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The highly efficient delivery of exogenous proteins into cells mediated by biodegradable chimaeric polymersomes

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ABSTRACT

Biodegradable chimaeric polymersomes based on asymmetric PEG-PCL-PDEA triblock copolymers were prepared and investigated for delivery of exogenous proteins into cells. PEG-PCL-PDEA copolymers with $M_{n\ PEG}$ = 5 kg/mol, $M_{n\ PCL}$ = 18.2 kg/mol, and short PDEA blocks ranging from 1.1, 2.7 to 4.1 kg/mol (denoted as copolymer **1**, **2** and **3**, respectively) were obtained by controlled reversible addition-fragmentation chain transfer (RAFT) polymerization. The direct hydration of copolymer thin films in MES buffer (pH 5.3) yielded uniform polymersomes with sizes of 130–175 nm. These polymersomes had close to neutral zeta potentials (−2 ~ +2.7 mV) at pH 7.4. The polymersomal structures were confirmed by confocal laser scanning microscopy (CLSM), transmission electron microscopy (TEM), and catalytic activity experiment on 3,3′,3″-phosphinidyne(trisbenzenesulfonic acid)-loaded polymersomes. MTT assays showed that these polymersomes were non-toxic up to a concentration of 0.5 mg/mL. These chimaeric polymersomes, in particular polymersome **2**, showed remarkably high protein loading efficiencies and loading contents for bovine serum albumin (BSA), cytochrome C (CC), lysozyme (Lys), ovalbumin (OVA) and immunoglobulin G (IgG). The encapsulation of proteins did not significantly alter the polymersome size distributions and zeta potentials. The protein release studies showed that both BSA and CC were released in a controlled manner. Importantly, the released CC fully maintained its activity. Notably, CLSM studies showed that FITC-CC loaded polymersomes efficiently delivered and released proteins into the cytoplasm of RAW 264.7 cells. Moreover, these chimaeric polymersomes were able to simultaneously load and transport proteins and doxorubicin into the cytoplasm as well as the cell nucleus. We are convinced that these biodegradable chimaeric polymersomes have great potentials in protein therapy.

1. Introduction

Protein drugs have emerged as potent medicines for various types of human diseases such as autoimmune diseases, certain cardiovascular and metabolic disorders, and cancers [1–4]. To elicit therapeutic effects, many proteins (e.g. BAX, cytochrome C, caspase-3, etc.) have to be directed to a proper cellular compartment like the cytoplasm [5,6]. Protein-based biotherapeutics encounter several challenges such as rapid elimination from the circulation, poor bioavailability, low cell permeability, and inefficient endosomal escape [7]. The clinical success of protein drugs is critically dependent on the advancement of non-toxic, efficient and economically viable delivery systems. In the past decades, hydrogels [8,9], microparticles [10,11], liposomes [12], polyion complex micelles [13], and carbon nanotubes [14] have been investigated as carriers for controlled protein delivery. These delivery approaches, however, require the use of organic solvents which may possibly lead to protein denaturation, involve chemical modifications of proteins, have low protein loading levels, and/or are not applicable for systemic applications.

Polymersomes are polymeric vesicles made of amphiphilic block copolymers [15–18]. Like liposomes, polymersomes contain an aqueous interior that is separated from the outer fluids by hydrophobic membranes. In the past decade, polymersomes, either degradable or non-degradable, with better stability and easier to modify as compared to liposomes have been explored for programmed delivery of hydrophobic and hydrophilic drugs [19,20]. Notably, several biodegradable polymersomes have been developed [21–24]. Recently, several groups have studied the encapsulation of proteins including myoglobin and TNF-α into polymersomes

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[25–27]. However, due to inefficient loading the protein contents were low.

In this paper, we report on biodegradable chimaeric polymersomes based on asymmetric poly(ethylene glycol)-*b*-poly(ϵ-caprolactone)-*b*-poly(2-(diethylamino) ethyl methacrylate) (PEG-PCL-PDEA) triblock copolymers for highly efficient encapsulation and delivery of exogenous proteins into cells (Scheme 1). These chimaeric polymersomes were designed on the basis of the following reasons: (1) the PEG block ($M_n = 5$ kg/mol), which is longer than the PDEA block ($M_n = 1.1$–4.1 kg/mol), will be preferentially oriented at the polymersome outlayer, thereby offering excellent biocompatibility and stability in the circulation; (2) the shorter cationic PDEA block will be preferentially located inside the polymersomes, which on one hand facilitates efficient encapsulation and stabilization of proteins and on the other hand may assist polymersomes escaping from endosomes via the "proton sponge effect" [28], resulting in efficient cytoplasmic delivery of proteins; (3) the hydrophobic PCL block is non-toxic and biodegradable; and (4) these chimaeric polymersomes may be capable of simultaneously delivering proteins and hydrophobic anticancer drugs such as doxorubicin (DOX). The combination cancer therapy has shown tremendous potentials in cancer treatment [29–32].

