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Synthesis and Characterization of Star-Shaped Poly(ε-Caprolactone)-*b*-Poly(2-Dimethylaminoethyl Methacrylate) for Co-delivery of Plasmid DNA and Doxorubicin

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Abstract

A star-shaped poly(ε-caprolactone)-*b*-poly(dimethylaminoethyl methacrylate) copolymer (S-PCL-PDMAEMA) was synthesized and applied to co-deliver pDNA and doxorubicin (DOX) into cancer cells. A linear-shaped L-PCL-PDMAEMA was prepared for comparison. A star-shaped PCL homopolymer (S-PCL) was synthesized through a ring-opening reaction of ε-caprolactone with pentaerythritol, followed by brominating the end hydroxyl groups of S-PCL to yield S-PCL-Br. The S-PCL-PDMAEMA was obtained via atom transfer radical polymerization using DMAEMA as a monomer and S-PCL-Br as a macroinitiator. Similar numbers of repeating units of PCL and PDMAEMA were controlled between L-PCL-PDMAEMA and S-PCL-PDMAEMA. The star-shaped copolymer formed uniform nano-sized micelles in water with lower cytotoxicity than the linear one and PDMAEMA. The L-PCL-PDMAEMA and S-PCL-PDMAEMA effectively formed polyplexes with pDNA at a low N/P ratio. The DOX-loaded S-PCL-PDMAEMA micelles showed a better cell-killing effect than the DOX-loaded L-PCL-PDMAEMA in four cell lines. The co-delivery of DOX and pDNA was confirmed using a confocal laser scanning microscope. The S-PCL-PDMAEMA delivered the drugs into the nuclei of U87 cells for 3 h of incubation but the L-PCL-PDMAEMA accumulated most of them in the cytoplasm. This result demonstrated the cationic S-PCL-PDMAEMA micelles are a promising co-delivery system for therapeutic pDNA and hydrophobic anticancer drugs.

Keywords: Poly(ε-caprolactone), Poly(dimethylaminoethyl methacrylate), Doxorubicin, Co-delivery, Atom transfer radical polymerization

Introduction

Amphiphilic block copolymers consist of hydrophilic and hydrophobic segments and self-assemble into polymeric micelles, cylinders, vesicles, and lamellae.[1] Because of their intriguing properties, the amphiphilic block copolymers have been extensively applied in drug delivery systems, cosmetics, fragrances, flavor-masking, pesticides, pollution remediation, and colloid stabilization.[2, 3]

Chemotherapy is an important approach to treat various types of tumor in clinics, but it has disadvantages that need to be overcome such as drug-induced side effects and multidrug resistance (MDR).[4] In contrast, gene therapy exhibits low toxicity and high specificity; however, it has defects like rapid degradation in culture and in vivo, low cellular internalization because of a negatively charged surface, the type I interferon responses and the pro-inflammatory cytokines production.[5] Co-delivery of gene drugs and chemical drugs has a potential to efficaciously treat human diseases via their synergetic effects because of different treatment modalities. Construction of a highly efficient multifunctional drug carrier combining chemotherapy and gene therapy attracts many researchers' attention because this enhances the anti-tumor effects.

Poly(ε-caprolactone) (PCL) is an US Food and Drug Administration (FDA)-approved hydrophobic polymer. It is biodegradable and widely used in various biomedical applications because of its excellent biocompatibility and degradability.[6, 7] The alkyl segments of PCL are advantageous for drug delivery systems because they efficiently encapsulate hydrophobic compounds, slow degradation and provide a sustained release of drugs.[8] Poly(2-dimethylamino ethyl methacrylate) (PDMAEMA) is a temperature and pH sensitive polymer and has excellent biocompatibility.[9] The tert-amino groups of PDMAEMA form a cationic polyelectrolyte with DNA via electrovalent bonds. This benefits PDMAEMA as a gene delivery carrier.[10, 11]

The amphiphilic block copolymers based on PCL and PDMAEMA have been extensively studied for different architectures such as diblock,[12] triblock,[13] miktoarm star,[14] brush/graft[15, 16] and multi-arm star-shaped types.[17] A star-shaped poly(ε-caprolactone)-*b*-poly(2-(dimethylamino)ethyl methacrylate) (HPs-Star-PCL-*b*-PDMAEMA) has been synthesized by ring-opening polymerization and reversible addition–fragmentation chain transfer (RAFT) polymerization. The star copolymer had pH- and temperature-responsive properties and aspirin was used as a drug model to test its release behaviors from the star copolymer.[17] The change in morphology and size of the star-shaped PCL-PDMAEMA mixing with different weight ratios of mPEG-PCL has been studied as well.[18]

Most of the hydrophobic PCL modified with cationic polymers was developed to deliver gene drugs,[19-21] but a few co-deliver anticancer drugs and gene drugs simultaneously. Qiu et al. prepared polyethylenimine-*graft*- poly(ε-caprolactone) micelles as potential co-delivery carriers of anticancer drugs and genes. The authors demonstrated the co-delivery system shows a synergistic effect in conquering the MDR problem.[22] Amphiphilic methoxy-poly(ethylene glycol)-*b*-poly (ε-caprolactone)-*b*-poly(2-dimethylaminoethyl methacrylate) (MPEG-*b*-PCL-*b*-PDMAEMA) linear cationic nanoparticles were used as a co-delivery system for pDNA and paclitaxel,[23] and linear PDMAEMA-PCL-PDMAEMA triblock copolymers for siRNA and a paclitaxel to cancer cells,[24] showing synergistic/combined effects on cancer therapies.

Many star-shaped polymers based on PDMAEMA have been used for gene delivery.[25-30] Compared with linear amphiphilic block copolymers of similar composition and molecular weight, the star-shaped ones often exhibited distinct properties such as smaller hydrodynamic diameters, lower inherent viscosities, denser functional groups,[31, 32] and better gene transfection.[33] Since no one has used star-shaped PDMAEMA to co-deliver anticancer drugs and gene drugs and compared its performance with the linear one with a similar length of DMAEMA, thus, we synthesized a star-shaped four-arm PCL-*b*-PDMAEMA copolymer (S-PCL-PDMAEMA) by ring-opening polymerization (ROP) and atom

transfer radical polymerization (ATRP). A linear PCL-*b*-PDMAEMA copolymer (L-PCL-PDMAEMA) with similar numbers of repeating segment lengths of PCL and PDMAEMA was synthesized for comparison. The effects of polymer architecture on self assembling properties, DNA complex formation, in vitro transfection efficiency, and drug loading efficiency were studied. Doxorubicin (DOX) and pDNA were used as co-delivery drug models. The co-delivery of DOX and pDNA was confirmed using a confocal laser scanning microscope (CLSM). The chemotherapeutic effect of DOX and gene transfection efficiency of pDNA were studied and compared between the star and linear polymers as a drug delivery carrier.

