potential to simultaneously deliver genes and hydrophobic anti-cancer drugs [22–25]. The co-delivery of paclitaxel and an interleukin-12-encoded plasmid DNA from self-assembled cationic core-shell nanoparticles have shown to efficiently suppress cancer growth in a 4T1 mouse breast cancer model [23].

In this paper, we report on cationic micelles based on well-defined PDMAEMA-PCL-PDMAEMA triblock copolymers for the combinatorial delivery of siRNA and paclitaxel (Scheme 1). These triblock copolymers were designed on the basis of the following reasons: (i) PDMAEMA has received a lot of attention for gene delivery due to its relatively low toxicity and high buffer capacity [26–30], (ii) combination of biodegradation and low molecular weight PDMAEMA will further reduce their *in vitro* and *in vivo*

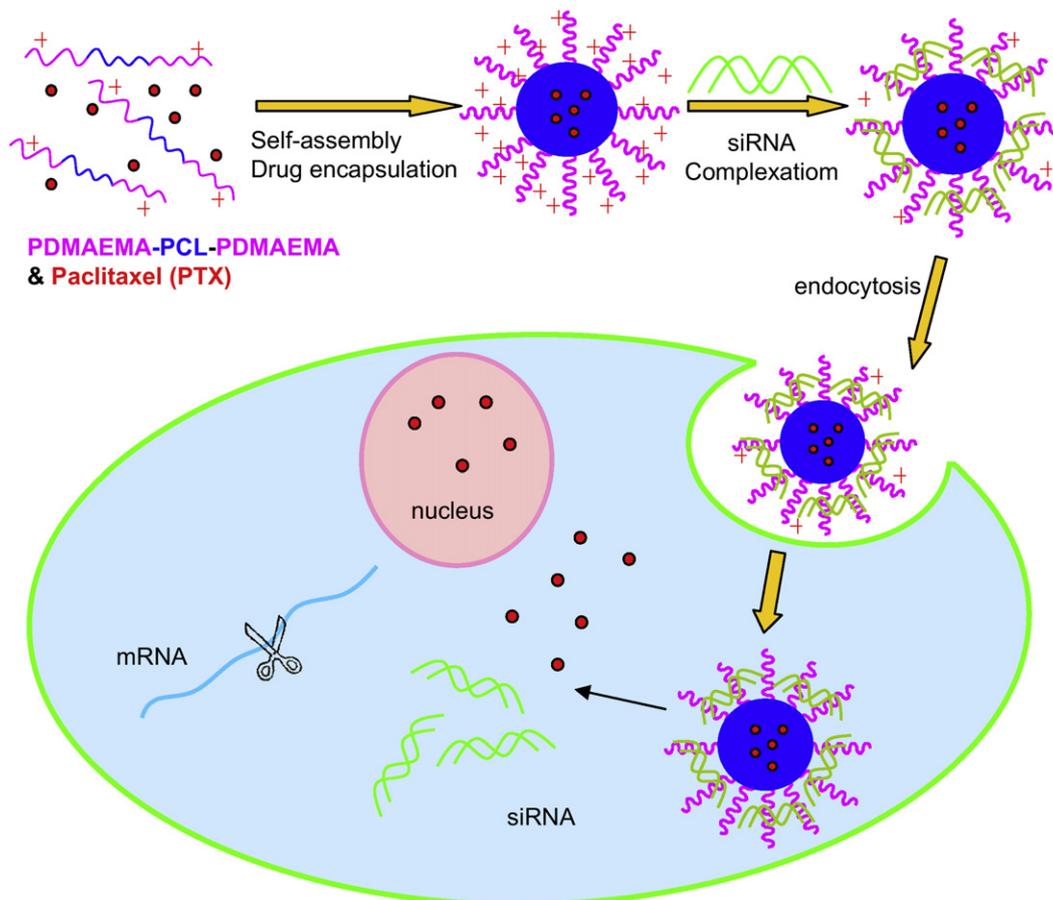
toxicity, (iii) formation of micelles may result in largely improved siRNA condensation ability and thereby transfection activity, (iv) micelles are one of the most efficient nano-carriers for hydrophobic anti-cancer drugs, and (v) they can be conveniently prepared with controlled macromolecular structures and molecular weights by living radical polymerization.

## 2. Materials and methods

### 2.1. Materials

Ethylene glycol (99%, Alfa Aesar) was dried over CaO and distilled under vacuum.  $\epsilon$ -Caprolactone ( $\epsilon$ -CL, 99%, Alfa Aesar) was dried over CaH<sub>2</sub> for 48 h at room temperature and distilled under reduced pressure before use. Toluene and methylene dichloride were dried by refluxing over sodium wire and CaH<sub>2</sub>, respectively, and distilled prior to use. 2-(*N,N*-dimethylaminoethyl) methacrylate (DMAEMA, 97%, Alfa Aesar) was purified by passing through a basic alumina column before use. 4-Cyanopentanoic acid dithionaphthalenoate (CPADN) was synthesized according to the described procedure for 4-cyanopentanoic acid dithiobenzoate [31]. Stannous octoate (Sn(Oct)<sub>2</sub>, 95%, Sigma), 4-dimethylamino pyridine (DMAP, 99%, Alfa Aesar), dicyclohexyl carbodiimide (DCC, 99%, Alfa Aesar), paclitaxel (>99%, TCI, Tokyo, Japan), and Nile red (>99%, Sigma) were used as received.