2. Materials and methods

2.1. Materials

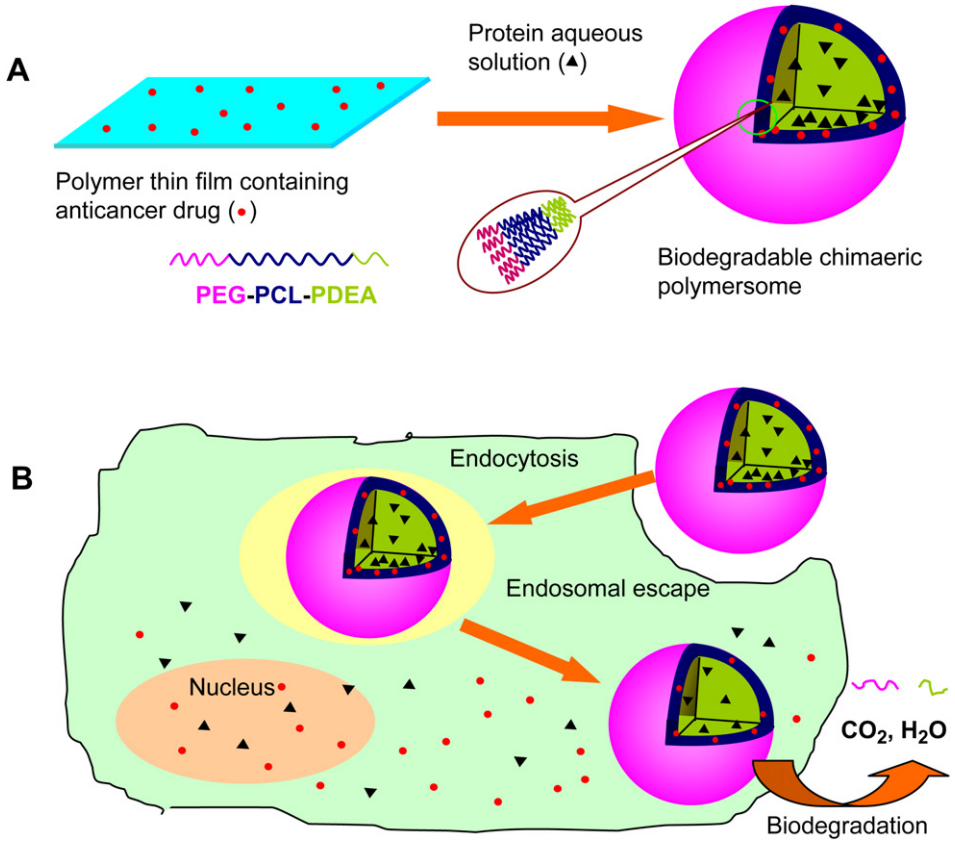
2-(Diethylamino)ethyl methacrylate) (DEA, 99%, Aldrich) was purified by passing through a basic alumina column. Poly(ethylene glycol) monomethyl ether (CH_3O-PEG, $M_n = 5000$, Fluka) was dried by azeotropic distillation from dry toluene. Azobisisobutyronitrile (AIBN, 98%, J&K) was recrystallized twice from methanol. ϵ-Caprolactone (ϵ-CL, 99%, Alfa Aesar) and dioxane were dried over CaH_2 and distilled under reduced pressure prior to use. Toluene and tetrahydrofuran (THF) were dried by refluxing over sodium wire and distilled prior to use. Stannous octoate (95%, Sigma), nile red (99%, Sigma), fluorescein isothiocyanate (95%, Fluka), pyrene (97%, Fluka), cytochrome C from equine heart (Sigma), bovine serum albumin V fraction (>98%, Roche), ovalbumin (Sigma), γ-IgG from bovine (Sigma), lysozyme (Amresco), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)diammonium salt (ABTS, Amresco), 3,3′,3″-phosphinidynetris-benzenesulfonic acid (PH, 95%, J&K), 5,5′-dithiobis(2-nitrobenzonic acid) (DTNB, 99%, Alfa Aesar), dicyclohexyl carbodiimide (DCC, 99%, Alfa Aesar), 4-(dimethylamino) pyridine (DMAP, 99%, Alfa Aesar) and doxorubicin hydrochloride (99%, Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd.) were used as received. 4-cyanopentanoic acid dithiobenzoate (CPADN) was synthesized according to literature [33].