Experimental section

Materials

2,2'- Bipyridine (Bpy), 2-bromo-2-methylpropionyl bromide, Amberlite® IR120, 2-(dimethylamino)ethyl methacrylate (DMAEMA), pyrene, ε-caprolactone (CL), and copper(I) bromide (CuBr) were purchased from Acros (Morris Plains, NJ). Aluminum oxide neutral (Al_2O_3) was from Seedchem Company PTY. LTD (Melbourne, Australia). Ethidium bromide (EtBr) was purchased from MP Biomedicals (Verona, Italy). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) was from Sigma-Aldrich (St. Louis, MO). The pGL3-control and luciferase assay kits were from Promega (Madison, WI). Trypsin-EDTA and fetal bovine serum (FBS) were from Biological Industries (Beit Haemek, Israel). Minimum Essential Medium (MEM) and Dulbecco's modified Eagle medium (DMEM) were from Invitrogen (Carlsbad, CA). All other unstated chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification.

Preparing star-shaped and linear-shaped poly(ε-caprolactone) (S-PCL and L-PCL)

Pentaerythritol (0.50 g, 3.68 mmol) and ε-caprolactone (16.76 g, 147.02 mmol) for S-PCL and methanol (0.1 mL, 2.47 mmol) and ε-caprolactone (10.95 g, 96.05 mmol) for L-PCL were separately introduced to a round-bottom flask. The flask was heated to 130 °C in an atmosphere of argon and 59 and 40 μL of stannous octoate ($Sn(Oct)_2$) was separately added into S-PCL and L-PCL. The reaction was continued for 3 h, and stopped by cooling with an ice batch. Twenty mL of dichloromethane was added to the reaction, and the product was precipitated in an excess of cold methanol. The obtained polymer was purified by three successive precipitations using dichloromethane as a solvent and methanol as a non-solvent and then dried under vacuum.

Brominating S-PCL and L-PCL

The S-PCL/L-PCL polymer (1.1 mmol, 1 equiv) was dissolved in 40 mL of anhydrous tetrahydrofuran (THF) in a round-bottom flask and degassed by three consecutive cycles of freeze-pump-thaw. Next, triethylamine (TEA, 8.8/2.2 mmol, 4/1 equiv) was added to the solution within 30 mins, and the solution was cooled to 0 °C. 2-Bromoisobutyryl bromide (8.8/2.2 mmol, 4/1 equiv) was added dropwise into the solution. The reaction was carried out under an argon atmosphere with stirring at room temperature for 48 h. The solution was precipitated in an excess of cold methanol and the white product was collected. The product was dissolved in dichloromethane and extracted with 0.1 M HCl to remove salts and TEA. Finally, the polymer (S-PCL-Br/L-PCL-Br) was precipitated in an excess of cold methanol and dried under vacuum.

Preparing S-PCL-PDMAEMA and L-PCL-PDMAEMA

S-PCL-Br/L-PCL-Br (200/200 mg) and DMAEMA (1.45/1.33 mL) was dissolved in 2 mL acetone in a dry round-bottom flask, and degassed by five consecutive cycles of freeze-pump-thaw. CuBr (13.3/12.0 mg), and Bpy (28.8/26.0 mg) were added into the solution. The polymerization was done with continuous stirring and re-fluxing at 40 °C for 8 h to produce S-PCL-PDMAEMA and at 60 °C for 6 h to produce L-PCL-PDMAEMA. The reaction solution was cooled down to room temperature and exposed to the air. The polymer was precipitated in an excess of cold hexane and dried in vacuum. The polymer solution was dissolved in dichloromethane and an acid cationic exchange resin (AMBERLITE IR-120) was added into the solution to remove Bpy. The solution was also passed through a column of aluminum oxide to remove the copper catalyst. Finally, the concentrated polymer solution was precipitated in excess cold hexane and the precipitate was collected and dried under reduced pressure.

Preparing L-PCL-PDMAEMA and S-PCL-PDMAEMA micelles and L-PCL-PDMAEMA/pDNA and S-PCL-PDMAEMA/pDNA polyplexes

PCL-PDMAEMA micelles were prepared by directly dispersing S-PCL-PDMAEMA or L-PCL-PDMAEMA in pH 3.0 buffer solutions at a concentration of 1.0 mg/mL with sonication for 30 min. The sample was collected by freeze-drying.

To prepare PCL-PDMAEMA/pDNA polyplexes, each PCL-PDMAEMA polymer was dispersed in double deionized (DD) water to a final concentration of 1 mg/mL at pH 6.0 to ensure the protonated amino groups of PDMAEMA. The pDNA concentration was fixed at 3 μg/100 μL in DD water to measure DNA binding ability and 4 μg/500 μL for other measurements. Equal volumes of S-PCL-PDMAEMA or L-PCL-PDMAEMA, and pDNA solutions were mixed and immediately vortexed at high speed for 60 s.

Characterization

The structures of copolymers were determined by ^{1}H NMR spectroscopy using a Varian Mercury plus-200 (200 MHz) Fourier-transform (FT) nuclear magnetic resonance (NMR) spectrometer in $CDCl_3$ at a concentration of 10 mg/mL. The molecular weight of copolymers were measured using gel permeation chromatography (GPC, Agilent 1100, Sata Clara, CA), composed of an Agilent G1310A pump, an Agilent refractive index detector, and a Shodex GPC KF-804 column. THF was used as a mobile phase at a flow rate of 1 mL/min at 45 °C. The column setting was calibrated using ten monodisperse polystyrene standards. Hydrodynamic diameters and zeta potentials were measured using laser Doppler anemometry with a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK). Light scattering measurements were done with a laser at 633 nm and a 90^{o} scattering angle. Polystyrene nanospheres (220 ± 6 nm and −50 mV, Duke Scientific, Palo Alto, CA) were used to verify

the performance of the instrument. The particle size and zeta potential of each sample was measured three times. The size and morphology were also observed using a transmission electron microscope (TEM, JEM-2000 EXII; JEOL, Japan). A carbon-coated 200-mesh copper specimen grid (Agar Scientific, Essex, UK) was glow-discharged for 1.5 min. Ten microliters of samples (1mg/mL) were deposited on a TEM grid and allowed to dry for one week at room temperature before examining the samples with the TEM. Pyrene was used as a fluorescent probe to determine the critical micelle concentration (CMC) of S-PCL-PDMAEMA and L-PCL-PDMAEMA. The polarity of the microenvironment surrounding pyrene was estimated by examining the III(383 nm)/I(373 nm) vibronic band ratio (I_3/I_1) of the fluorescence spectrum. The pyrene solution in acetone (3.08×10^{-5} M) was added in bottles, and then acetone was removed. Three milliliters of samples in DD water with various concentrations were added into the bottles containing pyrene. The final concentration of pyrene was 6.0×10^{-7} M. The solutions were kept at room temperature for 24 h. Excitation spectra were monitored at 336 nm, and emission spectra were recorded ranging from 350 to 450 nm. Both excitation and emission bandwidths were 8 nm.

Drug loading and releasing

Doxorubicin-loaded PCL-PDMAEMA micelles. Doxorubicin hydrochloride (DOX·HCl) was converted to a water-insoluble base form (DOX). Two milligram of DOX·HCl were dissolved in 1 mL of THF containing 18 mg of triethylamine (TEA) and vortexed for 1 min. The DOX solution was mixed with 1 mL of PCL-PDMAEMA solution in THF (10 mg/mL) and added dropwise to 10 mL DD water. The solution was sonicated for 30 mins to allow partitioning the drug into micelles. THF was evaporated under reduced pressure. The unloaded DOX was removed using a filter paper. The yield of the DOX-loaded micelle was > 95%. To evaluate the DOX loading efficiency, a dried sample was dissolved in DMSO and the absorbance was measured using a UV-Vis spectrometer at 485 nm.