Green fluorescence protein (GFP) siRNA (sense: 5'-AACUUCAGGGUCAGCUUG-CdTdT-3', anti-sense: 5'-GCAAGCUGACCCUGAAGUdTTdT-3') was obtained from Bioneer Co. (Daejeon, Korea). Targeting human VEGF siRNA (sense: 5'-GGAGUACCCUGAUGAGAUcTTdT-3', anti-sense: 5'-GAUCUCAUCAGGGUACUCCdTT-3') was obtained from Qiagen Inc. (Valencia, CA). MDA-MB-435-GFP (human breast cancer cell) cells stably expressing GFP protein donated by Samyang Corp. (Daejeon, South Korea) were maintained in a DMEM medium containing 10%FBS, penicillin (100 U/mL), and streptomycin (100  $\mu$ g/mL) at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. PC-3 (human prostate carcinoma cells, Korean Cell Line Bank, KCLB, Seoul, Korea) were maintained in an RPMI1640 medium containing 10%FBS, penicillin (100 U/mL),



**Scheme 1.** Illustration of biodegradable cationic micelles self-assembled from PDMAEMA-PCL-PDMAEMA triblock copolymers for combinatorial delivery of hydrophobic anti-cancer drugs and siRNA into cancer cells.

and streptomycin (100 µg/mL) at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. FBS (Fetal bovine serum), DMEM (Dulbecco's Modified Eagle Medium) and RPMI 1640 (Roswell Park Memorial Institute 1640 medium) were obtained from Invitrogen (Carlsbad, CA) and penicillin/streptomycin solution was purchased from Sigma-Aldrich (St. Louis, MO). Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan).

## 2.2. Synthesis of PDMAEMA–PCL–PDMAEMA triblock copolymers

### 2.2.1. Synthesis of $\alpha,\omega$ -dihydroxy poly( $\epsilon$ -caprolactone) (HO–PCL–OH)

Under a nitrogen atmosphere, to a dried reaction flask equipped with a magnetic stir bar, ethylene glycol (72.5 mg, 1.17 mmol), Sn(Oct)<sub>2</sub> (118.4 mg, 0.29 mmol),  $\epsilon$ -CL (4 g, 35.1 mmol) and toluene (5 mL) were added. The flask was sealed and placed into an oil bath thermostated at 100 °C and the reaction was allowed to proceed for 24 h. The polymerization was terminated with excess hydrochloride, and the resulting polymer was isolated by precipitation in cold diethyl ether, filtration, and drying in vacuo. Yield: 83.3%. <sup>1</sup>H NMR end group analysis revealed  $M_n = 3600$ .  $M_n$  (GPC) = 7000, PDI (GPC) = 1.26. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  4.27 (s, –OCH<sub>2</sub>CH<sub>2</sub>O–), 4.06 (t, –COCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>O–), 2.30 (t, –COCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>O–), 1.64 (m, –COCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>O–), 1.38 (m, –COCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>O–), 3.64 (t, –COCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>OH).

### 2.2.2. Synthesis of macro-RAFT agent CPADN–PCL–CPADN

To a stirred solution of HO–PCL–OH (360 mg, 0.1 mmol), CPADN (150 mg, 0.4 mmol) and catalytic amount of DMAP (2.4 mg, 0.02 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (15 mL) was added dropwise a solution of DCC (100 mg, 0.45 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10 mL). The reaction was allowed to proceed at room temperature for 48 h in the dark. The precipitate was removed by filtration. The polymer was isolated by precipitation in excess diethyl ether, filtration, and drying in vacuo. Yield: 74.6%. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  4.27 (s, –OCH<sub>2</sub>CH<sub>2</sub>O–), 4.06 (t, –COCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>O–), 2.30 (t, –COCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>O–), 1.64 (m, –COCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>O–), 1.38 (m, –COCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>O–), 1.99 (s, –OCOCH<sub>2</sub>CH<sub>2</sub>C(CN)(CH<sub>3</sub>)SCSC<sub>10</sub>H<sub>7</sub>), 7.49, 7.90, and 8.14 (–OCOCH<sub>2</sub>CH<sub>2</sub>–C(CN)(CH<sub>3</sub>)–SCSC<sub>10</sub>H<sub>7</sub>), 2.67 (m, –OCOCH<sub>2</sub>CH<sub>2</sub>C(CN)(CH<sub>3</sub>)SCSC<sub>10</sub>H<sub>7</sub>), 2.44 (m, –OCOCH<sub>2</sub>CH<sub>2</sub>–C(CN)(CH<sub>3</sub>)SCSC<sub>10</sub>H<sub>7</sub>).

### 2.2.3. Synthesis of PDMAEMA–PCL–PDMAEMA triblock copolymers by RAFT polymerization

PDMAEMA–PCL–PDMAEMA triblock copolymers were prepared by RAFT polymerization of DMAEMA using CPADN–PCL–CPADN as a macro-RAFT agent. In a typical example, under a nitrogen atmosphere, DMAEMA (0.19 g, 1.24 mmol), CPADN–PCL–CPADN (0.13 g, 0.031 mmol), AIBN (1.0 mg, 0.0062 mmol) and 1.5 mL

THF was added into a 10 mL Schlenk flask. The flask was sealed and placed into oil bath thermostated at 60 °C. The mixture proceeded with magnetic stirring for 24 h. The resulting copolymer was isolated by precipitation in n-hexane, filtration and drying in vacuo. Yield: 72–80%. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  7.52, 7.85, and 8.16 (naphthalene protons); 4.27 (s, –OCH<sub>2</sub>CH<sub>2</sub>O–); 1.05/0.89, 1.86, 2.28, 2.56, and 4.07 (PDMAEMA block); 1.39, 1.65, 2.28, and 4.07 (PCL block).

## 2.3. Characterization

<sup>1</sup>H NMR spectra of the polymers were recorded on an INOVA 400 MHz nuclear magnetic resonance instrument using deuterated chloroform (CDCl<sub>3</sub>) as a solvent. The chemical shifts were calibrated against solvent signal of CDCl<sub>3</sub>. The molecular weight and polydispersity (PDI) of the polymers were determined with a Waters 1515 gel permeation chromatography (GPC) instrument equipped with a Styragel HR 5E THF column (Waters Chromatography) following a INLINE precolumn and a differential refractive index detector. The measurements were performed using THF as eluent at a flow rate of 0.6 mL/min at 35 °C and a series of polystyrene as standards.