2.2. Characterization

^{1}H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using $CDCl_3$ or D_2O as solvent. The chemical shifts were calibrated against residual solvent signals of $CDCl_3$ or D_2O. The molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with two linear PLgel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector. The measurements were performed using THF as the eluent at a flow rate of 1.0 mL/min at 30 °C and a series of narrow polystyrene standards for the calibration of the columns. The size of polymersomes was determined using dynamic light scattering (DLS). Measurements were carried out at 25 °C using a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He–Ne laser using back-scattering detection. The zeta potential of the polymersomes was determined with a Zetasizer Nano-ZS from Malvern Instruments. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 μL of 0.1 mg/mL polymersome suspension on the copper grid followed by staining with phosphotungstic acid (1 wt.%).

2.3. Synthesis of PEG-PCL-CPADN

The macro-RAFT agent was synthesized by coupling 4-cyanopentanoic acid dithiobenzoate (CPADN) to PEG-PCL-OH. PEG-PCL-OH was prepared by ring-opening polymerization of ϵ-caprolactone (3.79 g, 0.026 mol) using monomethoxy



Scheme 1. Schematic presentation of biodegradable chimaeric polymersomes. (A) PEG-PCL-PDEA forms nano-sized polymersomes directly in aqueous solution via the film rehydration method; both proteins and DOX can be readily loaded into polymersomes with high loading efficiency; (B) these polymersomes after being taken up by cancer cells, can escape from endosomes through the buffer effect of PDEA; proteins and DOX are released into the cytoplasm and/or cell nucleus.

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PEG (1.05 g, 0.21 mmol) as an initiator and stannous octoate (0.03 g, 0.074 mmol) as a catalyst at 100 °C in toluene (20 mL). The polymerization mixture was cooled to r.t. after 24 h reaction, and an aliquot was taken to determine the monomer conversion and the molecular weight of the resulting copolymer by ^{1}H NMR measurements. The monomer conversion was close to 100%. M_n (^{1}H NMR): 23.2 kg/mol.

To the rest of the reaction mixture was added a CH_2Cl_2 solution (30 mL) of CPADN (0.138 g, 0.42 mmol) and a catalytic amount of DMAP (2.56 mg, 0.021 mmol) and a CH_2Cl_2 solution (10 mL) of DCC (0.058 g, 0.28 mmol). The reaction was carried out for 48 h. The resulting macro-RAFT agent, PEG-PCL-CPADN, was isolated by filtration to remove insoluble by-products, concentration by rotary evaporation, precipitation in cold diethyl ether, filtration and drying in vacuo for 2 days. Yield: 67%. ^{1}H NMR (400 MHz, $CDCl_3$): PEG (δ 3.38, CH_3O; δ 3.65, CH_2CH_2O), PCL (δ 4.05 (CH_2CH_2OCO), 2.28 (CH_2CH_2COO), 1.62 ($CH_2CH_2CH_2CH_2CH_2OCO$), and 1.34 ($CH_2CH_2CH_2CH_2OCO$)), and naphthalene ring (δ7.52–8.15). M_n (^{1}H NMR) = 23.2 kg/mol, PDI (GPC) = 1.24. The CPADN substitution is close to 100% as calculated from the NMR integration ratio of the naphthalene and PEG methoxy peaks.

2.4. Synthesis of PEG-PCL-PDEA triblock copolymers

The synthesis of PEG-PCL-PDEA (2.7 kDa) is given as an example. To a Schlenk bottle equipped with a magnetic stir bar were charged DEA (0.065 g, 0.35 mmol), AIBN (0.7 mg, 4.3 μmol), PEG-PCL-CPADN (0.5 g, 0.022 mmol) and THF (3.5 mL). After 30 min degassing with nitrogen flow, the reaction vessel was sealed and immersed in an oil bath thermostated at 70 °C. The polymerization was allowed to proceed for 24 h. The resulting copolymer was isolated by precipitation in cold diethyl ether, filtration and drying in vacuo. Yield: 68%. ^{1}H NMR (400 MHz, $CDCl_3$): PEG (δ 3.65, 3.38), PCL (δ 4.05, 2.28, 1.62, and 1.34), and PDEA (δ 0.88 (methyl, $CHCCH_3CO$), δ 1.03 (methyl, $N(CH_2CH_3)_2$), δ 2.56 (methylene, $N(CH_2CH_3)_2$), δ 2.70 (methylene, OCH_2CH_2N), δ 4.03 (methylene, OCH_2CH_2N)). M_n (^{1}H NMR) = 25.9 kg/mol; PDI (GPC) = 1.43.