Loading efficiency (LE, %) = (encapsulated drug weight/nanoparticle weight) x 100 (1)

In vitro *DOX release.* One mg of DOX-loaded micelles was dissolved in a tube containing 2.0 mL PBS (0.1 M, pH 7.4) and the tube was kept at 37 ^{o}C with 150 rpm stirring. At several time intervals, the upper solution was collected by centrifugation and the fresh buffer solution was refilled into the tube. One mL of the solution was withdrawn for UV-Vis analysis and the DOX concentration was calculated based on the absorbance at 485 nm.

Cell-line experiments

Cell culture. Non-small cell lung carcinoma cell lines (CRL-5802 and NCI-H358 cells), human breast adenocarcinoma cell line (MCF-7 cells) and human embryonic kidney cell line (293T cells) were cultivated at 37 °C under humidified 5% CO_2 in DMEM, and a human glioblastoma cell line (U87 cells) in MEM, supplemented with 10% FBS and 100 μg/mL penicillin-streptomycin. The medium was replenished every three days, and the cells were sub-cultured when they had reached confluence.

Cytotoxicity assay of micelles. Cells were seeded in 96-well tissue culture plates at a density of 5 × 10^3/well in medium containing 10% FBS. The cell viabilities of PCL-PDMAEMA micelles were determined after 24 h of incubation with various concentrations of micelles (1 - 100 μg/mL) using the tetrazolium-based colorimetric method.[34] Fifty μL/well of PBS containing MTT (2 mg/mL) was added into each well and incubated for 3 h. The solution was removed and 100μL/well DMSO was added. The number of active cells was estimated by measuring the absorbance at 540 nm.

Cytotoxicity assay of DOX-loaded micelles. The antiproliferative effect of DOX was analyzed using the MTT method. Cells were seeded in 96-well culture plates at a density of 5 × 10^3/well in medium containing 10% FBS. One day later, the experiments were performed with

increasing equivalent DOX concentrations of 0.2 - 20 μg/mL. After the cells had been post-incubated for 24 h, the DOX solutions were removed and washed with PBS. The cells were incubated for another 24 h in 100 μL fresh media. Following the incubation, 50 μL/well of PBS containing 2 mg/mL MTT were added, and the plates were incubated for additional 3 h. The solution was removed and 100 μL/well of DMSO were added. The number of active cells was estimated by measuring the absorbance at 540 nm.

Transfection efficiency. HEK 293T cells were seeded at a density of 1×10^5 /well in 12 well plates and incubated in DMEM containing 10% FBS for 24 h before transfection. Polyplexes with N/P ratios of 1, 3, 5, and 7 were prepared using different amounts of polymers and a fixed pDNA amount of 4 μg to a final volume of 500 μL. The polyplexes were left to stand for 30 min and added into the wells. After 3 h of incubation, the medium was replaced with 1 mL of fresh complete medium, and the cells were post-incubated for 72 h. The green fluorescence protein (GFP) expression was directly visualized using a fluorescence microscope (Zeiss Axiovert 200, Gottingen, Germany).

For the luciferase assay, the procedures stated above were repeated to determine the transfection efficiency of pGL3 plasmid in 293T cells cultured in DMEM. The transfection efficiencies of polyplexes were compared with naked DNA (as a negative control), Lipofectamine (a positive control). To quantify the luciferase expression, transfected cells were twice rinsed gently with 1 mL of 0.1 M PBS, added to a 200-μL/well of lysis buffer (0.1 M Tris-HCl, 2 mM EDTA, and 0.1% Triton X-100, pH 7.8), and allowed to stand overnight at –20 °C. The luciferase activity was monitored using a microplate scintillation and luminescence counter (Perkin-Elmer, NJ) after mixing the contents of a 50-μL/well of supernatant with the contents of 50-μL/well of luciferase assay reagent (Promega, Madison, WI). The total protein content of the cell lysate was examined using a BCA protein assay kit and done according to the manufacturer's instructions (Pierce Rockford, IL).

Confocal laser scanning microscope (CLSM). The intracellular delivery of pDNA and DOX was observed using CLSM. U87 cells were seeded at a density of 1.5×10^5/well in 12-well plates containing one glass coverslip in MEM supplemented with 10% FBS and incubated for 24 h. The polyplex was prepared at an N/P ratio of 5 of PCL-PDMAEMA with DOX and enhanced green fluorescence protein plasmid (pEGFP). The cells were exposed to the polyplex at 37 ^{o}C for 3 h. After 3 h of incubation, the medium was removed and the coverslips containing cells were washed with 1 mL of 0.1 M PBS at pH 7.4 and treated with 1 mL of Hoechest at 37 ^{o}C for 15 mins. Next, the coverslips were removed, washed gently 3 times with 1 mL of 0.1 M PBS, placed in a new empty well, and treated with 1 mL of 3.7% paraformaldehyde in 0.1 M PBS for 10 mins to fix the cells. The cells on the coverslips were washed 3 times with PBS and mounted with a fluorescent mounting medium on glass slides. CLSM (Fv 1000; Olympus, Tokyo, Japan) was used for cell imaging.

Results and discussion

Synthesizing star-shaped PCL-*b*-PDMAEMA copolymer (S-PCL-PDMAEMA)

The star-shaped poly(ε-caprolactone) homopolymer (S-PCL) was synthesized by ring-opening polymerization of CL with pentaerythritol at 130 ^{o}C in the presence of tin (II) octanoate ($SnOct_2$). The ^{1}H NMR spectrum of S-PCL shows the methylene (e) protons peaked at 4.05 ppm and the terminal methylene (e') at δ= 3.65 ppm (Figure 1). The degree of polymerization (DP) of S-PCL was calculated from the integration ratio of the repeated methylene protons at δ= 2.25 ppm (b) and the terminal ones (e') and the result was listed in Table 1. The DP of PCL segments in S-PCL of 35 (87.5% conversion) was controlled to close to that of L-PCL of 40 (100% conversion). PCL is a highly crystallized polymer and its crystallinity increases with increasing molecular weight. This fact slows the drug release.[35] Based on our previous study, the molecular weight of PCL ~ 2000 g/mol was good enough for self-assembled micelles when PCL was grafted onto chondroitin sulfate.[36] Thus, the DP of PCL ~ 40 was selected in this study.

The end hydroxyl groups of S-PCL and L-PCL were brominated with 2-bromoisobutyryl bromide in THF and products were precipitated in an excess of methanol with >90% yield. The ^{1}H NMR spectrum of S-PCL-Br was shown in Figure 1. The protons of the terminal methylene (e') shifted from δ= 3.65 to 4.19 ppm and a new peak attributable to methyl protons of 2-bromoisobutyryl group appeared at δ= 1.97 ppm (f). The degree of bromination of PCL-Br was close to ~ 100% in both the linear and star-shaped polymers. The number average molecular weights (M_n) measured by GPC were 4400 g/mol of S-PCL-Br, and 4800 g/mol of L-PCL-Br, respectively.