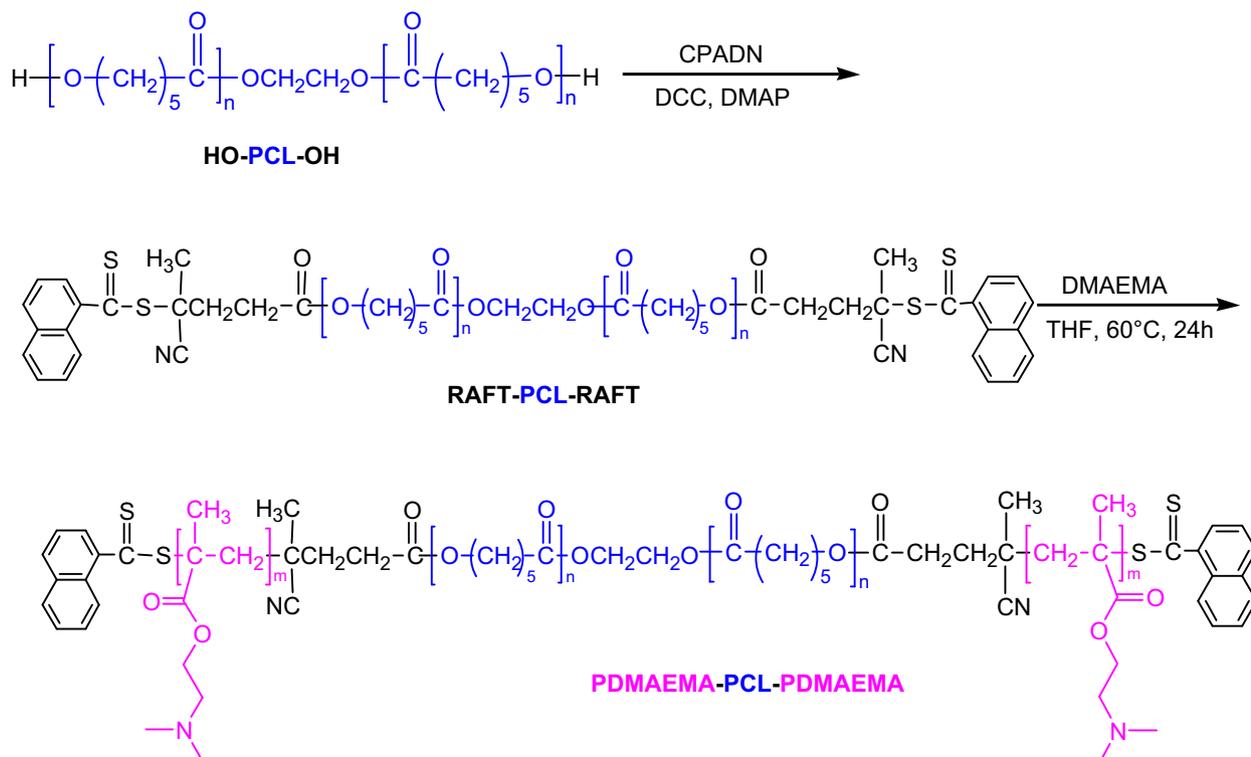
## 2.4. Preparation and characterization of micelles

Micelles were prepared by directly dissolving PDMAEMA–PCL–PDMAEMA copolymers in HAC–NaAc buffer (20 mM, pH 5.0) overnight at a concentration of 0.5 mg/mL with magnetic stirring followed by sonication for 30 min. The sizes and the zeta potentials of the self-assembled micelles were determined at 25 °C by Zetasizer Nano ZS (Malvern Instruments).

The critical micelle concentration (CMC) was determined using pyrene as a fluorescence probe. The concentration of block copolymer varied from 1 × 10<sup>–5</sup> to 0.5 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<sub>372</sub>/I<sub>383</sub> at low and high concentration regions.

## 2.5. Preparation and characterization of drug-loaded micelles

PTX was loaded into micelles by dropwise adding 5 mL HAC–NaAc buffer (20 mM, pH 5.0) to 0.5 mL of THF solution of block copolymer (5 mg/mL) and 200 µL of PTX solution in DMSO (5 mg/mL), sonicating for 1 h, and dialysis against HAC–NaAc buffer (20 mM, pH 5.0) with an MWCO of 3500 at r.t. The final concentration of micelles was ca. 0.5 mg/mL. 0.2 mL of micelle solution was freeze-dried, the residue was dissolved in acetonitrile, and the amount of PTX was determined by HPLC (1100