2.5. Formation of polymersomes via the film hydration method

A copolymer solution in chloroform (0.5 mg/mL, 1 mL) in a pear shaped flask was slowly evaporated on a rotary evaporator to form a thin film, which was further dried under high vacuum (0.3 mbar) for 3–6 h applying a nitrogen flush every hour. After adding 1 mL of MES buffer (20 mM, pH 5.4) or protein solution in MES buffer (20 mM, pH 5.4) to the dry film, the system was first sonicated for 3 min at room temperature, and then stirred at 60 °C for 12 h. The size and size distribution of the formed polymersomes were determined by DLS. The average sizes of polymersomes **1, 2,** and **3** were between 125 and 175 nm, and the average size of PEG-PCL polymersomes was 235 nm.

2.6. Critical aggregation concentration (CAC) determination

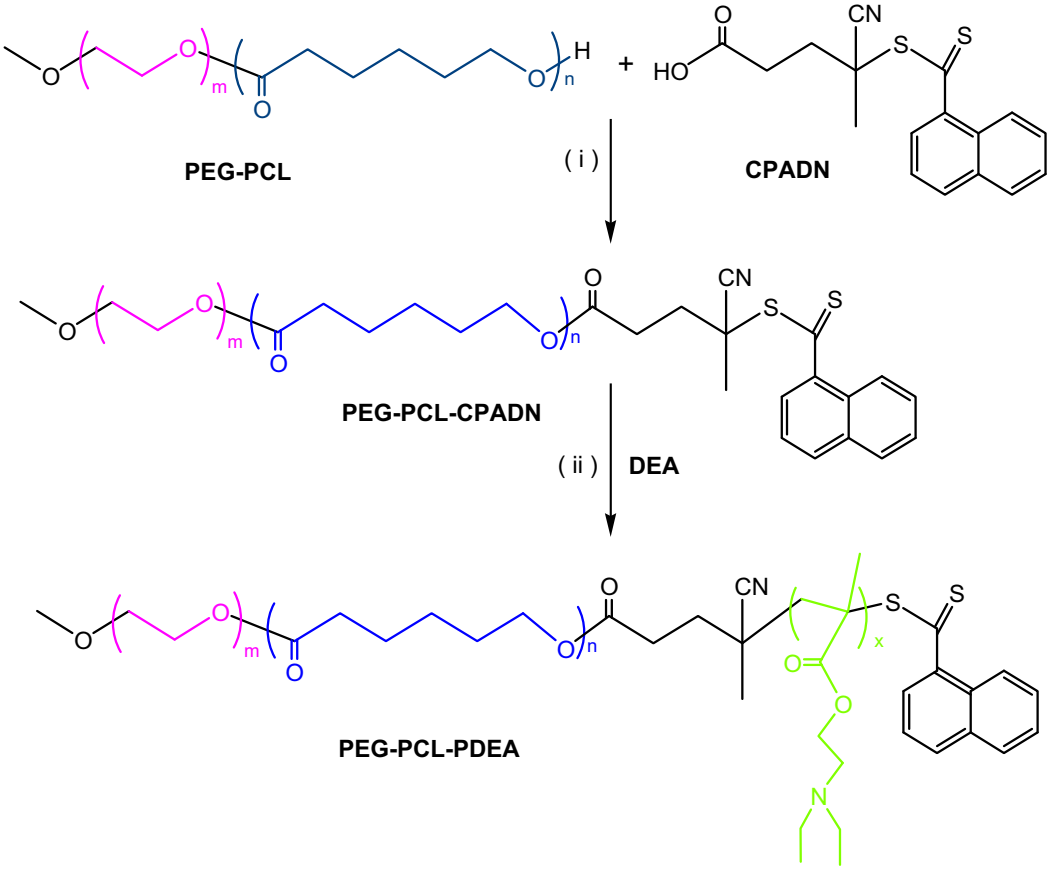
CAC was determined using pyrene as a fluorescence probe. The polymersomes (2 mL in MES) were prepared using the film hydration method with initial copolymer concentrations varying from 6.0×10^{-6} to 0.02 mg/mL. Pyrene in acetone (10 μL) was added. After stirring for 30 min, acetone was evaporated and the final concentration of pyrene was fixed at 0.6 μM. The fluorescence spectra (FLS920) were recorded with an excitation wavelength of 330 nm. The emission fluorescence at 372 and 383 nm were monitored. The CAC was estimated as the cross-point when extrapolating the intensity ratio I_{372}/I_{383} at low and high concentration regions.