Several parameters were optimized to produce a similar DMAEMA segment length between S-PCL-Br and L-PCL-Br using an ATRP technique. We selected Bpy as a ligand, acetone as a solvent and did the reaction at 40 ^{o}C for 8 h to produce S-PCL-PDMAEMA. L-PCL-PDMAEMA was prepared

according to the synthesis of MPEG-PCL-PDMAEMA,[23] except acetone was used as a solvent instead of a bulk reaction. When toluene or N,N-dimethylformamide was chosen as a solvent and 1,1,4,7,7-pentamethyldiethylenetriamine (PMDETA) as a ligand, commonly used for linking DMAEMA with a polymer via ATRP,[23, 37] we obtained a long DMAEMA segment length (> 100) in S-PCL-PDMAEMA. However, the polydispersity of molecular weight was poor (>1.5, data not shown).

The ^{1}H NMR spectrum of S-PCL-PDMAEMA displayed the characteristic peaks of DMAEMA (Figure 1). The DP values of PDMAEMA were estimated from the peak intensities of the methylene protons of PCL (d, δ= 1.29 ppm) and the methylene protons next to the amino groups of PDMAEMA (j, δ= 2.56 ppm). The DP of PDMAEMA in S-PCL-PDMAEMA was 97, close to that of L-PCL-PDMAEMA (DP = 95). Although L-PCL-PDMAEMA and S-PCL-PDMAEMA had a moderate molecular weight distribution, the polydispersity of S-PCL-PDMAEMA (1.41) was worse than that of L-PCL-PDMAEMA (1.27) (Table 1, Figure S1). The increase in polydispersity of the star polymer is possibly because of star-star coupling reactions occurring at the stage of high conversion.[38]

S-PCL-PDMAEMA/pDNA and L-PCL-PDMAEMA/pDNA polyplexes

S-PCL-PDMAEMA and L-PCL-PDMAEMA micelles were prepared by directly dispersing S-PCL-PDMAEMA and L-PCL-PDMAEMA polymers in pH 3.0 solutions at a concentration of 1.0 mg/mL with sonication for 30 min. The hydrodynamic diameters of S-PCL-PDMAEMA and L-PCL-PDMAEMA were 160.0 ± 10.0 nm and 210.6 ± 1.3 nm, respectively. The star polymer formed a more compact structure than the linear one.

The zeta potentials of two PCL-PDMAEMA micelles were similar and close to ~30 mV (Table 2). A critical micelle concentration (CMC) was determined using pyrene as a fluorescence probe. The

CMC value of S-PCL-PDMAEMA was 8.01 × 10^{-3} mg/mL, smaller than 2.12 × 10^{-2} mg/mL of L-PCL-PDMAEMA (Figure S2).

The binding ability of S-PCL-PDMAEMA and L-PCL-PDMAEMA with pDNA was studied using an agarose gel electrophoresis retardation assay. The complete complexation of S-PCL-PDMAEMA/pEGFP was observed at an N/P $\geq$ 3 and that of L-PCL-PDMAEMA/pDNA was at an N/P $\geq$ 1 because no exposed pDNA was stained by EtBr (Figure 3). The different shapes of PCL-PDMAEMA slightly affected the binding ability with pDNA.

The hydrodynamic diameters and zeta potentials of PCL-PDMAEMA/pDNA at an N/P ratio of 5 were tabulated in Table 2 as well. At this N/P ratio, the hydrodynamic diameter of S-PCL-PDMAEMA/pEGFP was approximately half that of L-PCL-PDMAEMA/pEGFP. They were 128.4 ± 6.4 nm and 283.4 ± 15.4, respectively. Although the star polymer had a similar DP of DMAEMA with the linear polymer, however, the four arms structure would bring thinner cationic density than the linear one. The thinner cationic density might cause S-PCL-PDMAEMA to form smaller complexes with DNA. The particle sizes are preferred to be >10 nm to escape renal clearance and < 200 nm to prevent sequestration from the RES of the spleen and liver.[39] The S-PCL-PDMAEMA micelles are in the right size range to prevent sequestration by the RES.

When S-PCL-PDMAEMA formed a complex with pEGFP, the zeta potential decreased from 33.3 ± 4.1 to 14.9 ± 3.6 mV. This value was smaller than that of L-PCL-PDMAEMA/pEGFP (25.6 ± 0.9 mV), implying the star polymer had a higher ability to load the larger amount of negatively-charged pDNA, which neutralized the positively-charged S-PCL-PDMAEMA.

Cytotoxicity of S-PCL-PDMAEMA and L-PCL-PDMAEMA micelles

The cytotoxicities of S-PCL-PDMAEMA and L-PCL-PDMAEMA micelles were evaluated using an MTT assay and compared with those of PDMAEMA in 293T cells. The cytotoxicity of PDMAEMA dramatically increased when the concentration was > 20.0 μg/mL (Figure 4). The cell viability of PDMAEMA was > 90% at 10.0 μg/mL but decreased to < 40% at 25.0 μg/mL. In contrast, the cell viabilities of S-PCL-PDMAEMA and L-PCL-PDMAEMA slightly decreased at 25.0 μg/mL. The cell viability of S-PCL-PDMAEMA was higher than that of L-PCL-PDMAEMA. The dosage required to inhibit cell proliferation by 50% (IC_{50}) was 79.1 μg/mL for L-PCL-PDMAEMA but > 100 μg/mL for S-PCL-PDMAEMA. Indeed, the cytotoxicity was significantly affected by architecture of micelles. Compared the cytotoxicity of S-PCL-PDMAEMA with PDMAEMA-PCL-PDMAEMA at a similar molecular weight of PDMAEMA,[24] it seems the star polymer had lower cytotoxicity than the tri-block one.

Cytotoxicity of DOX/S-PCL-PDMAEMA and DOX/L-PCL-PDMAEMA

The hydrophobic anticancer drug, DOX, was used as a chemotherapeutic drug model. The drug was loaded into the micelles using an emulsion method. The loading amount of DOX into PCL-PDMAEMA micelles was determined based on a calibration curve of DOX in DMSO at 485 nm. The loading efficiencies (L.E.) of DOX/L-PCL-PDMAEMA and DOX/S-PCL-PDMAEMA were 15.8 ± 0.2 and 16.6 ± 0.2 % as the DOX feeding amount was controlled at 20%.

The release behaviors of DOX from DOX/L-PCL-PDMAEMA and DOX/S-PCL-PDMAEMA were done in pH 7.4 PBS at 37 °C. The final encapsulated DOX concentration was determined to check the mass balance. At 12 h, the total DOX release amounts from DOX/L-PCL-PDMAEMA and DOX/S-PCL-PDMAEMA micelles were 82.3% and 74.6%, respectively (Figure S3). The compact structure of S-PCL-PDMAEMA showed a good drug-sustained release behavior.