**Scheme 2.** Synthesis of PDMAEMA–PCL–PDMAEMA triblock copolymers.

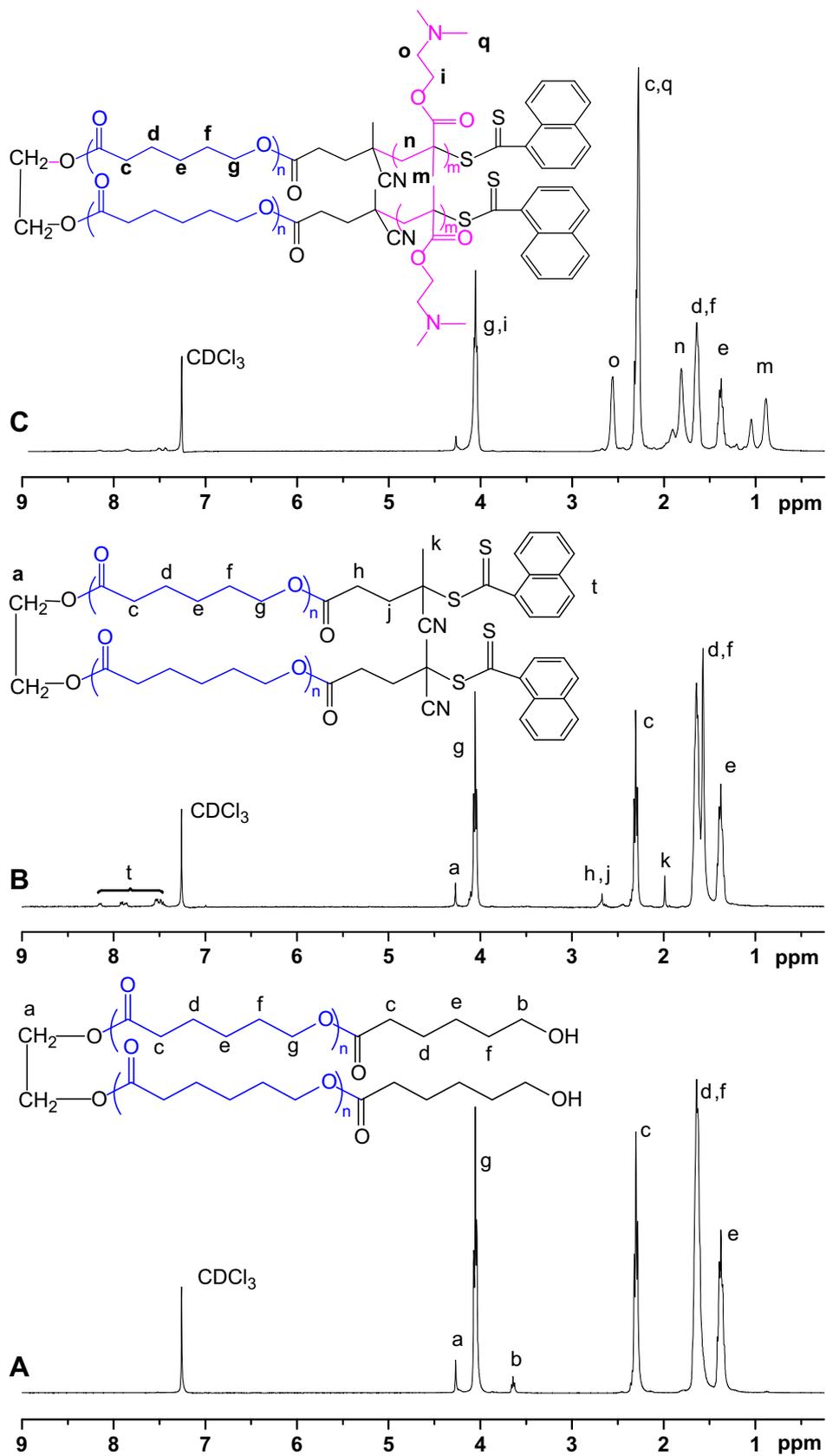


Fig. 1.  $^1\text{H}$  NMR spectra ( $\text{CDCl}_3$ , 400 MHz) of HO-PCL-OH (A); CPADN-PCL-CPADN (B); and PDMAEMA-PCL-PDMAEMA (polymer 1) (C).

**Table 1**  
Characterization of PDMAEMA–PCL–PDMAEMA triblock copolymers and micelles.

Polymer	M/CPADN feed ratio <sup>a</sup>	$M_n$ /kDa ( <sup>1</sup> H NMR) <sup>b</sup>	$M_n$ /kDa (GPC) <sup>c</sup>	PDI <sup>c</sup> (GPC)	Size (nm) <sup>d</sup>	PDI <sup>d</sup>	Zeta (mV) <sup>d</sup>	CMC <sup>e</sup> (mg/L)
<b>1</b>	40/1	2.7–3.6–2.7	9.2	1.15	94.7 ± 0.1	0.22	35.5 ± 0.7	24.0
<b>2</b>	76/1	4.8–3.6–4.8	14.2	1.31	53.6 ± 0.6	0.26	29.5 ± 1.6	21.9
<b>3</b>	152/1	9.1–3.6–9.1	21.1	1.42	132.2 ± 2.6	0.18	29.3 ± 1.2	15.8

<sup>a</sup> Molar feed ratio of DMAEMA to CPADN–PCL–CPADN macro-RAFT agent.

<sup>b</sup> Determined from <sup>1</sup>H NMR by comparing the integrals of signals at  $\delta$  4.07 and 2.56.

<sup>c</sup> Determined by GPC measurements in THF.

<sup>d</sup> Determined by dynamic light scattering (DLS). Micelles were prepared by directly dissolving copolymers in pH 5.0 HAC–NaAc buffer at a concentration of 0.5 mg/mL overnight followed by 30 min sonication.

<sup>e</sup> Determined using pyrene as a fluorescence probe.

series, Agilent Technologies, Palo Alto, CA, USA) with UV detection at 227 nm using a 1/1 (v/v) mixture of acetonitrile and water as a mobile phase. Nile red was loaded into the micelles in a similar way as for PTX except that the whole procedure was performed in the dark.

## 2.6. Gel retardation assay

The siRNA binding ability of micelles was studied by agarose gel electrophoresis. The complexes were prepared at different N/P ratios (1/1, 2/1, 4/1, and 6/1). Electrophoresis was carried out on 1% agarose gel with a current of 100 V for 10 min in TAE buffer solution (40 mM Tris–HCl, 1 v/v % acetic acid, and 1 mM EDTA). The retardation of the complexes was visualized by staining with ethidium bromide. The final siRNA concentration is 1  $\mu$ g per well.

## 2.7. In vitro cytotoxicity measurements

The cytotoxicity of micelles and PTX-loaded micelles was assessed with CCK-8 viability assay against MDA-MB-435-GFP (human breast cancer cell) and PC3 (human prostate carcinoma cell) cells. MDA-MB-435-GFP cells (5000 cells/well) and PC3 cells (5000 cells/well) were seeded in a 96-well plate at 37 °C for 24 h in 100  $\mu$ L DMEM and RPMI1640 medium containing 10% (v/v) FBS, respectively. The cells were incubated with different amounts of micelles or PTX-loaded micelles for 24 h at 37 °C. The amount of viable cells was determined by CCK-8 cell viability assay.

## 2.8. Preparation of siRNA complexes

100  $\mu$ L of micelle or drug-loaded micelle solution in HAC–NaAc buffer at different concentrations ranging from 39.9  $\mu$ g/mL to 145.1  $\mu$ g/mL was added to 100  $\mu$ L siRNA solution in HAC–NaAc buffer (5  $\mu$ g/mL). The solution was gently vortexed and incubated at r.t. for 15 min.