The anti-proliferation effect of DOX/PCL-PDMAEMA was tested in four cell lines, CRL-5802, NCI-H358, MCF-7, and 293T cells. The cells were incubated with equivalent DOX concentrations ranging 0.1 - 20 μg/mL for 24 h. The cytotoxicities of free DOX and DOX/PCL-PDMAEMA were all dose-dependent in the cell lines (Figure S4). The cell-killing effect of DOX/S-PCL-PDMAEMA was better than that of DOX/L-PCL-PDMAEMA against 293T, CRL-5802, MCF-7, and NCI-H358 cells (Figure 5). The IC_{50} values of DOX/S-PCL-PDMAEMA were 2.2, 1.4, 4.9 and 2.2 μg/mL for 293T, CRL-5802, MCF-7, and NCI-H358 cells, larger than those of free DOX (0.4, 0.9, 1.3 and 1.5 μg/mL), but much smaller than those of DOX/L-PCL-PDMAEMA (7.5, 6.6, 7.7 and 3.7 μg/mL), respectively. The impact of particle size on cellular uptake is widely reported.[40] The smaller particle size of S-PCL-PDMAEMA than L-PCL-PDMAEMA as well as the star structure[41] enhanced the cellular uptake of nanoparticles and increased the cell-killing effect when DOX was loaded.

In vitro gene transfection of S-PCL-PDMAEMA/pDNA and L-PCL-PDMAEMA/pDNA

The transfection efficiencies of PCL-PDMAEMA/pDNA polyplexes were assayed using two different plasmid DNAs, pEGFP-C1 for fluorescence measurement and pGL3-control for luminescence measurement. The relative green fluorescence expression of pEGFP-C1 was observed using a fluorescence microscope. The green fluorescence expression was clearly seen as the N/P ratio of PCL-PDMAEMA/pEGFP was ≥ 3 in both the PCL-PDMAEMA polyplexes (Figure 6). A similar green fluorescence expression effect with Lipofectamine was observed at the N/P ratio of 3 for S-PCL-PDMAEMA/pEGFP and 5 for L-PCL-PDMAEMA/pEGFP.

To quantitatively analyze the transfection efficiency of polyplexes, the amount of pGL3 expression was measured at different N/P ratios and compared with that of Lipofectamine. In Figure 7, the transfection efficiency of S-PCL-PDMAEMA/pGL3 and L-PCL-PDMAEMA/pGL3 increased with

increasing N/P ratios. At the same N/P ratio, S-PCL-PDMAEMA/pGL3 always showed higher transfection efficiency than did L-PCL-PDMAEMA/pGL3. The luciferase activity of pGL3 expression of S-PCL-PDMAEMA at N/P=5 was close to those of Lipofectamine and PEI at N/P=10. Nevertheless, the pGL3 expression of L-PCL-PDMAEMA at N/P=5 was approximately two orders lower than that of Lipofectamine. The higher gene expression found in S-PCL-PDMAEMA/pDNA might be because of the higher loading capacity of pDNA and protection of pDNA in the star-shaped architecture, agreeing with the previous report that star PDMAEMA vectors mediated a higher level of transfection than linear PDMAEMA precursors at the same weight ratio of polymer/pDNA.[33]

Co-delivery of pEGFP and DOX in U87 cells

The co-delivery effect of PCL-PDMAEMA was studied in U87 cells using pEGFP and DOX as model drugs. The cellular uptake and intracellular distribution of DOX and pEGFP in U87 cells were analyzed using confocal microscopic images after the cells had been incubated with DOX/PCL-PDMAEMA/pEGFP for 3 h. The CLSM result demonstrated DOX was transported inside U87 cells and pEGFP was highly expressed in both the polyplexes (Figure 8). The red fluorescence of DOX was clearly found in the nuclei of U87 cells incubated with DOX/S-PCL-PDMAEMA/pEGFP, but most of the DOX molecules were accumulated in the cytoplasm after the cells had been incubated with DOX/L-PCL-PDMAEMA/pEGFP for 3 h. The green fluorescence intensities of pEGFP for DOX/S-PCL-PDMAEMA/pEGFP and DOX/L-PCL-PDMAEMA/pEGFP in U87 cells were similar. The CLSM result reconfirmed DOX/S-PCL-PDMAEMA had better cell-killing effect against cancer cells.

Conclusion

We successfully prepared star-shaped S-PCL-PDMAEMA cationic micelles to co-deliver pDNA and DOX to cancer cells. Both S-PCL-PDMAEMA and S-PCL-PDMAEMA formed stable complexes with pDNA when the N/P ratio was $\geq$ 3. The S-PCL-PDMAEMA/pDNA polyplex showed similar transfection efficiency with Lipofectamine as the N/P ratio was $\geq$ 5. The DOX-loaded S-PCL-PDMAEMA micelles had higher drug loading ability and more efficiently chemotherapeutic effect than did the DOX-loaded L-PCL-PDMAEMA in four cancer cell lines. The CLSM result demonstrated S-PCL-PDMAEMA delivered the DOX molecules and pEGFP to the nuclei. The star-shaped S-PCL-PDMAEMA seems a promising co-delivery carrier of hydrophobic anticancer agents and therapeutic pDNAs for combinational therapy.

Acknowledgements

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Figure caption

Scheme 1. Synthesis of four-arm S-PCL-PDMAEMA copolymers.

Figure 1. ^{1}H NMR spectra ($CDCl_3$, 200 MHz) of S-PCL; S-PCL-Br and S-PCL-PDMAEMA.

Figure 2. Transmission electron microscope (TEM) images of S-PCL-PDMAEMA and L-PCL-PDMAEMA.

Figure 3. Electrophoretic mobility of pDNA complexed with S-PCL-PDMAEMA or L-PCL-PDMAEMA at N/P ratios of 1, 3, 5 and 7.

Figure 4. Cell viabilities of PDMAEMA, L-PCL-PDMAEMA and S-PCL-PDMAEMA in 293T cells. The cells were treated with the polymers for 24 h and metabolic activity was measured by MTT. (n = 3).

Figure 5. IC_{50} of 293T, CRL-5802, MCF-7, and NCI-H358 cells incubated with free DOX, DOX/S-PCL-PDMAEMA, and DOX/L-PCL-PDMAEMA for 24 h.

Figure 6. Images of green fluorescent pEGFP expression in 293T cells as a function of N/P ratio.

Figure 7. Luciferase activity of pGL3-Control expression in 293T cells normalized with protein amounts in the presence of 10% FBS (n = 3).

Figure 8. CLSM images of U87 cells incubated with DOX/L-PCL-PDMAEMA/pEGFP and DOX/S-PCL-PDMAEMA/pEGFP at 37 °C for 3 h.