## 2.9. In vitro transfection and gene silencing

### 2.9.1. Transfection with GFP siRNA

MDA-MB-435-GFP cells were plated on a 12-well plate at a density of  $1.5 \times 10^5$  cells/well. After 24 h of culture, the medium was removed, the cells were washed with PBS, and 400  $\mu$ L of serum-free medium and 100  $\mu$ L of siRNA complexes solution containing 0.25  $\mu$ g siRNA were added. The final concentration of siRNA in each well was 0.5  $\mu$ g/mL. The cells were incubated with siRNA complexes for 4 h at 37 °C. Then, the transfection medium was discarded and supplemented with fresh medium containing 10% FBS. The cells were cultured for another 48 h, washed twice with cold PBS and lysed with 1% Triton-X100. The GFP expression level of cell lysates

was analyzed with a spectrofluorophotometer (SLM-AMINCO 8100, SLM Instruments Inc., Urbana, IL) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

### 2.9.2. Transfection with VEGF siRNA

PC-3 cells were plated on a 12-well plate at a density of  $1.5 \times 10^5$  cells/well. After 24 h of culture, the medium was removed, the cells were washed with PBS, and 400  $\mu$ L of serum-free medium and 100  $\mu$ L of siRNA complexes solution containing 0.25  $\mu$ g siRNA were added. The final concentration of siRNA in each well was 0.5  $\mu$ g/mL. The cells were incubated with siRNA complexes for 4 h at 37 °C. The transfection medium was discarded and supplemented with fresh medium containing 10% FBS. After additional 6 h of incubation, the medium was again replaced with fresh medium containing 10% FBS and 20  $\mu$ g/mL of heparin. The conditioned medium was collected after 18 h and subjected to analysis to determine the amount of VEGF secreted from the cells by enzyme-linked immunosorbent assay (Quantikine<sup>®</sup> human VEGF immuno-detection kit, R&D systems, Minneapolis, MN), following the manufacturer's instruction.

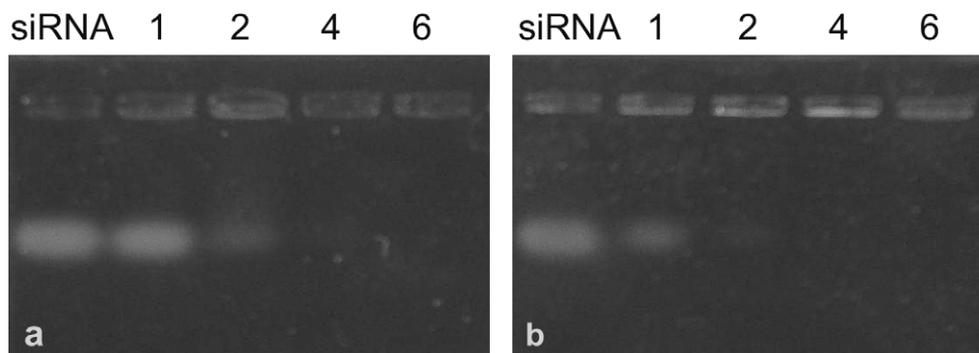
## 2.10. Confocal laser scanning microscopy

The simultaneous delivery of drug and gene was observed by confocal laser scanning microscope (LSM510, CARL-ZEISS INC., USA). For confocal microscopy observation, GFP siRNA was used to examine the GFP silencing and Nile red was used as a hydrophobic drug model. Firstly, MDA-MB-435 cells were seeded at a density of  $2.0 \times 10^5$  cells/well in two 4-well chamber slides overnight. The cells were transfected with Nile red-loaded micelle/siRNA complexes for 4 h at 37 °C in serum-free medium. One slide was taken out and washed with cold PBS four times, and the cells were fixed with 1% para-formaldehyde solution for 30 min at 4 °C. For the other slide, the culture medium was removed and replenished by fresh medium containing 10% FBS, and the cells were cultured for another 48 h. The slide was washed with PBS four times and fixed with para-formaldehyde solution. GFP inhibition and Nile red uptake by cells were imaged by confocal laser scanning microscope.

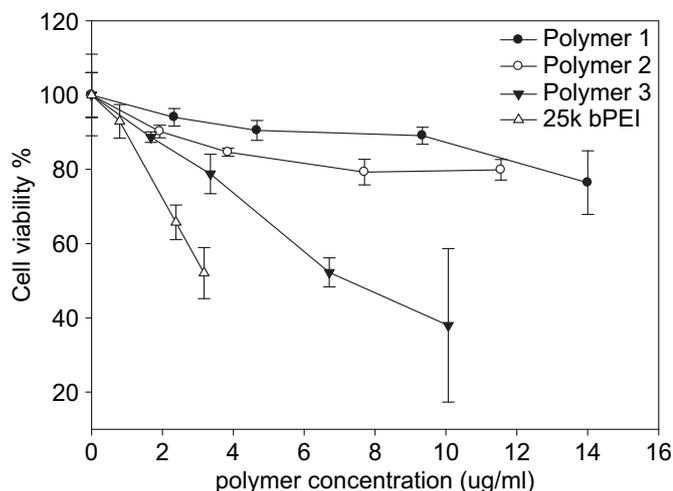
## 3. Results and discussion

### 3.1. Synthesis of PDMAEMA–PCL–PDMAEMA triblock copolymers

PDMAEMA–PCL–PDMAEMA triblock copolymers were prepared by reversible addition-fragmentation chain transfer (RAFT) polymerization of dimethylaminoethyl methacrylate (DMAEMA) using

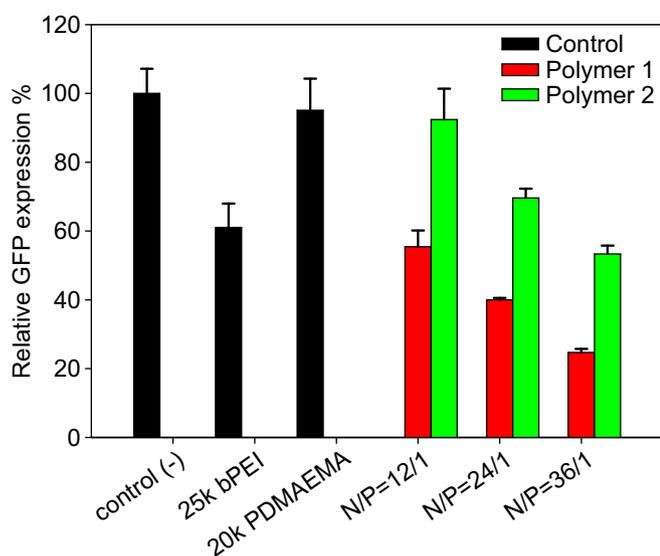


**Fig. 2.** Electrophoretic mobility of siRNA after complexation with PDMAEMA–PCL–PDMAEMA micelles at varying N/P ratios of 1, 2, 4 and 6. (a) micelle 1; (b) micelle 2.

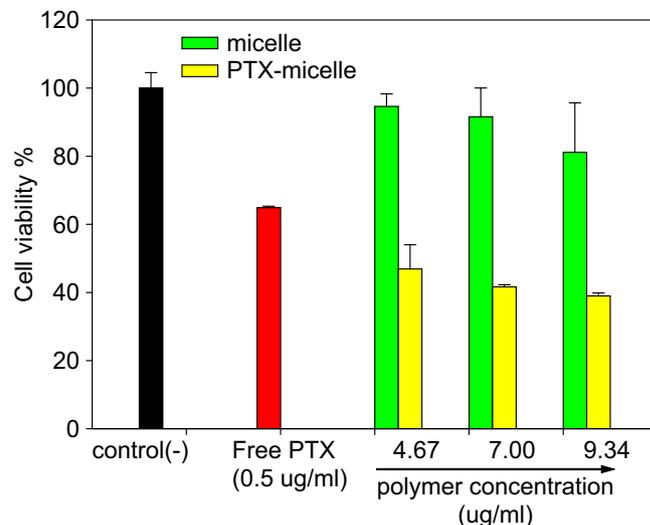


**Fig. 3.** Cytotoxicity of PDMAEMA-PCL-PDMAEMA micelles in MDA-MB-435-GFP cells. The cells were treated for 24 h and metabolic activity was measured by CCK assay. (mean  $\pm$  S. D.,  $n = 3$ ).

CPADN-PCL-CPADN (CPADN: 4-cyanopentanoic acid dithionaphthalenoate) as a macro-RAFT agent (Scheme 2). CPADN-PCL-CPADN was readily synthesized by coupling CPADN to dihydroxy PCL prepolymer (HO-PCL-OH) with a  $M_n$  of 3600 Da in the presence of DCC and catalytic amount of DMAP.  $^1\text{H}$  NMR showed complete disappearance of signals at  $\delta$  3.64 attributable to methylene protons neighboring to the hydroxyl end group of PCL prepolymer and a new set of signals at  $\delta$  7.49–8.14 and 2.44–2.67 assignable to CPADN (Fig. 1A and B). The integral ratio between resonances at  $\delta$  4.27 (ethylene protons in the middle of PCL prepolymer) and  $\delta$  1.99 (methyl protons of CPADN) was close to the theoretical value of 2:3, confirming successful synthesis of CPADN-PCL-CPADN. The polymerization of DMAEMA was performed in THF at 60 °C under nitrogen atmosphere for 24 h and the resulting block copolymers were isolated by precipitation from n-hexane. The results of polymerization are shown in Table 1.  $^1\text{H}$  NMR displayed clearly peaks

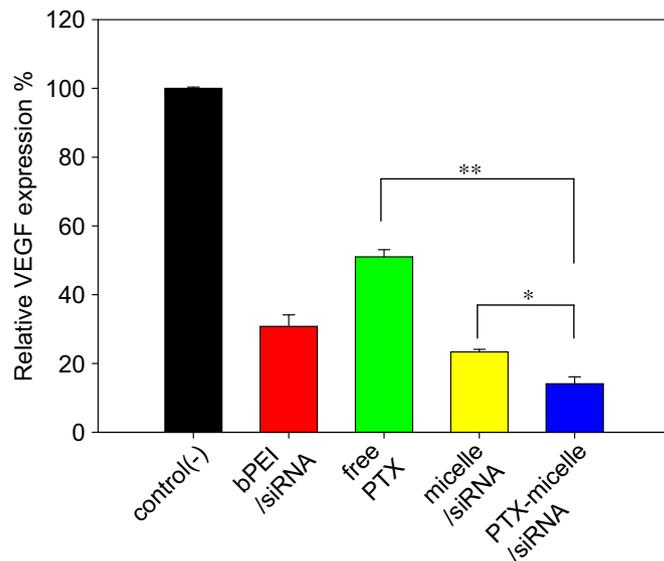


**Fig. 4.** Relative GFP silencing efficiency in GFP-expressed MDA-MB-435-GFP cells. The cells were incubated with siRNA complexes of micelles 1 and 2 at N/P ratios ranging from 12/1 to 36/1. 25 kDa bPEI at its optimal N/P ratios of 16/1 and 20 kDa PDMAEMA at an N/P ratio of 36/1 were used as positive controls. Dose of GFP siRNA was 0.5  $\mu\text{g}/\text{mL}$ . (mean  $\pm$  S.D.,  $n = 2$ ).



**Fig. 5.** Cytotoxicity of micelle 1 and PTX-loaded micelle 1 at different concentrations in PC3 cells. PTX-micelle had a drug loading content of 6.79 wt.%. The dose of free PTX was 0.5  $\mu\text{g}/\text{mL}$ , which had similar amount of PTX in PTX-loaded micelle 1 at a micelle concentration of 7.00  $\mu\text{g}/\text{mL}$ . The cells were treated for 24 h and metabolic activity was measured by CCK assay. (mean  $\pm$  S.D.,  $n = 3$ ).

characteristic of both PDMAEMA and PCL blocks (Fig. 1C). The  $M_n$  values of PDMAEMA could be estimated by comparing the intensities of signals at  $\delta$  4.07 and 2.56 which were attributable to the methylene protons neighboring to the ester bonds of PCL and PDMAEMA and the methylene protons next to amine group of PDMAEMA, respectively. Notably, results showed that these block copolymers had  $M_n$  PDMAEMA varying from 2700, 4800 to 9100 (denoted as polymer 1, 2 and 3, respectively), which correlated well with the monomer to CPADN-PCL-CPADN molar feed ratios (Table 1). Gel permeation chromatography (GPC) further showed that these block copolymers had controlled molecular weights with moderate polydispersities of 1.15–1.42 (Table 1). Hence, PDMAEMA-PCL-



**Fig. 6.** Relative gene silencing efficiency in PC3 cells using VEGF siRNA (dose: 0.5  $\mu\text{g}/\text{mL}$ ). The N/P ratio of micelle 1 or PTX-loaded micelle 1 (PTX-micelle) to VEGF siRNA was fixed at 24/1. PTX-micelle had a drug loading content of 6.79 wt.%. 25 kDa bPEI at its optimal N/P ratio of 16/1 was used as positive control. The dose of free PTX was 0.66  $\mu\text{g}/\text{mL}$ , which had similar amount of PTX in PTX-loaded micelle 1. (Student's *t*-test, \* $p < 0.05$ , \*\* $p < 0.01$ ).