Table 1. The properties of S-PCL-Br, L-PCL-Br, S-PCL-PDMAEMA, L-PCL-PDMAEMA

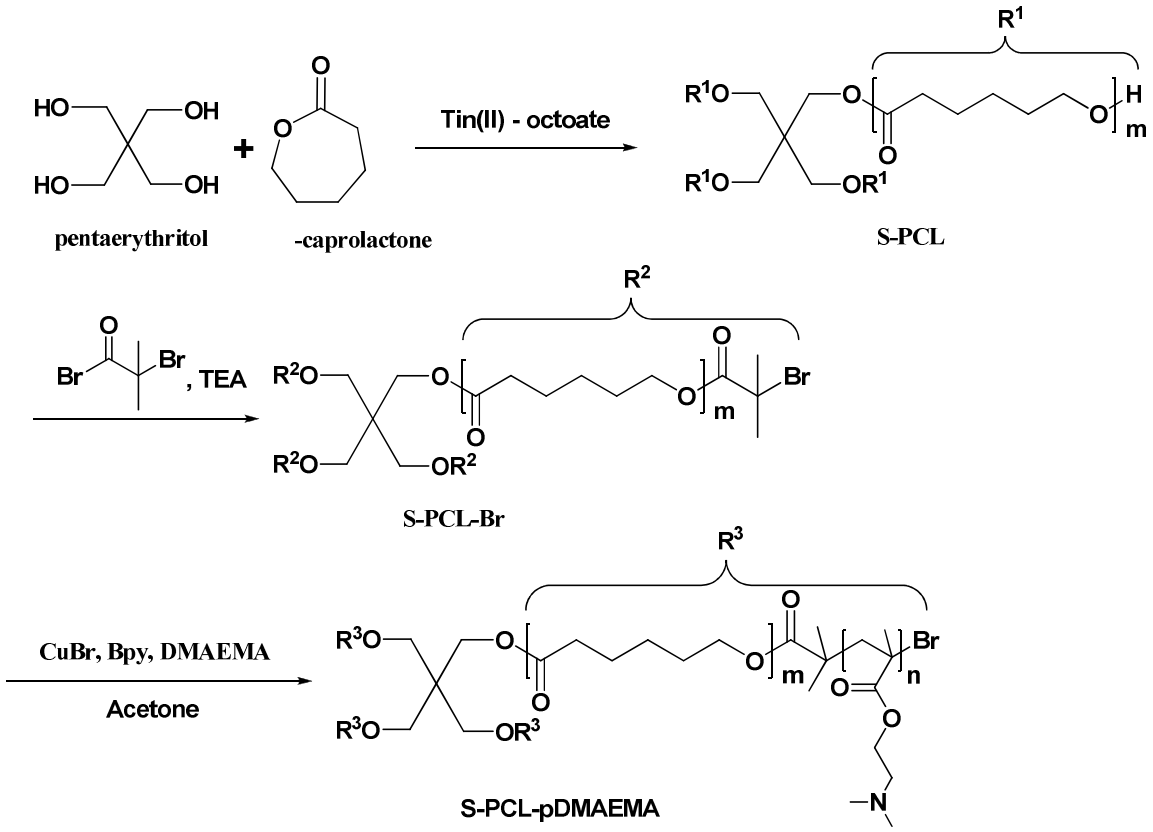
	DP_{CL}[a]	M_n (g/mol)[a]	M_n (g/mol)[b]	DP_{DMAEMA}	Conversion (%)	DPI[b]
S-PCL	35	4000	4100			1.27
L-PCL	40	4700	4600			1.27
S-PCL-Br	35	4600	4400			1.27
L-PCL-Br	40	4850	4800			1.27
S-PCL-PDMAEMA	35	19800	33500	97[a], 185[b]	65[a], 100[b]	1.41
L-PCL-PDMAEMA	40	19800	23500	95[a], 119[b]	63[a], 80[b]	1.27

a) Determined by ^{1}H NMR
b) Determined by GPC

Table 2. Hydrodynamic diameters (D_h), zeta potentials (V) and DOX loading efficiency (LE) of L-PCL-PDMAEMA and S-PCL-PDMAEMA micelles, and hydrodynamic diameters and zeta potentials of PCL-PDMAEMA/pDNA polyplex at an N/P ratio of 5.

	D_h (nm)[a]	PDI	V (mV)	LE
L-PCL-PDMAEMA	210.6 ± 1.3	0.24 ± 0.01	31.3 ± 2.1	15.8 ± 0.2
S-PCL-PDMAEMA	160.0 ± 10.0	0.27 ± 0.10	33.3 ± 4.1	16.6 ± 0.2
L-PCL-PDMAEMA/pEGFP (N/P= 5)	283.4 ± 15.4	0.58 ± 0.03	25.6 ± 0.9	
S-PCL-PDMAEMA/pEGFP (N/P= 5)	128.4 ± 6.4	0.29 ± 0.03	14.9 ± 3.6	

[a] Intensity percentage measured by DLS



Scheme 1

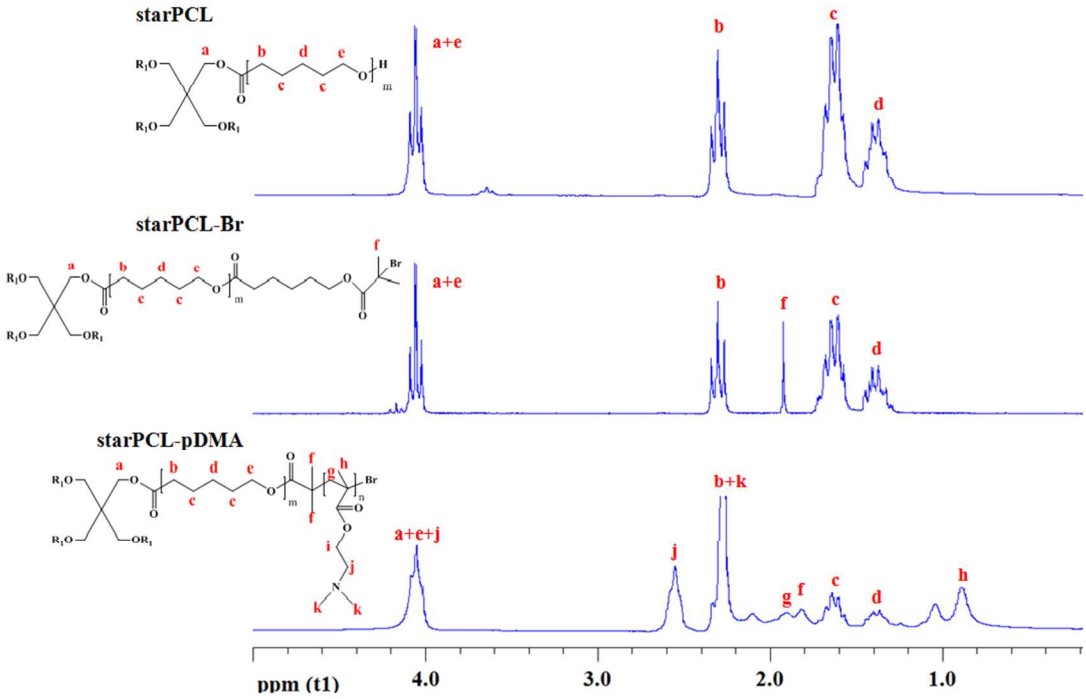
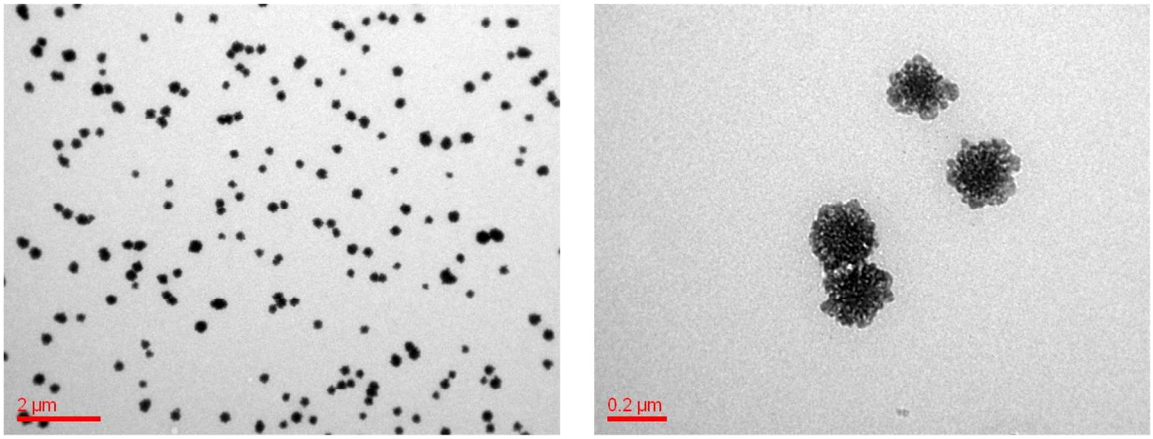