PDMAEMA triblock copolymers with different molecular weights can be easily obtained by controlling monomer to CPADN–PCL–CPADN molar feed ratios.

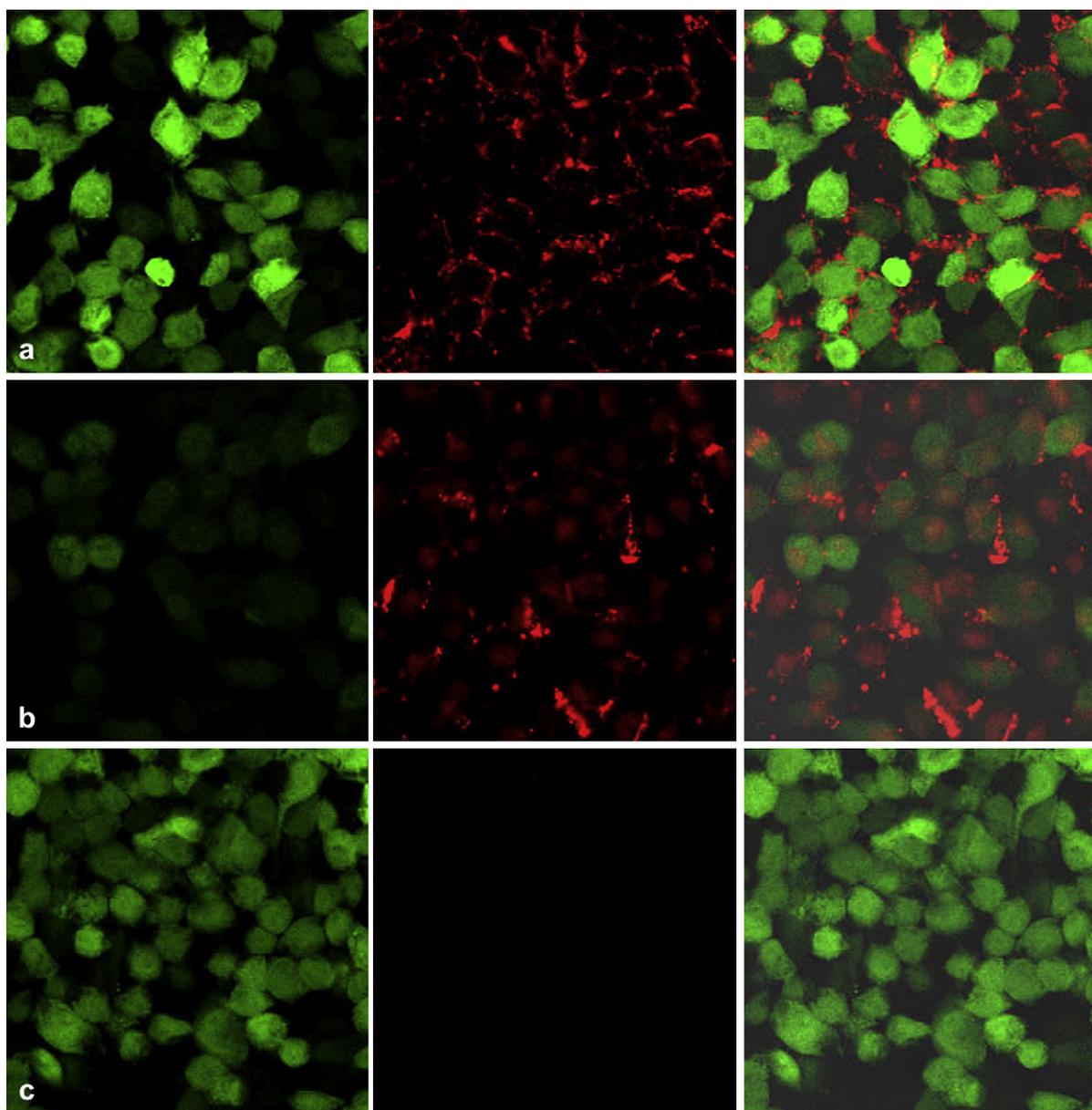
### 3.2. PDMAEMA–PCL–PDMAEMA micelles and siRNA complexation

PDMAEMA–PCL–PDMAEMA triblock copolymers were soluble in water to afford micelles with particle sizes ranging from 53.6 to 132.2 nm and positive surface charges ranging from +29.3 to +35.5 mV (Table 1). Notably, copolymer with the intermediate PDMAEMA (4.8 kDa) had the smallest particle size, and copolymer with the shortest PDMAEMA (2.7 kDa) revealed the highest surface charge. The critical micelle concentration (CMC) determined using pyrene as a fluorescence probe showed low CMC values of 15.8–24.0 mg/L (Table 1), which decreased with increasing PDMAEMA block length.

To investigate interactions of PDMAEMA–PCL–PDMAEMA micelles with siRNA, agarose gel electrophoresis studies were carried out at different N/P ratios (Fig. 2). The results showed that for micelle 1, significant interaction with siRNA was achieved starting from N/P ratio of 2/1 and complete complexation was observed at and above N/P ratio of 4/1. Micelle 2 showed further enhanced siRNA complexation ability, most likely due to its longer cationic PDMAEMA block as compared to micelle 1.

### 3.3. Cytotoxicity of PDMAEMA–PCL–PDMAEMA micelles

The cytotoxicity of PDMAEMA–PCL–PDMAEMA micelles was evaluated by CCK assay using MDA-MB-435-GFP cells. The cells were incubated with varying amount of micelles for 24 h. The commonly used polymeric transfer agent, 25 kDa bPEI, was used as a control. The results showed that all three micelles were less toxic than 25 kDa bPEI



**Fig. 7.** Confocal microscopic images of GFP-expressed MDA-MB-435-GFP cells treated with Nile red-loaded micelle 1/siRNA complexes at 37 °C. (a) cells incubated with Nile red-loaded micelle 1/siRNA complexes in serum-free media for 4 h; (b) cells incubated with Nile red-loaded micelle 1/siRNA complexes in serum-free media for 4 h, culture media was removed and replenished with serum media, and cells were cultured for another 48 h; (c) cells received no treatment (control).

and that the cytotoxicity of PDMAEMA–PCL–PDMAEMA micelles decreased with decreasing the molecular weights of PDMAEMA block (Fig. 3). It should be noted that both micelles **1** and **2** maintained over 80% cell viability at a concentration of 10  $\mu\text{g}/\text{mL}$ , indicating that these cationic micelles have low cytotoxicity. Furthermore, compared to 25 kDa bPEI, these triblock copolymers have low molecular weights and are biodegradable, which will result in largely decreased long-term toxicity. In the following studies, micelles **1** and **2** were selected for siRNA and paclitaxel delivery.

### 3.4. *In vitro* siRNA transfection

The siRNA transfection activities of PDMAEMA–PCL–PDMAEMA micelles were investigated using MDA-MB-435-GFP cells and GFP siRNA (GFP siRNA 0.5  $\mu\text{g}/\text{mL}$ ). The transfection was performed at different N/P ratios varying from 12/1 to 36/1, and cells were treated with siRNA complexes for 48 h. 25 kDa bPEI and 20 kDa PDMAEMA homopolymer were employed as positive controls. Remarkably, micelles showed largely enhanced siRNA transfection activity as compared to PDMAEMA homopolymer (Fig. 4). The silencing efficiency increased with increasing N/P ratios. It is interesting to note that micelle **1** though with shorter PDMAEMA block (2.7 kDa) displayed much higher siRNA transfection activity than micelle **2**, achieving over 70% silencing efficiency at an N/P ratio of 36/1. In contrast, 20 kDa PDMAEMA revealed practically no silencing effect at an N/P ratio of 36/1 under otherwise the same transfection conditions. The other often applied control, 25 kDa bPEI, showed only 40% silencing efficiency at its optimal N/P ratio of 16/1, which was much lower than micelle **1**. The largely enhanced siRNA transfection of PDMAEMA–PCL–PDMAEMA micelles as compared to PDMAEMA homopolymer is most likely due to their improved siRNA condensation and endosomal escaping ability.

### 3.5. CCK assay and *in vitro* delivery of paclitaxel

Micelles have been extensively investigated as nano-carrier systems for the delivery of potent hydrophobic anti-cancer drugs such as doxorubicin and paclitaxel (PTX) [32,33]. In this study, PTX was used as a model drug and loaded into micelle **1** with a drug loading content of 6.8 wt.%. The drug efficacy of PTX-loaded micelle was evaluated in PC3 cells by CCK assay. The cells were incubated with varying amount of PTX-loaded micelles for 24 h. Interestingly, PTX-loaded micelles demonstrated clearly higher drug efficacy as compared to free PTX (Fig. 5), which is most likely due to enhanced endocytosis of micelles.

### 3.6. Combinatorial delivery of VEGF siRNA and paclitaxel in PC3 cells

PDMAEMA–PCL–PDMAEMA micelles capable of efficiently delivering both siRNA and paclitaxel into cells are of particular interest for combinational cancer therapy. Herein, co-delivery of VEGF siRNA and paclitaxel in human prostate carcinoma PC-3 cells was investigated. VEGF has a predominant role in tumor angiogenesis [34,35]. VEGF siRNA has shown to effectively suppress neovascularization and tumor growth [36,37]. As for GFP siRNA, VEGF siRNA complexed with cationic micelles at an N/P ratio of 24/1 showed higher gene silencing efficiency in PC-3 cells than 25 kDa bPEI at its optimal formulation (Fig. 6). The combinatorial delivery of VEGF siRNA and paclitaxel using PDMAEMA–PCL–PDMAEMA micelles resulted in significantly lower VEGF expression as compared to delivery of VEGF siRNA alone, reaching a high silencing efficiency of ca. 85% (Fig. 6). The confocal microscopy studies using GFP siRNA and Nile red as a hydrophobic drug model in MDA-MB-435-GFP cells showed clearly that Nile red was transported inside cells and GFP protein expression was

significantly down-regulated (Fig. 7). These cationic micelles capable of efficiently co-delivering siRNA and hydrophobic anti-cancer drugs into cancer cells are highly promising for combination cancer therapy.

## 4. Conclusions

We have demonstrated that cationic biodegradable micelles based on low molecular weight PDMAEMA–PCL–PDMAEMA triblock copolymers mediate efficient *in vitro* siRNA transfection, affording a significantly higher gene knockdown efficiency than 25 kDa bPEI at its optimal N/P ratio and 20 kDa PDMAEMA homopolymer under otherwise the same conditions. Furthermore, we have also shown that these micelles are capable of efficiently delivering paclitaxel into cancer cells, resulting in an enhanced drug efficacy as compared to free paclitaxel. The co-delivery of VEGF siRNA and paclitaxel has revealed a further improved gene knockdown efficiency. Importantly, these micellar carriers are easy to prepare with controlled molecular characteristics, are biodegradable, and have low cytotoxicity. These biodegradable micellar carriers are promising in combination cancer therapy with therapeutic siRNA and chemotherapeutics.

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## Appendix

Figures with essential color discrimination. Figs. 1, 4–7 in this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at [doi:10.1016/j.biomaterials.2009.11.077](https://doi.org/10.1016/j.biomaterials.2009.11.077).

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