Figure 1

S-PCL-pDMAEMA



L-PCL-pDMAEMA

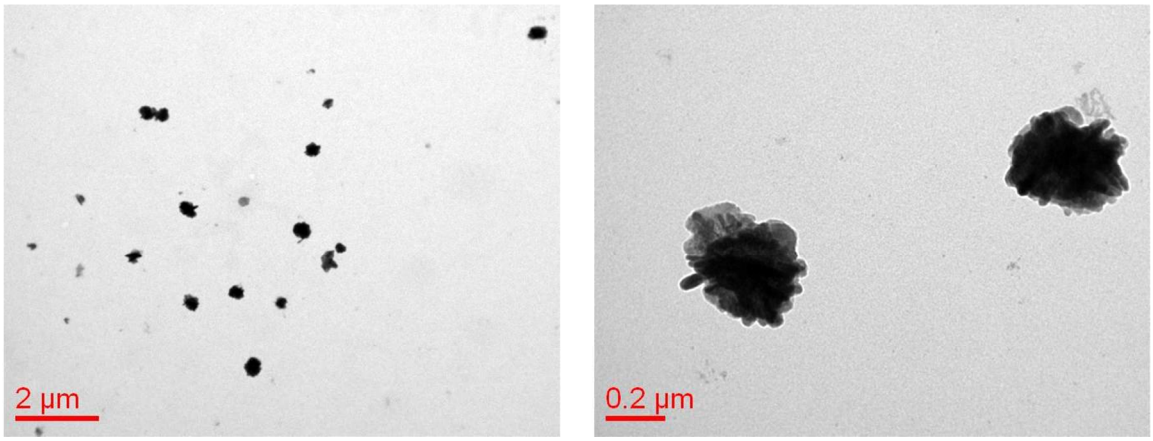


Figure 2

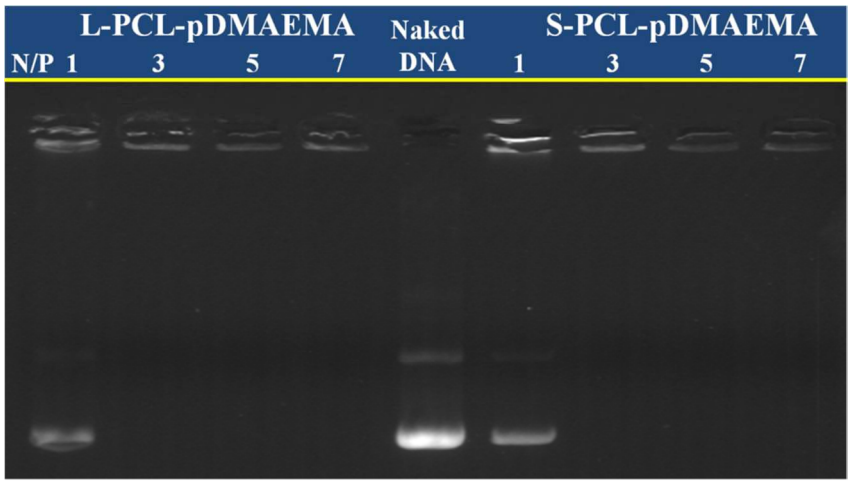


Figure 3.

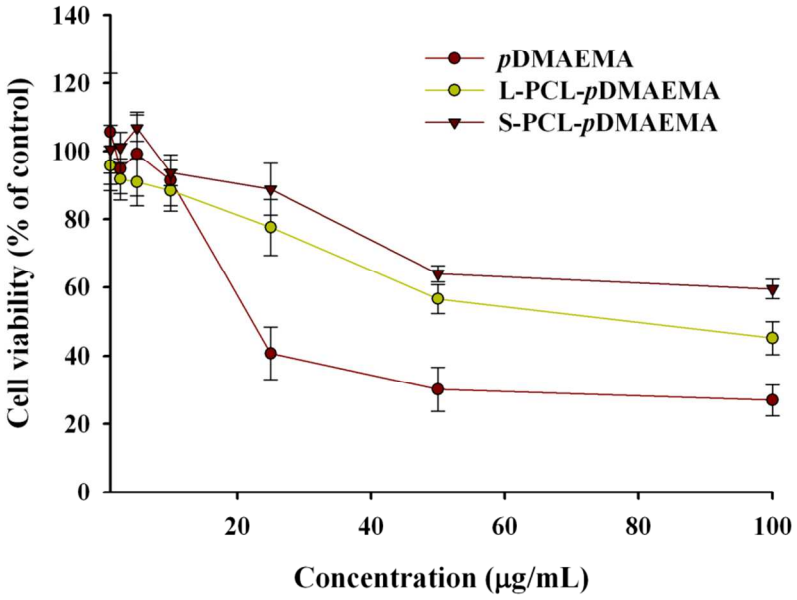


Figure 4

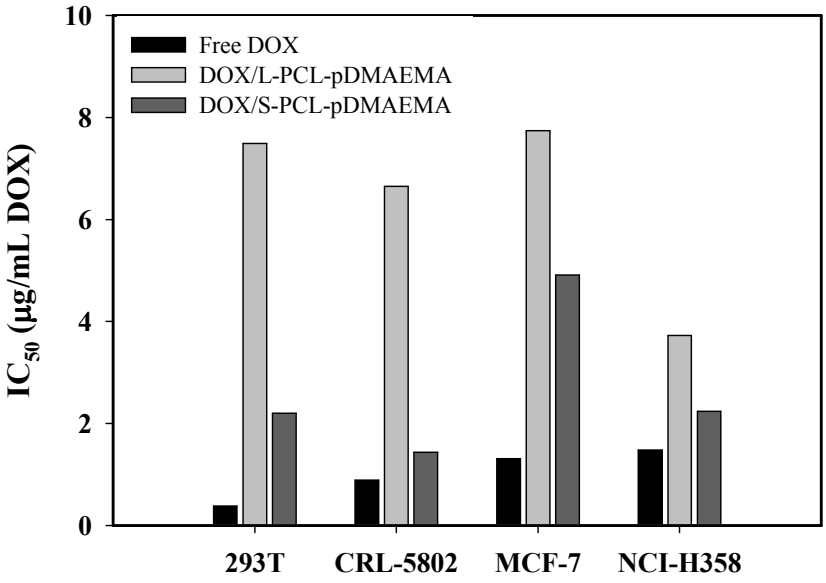


Figure 5

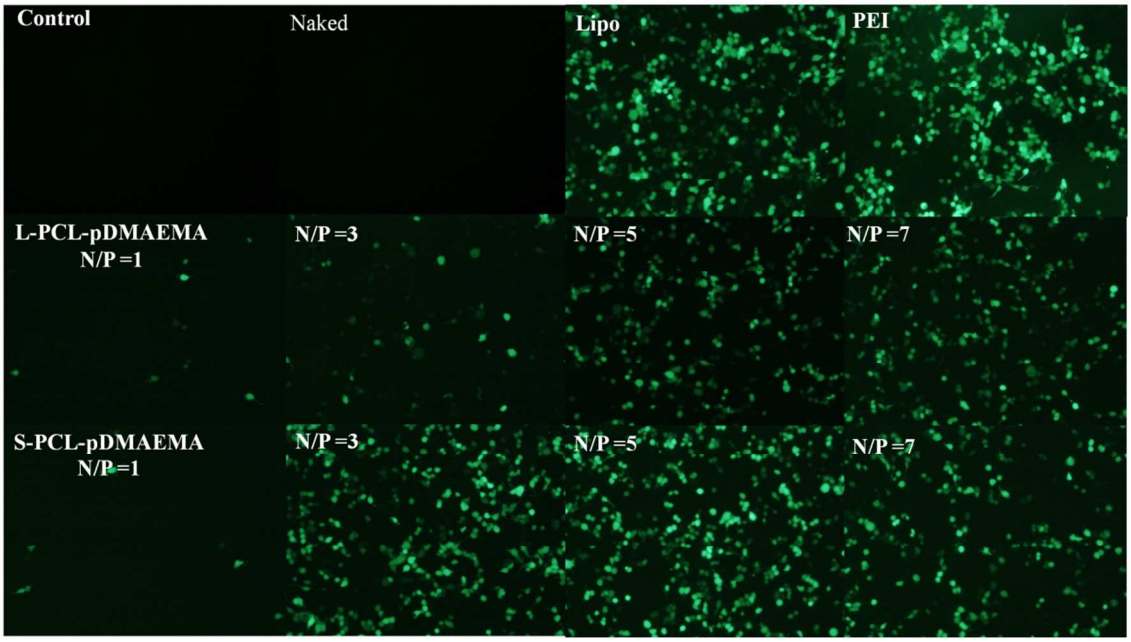


Figure 6

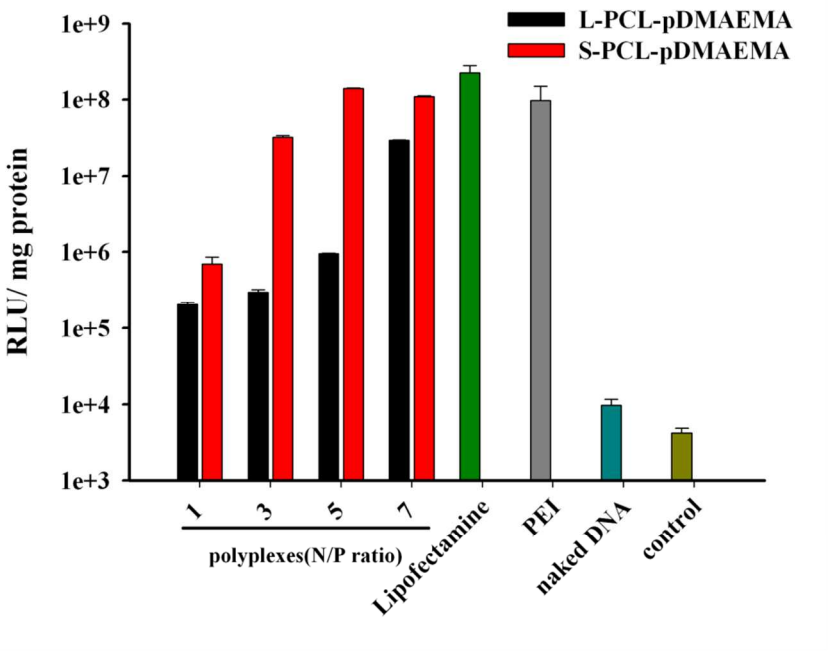


Figure 7

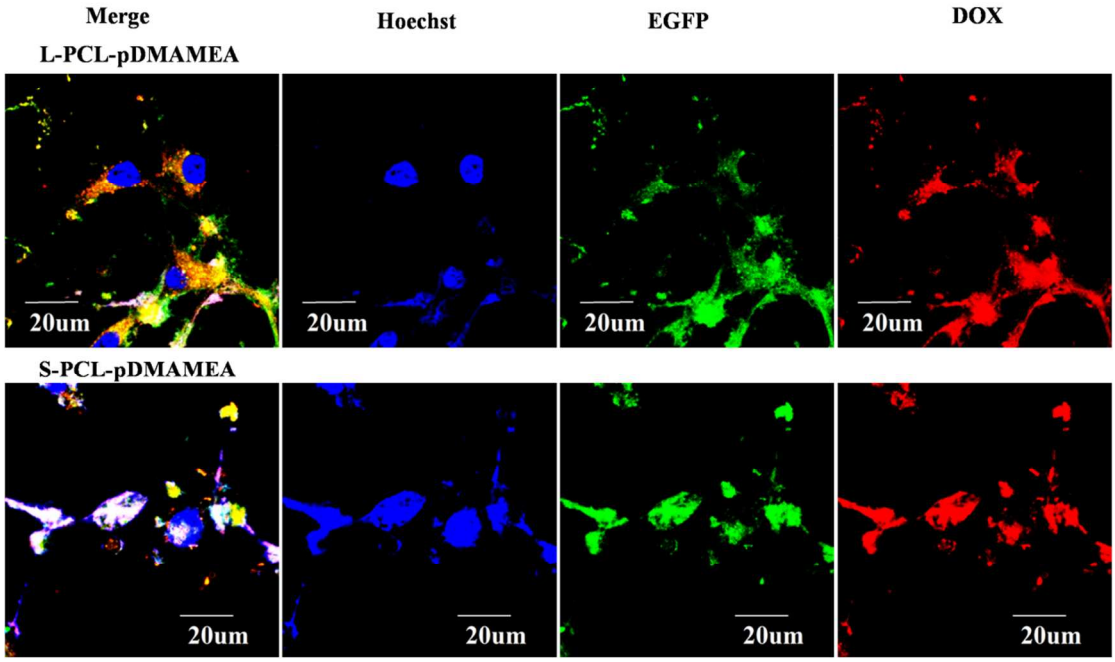


Figure 8