2.7. Confocal laser scanning microscopy (CLSM) of polymersomes loaded with nile red and FITC-CC

FITC-CC was loaded into polymersomes **2** using the film hydration method. Free FITC-CC was removed by dialysis (MWCO 500 kDa). Then, 10 μL of nile red solution in acetone was added to give a final nile red concentration of 10^{-5} M. The solution was stirred for 3 h at 37 °C before removal of acetone by evaporation. Fluorescence images were obtained using a confocal microscope (TCS SP2). To prepare giant polymersomes, a shorter stirring time of 4 h at 60 °C was applied. The slice thickness of the confocal image of Fig. 2B was 100 nm.

2.8. Reduction of DTNB using 3,3′,3″-phosphinidyne tris(benzenesulfonic acid) (PH) loaded polymersomes

PH-loaded polymersomes were prepared by rehydrating thin films of copolymer **2** using a solution of PH (1 mg, 1.5 μmol) in MES buffer (2 mL), sonicating at 60 °C for 5 h to obtain a homogeneous suspension, and extensive dialysis (two days) to remove free PH. A solution (40 μL) of DTNB (25 mg/mL) was added to a cuvette containing PH-loaded polymersomes. The kinetics of the reaction between DTNB and PH-loaded polymersomes were monitored by UV. A solution of DTNB and empty polymersomes was used as control. The results showed that DTNB was quickly



Scheme 2. Synthetic route to PEG-PCL-PDEA triblock copolymers. (i)DCC/DMAP, toluene/CH_2Cl_2, r.t., 48 h (ii) AIBN, dioxane, 70 °C, 48 h.

Table 1
Synthesis of PEG-PCL and PEG-PCL-PDEA triblock copolymers.

Entry	copolymer[a]	$M_{n,design}$ (kg/mol)	$M_{n,NMR}$ (kg/mol)	M_w/M_n[b]	Size[c] (PDI)	CAC[d] (mg/L)	ζ[e] (mV)
1	PEG-PCL-PDEA(1.1 k)	5-18-1	5-18.2-1.1	1.20	130(0.23)	2.73	−2
2	PEG-PCL-PDEA(2.7 k)	5-18-3	5-18.2-2.7	1.43	154(0.19)	2.22	1.1
3	PEG-PCL-PDEA(4.1 k)	5-18-5	5-18.2-4.1	1.21	175(0.20)	2.24	2.7
4	PEG-PCL	5–18	5–18.2	1.24	230(0.21)	3.57	−1

[a] The molecular weights (M_n) of PEG and PCL were 5 and 18.2 kg/mol, respectively, and kept unchanged. Triblock copolymers were denoted as PEG-PCL-PDEA ($M_{n\ PDEA}$).
[b] Determined by GPC (eluent: THF, flow rate: 1.0 mL/min, standards: polystyrene, 30 °C).
[c] The average particle size (nm) and size distribution measured by DLS at 25 °C at pH 5.3.
[d] Critical aggregation concentration (CAC) determined at pH 5.3 using pyrene as fluorescence probe.
[e] Determined in PB buffer (pH 7.4, 20 mM).

reduced by PH-loaded polymersomes into 2-nitro-S-thiobenzoic acid (TNB) giving a characteristic absorption at 412 nm [34].

2.9. 2D-^{1}H-NOESY-NMR analysis of polymersomes in D_2O

In order to perform NMR measurements in D_2O, polymersome **2** was prepared by a solvent evaporation method. In brief, to a stirred copolymer **2** solution in THF (0.6 mL) was dropwise added 2.0 mL of D_2O (pH 4 adjusted by $FeCl_3$). THF was evaporated overnight under vacuum. After concentration, 0.6 mL of polymersome **2** solution with a concentration of ca. 0.6 wt.% was analyzed by ^{1}H NMR. 2D-^{1}H, ^{1}H-NOESY-NMR spectra were recorded at room temperature on an INOVA spectrometer operating at 400 MHz. The Varian standard software package vnmr6.1c was used for acquisition and processing of data.

2.10. MTT assay

Murine RAW 264.7 macrophages or HeLa cells were plated in a 96-well plate (5×10^3 cells/well) using DulbeCCo's modified Eagle medium (DMEM) or RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, antibiotics penicillin (100 IU/mL) and streptomycin (100 μg/ml). After 24 h, prescribed amounts of polymersome **1**, **2** or **3** were added and incubated for 24 h at 37 °C in an atmosphere containing 5% CO_2. Then 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) solution in PBS (5 mg/mL) was added and incubated for another 4 h. The medium was aspirated, the MTT-formazan generated by live cells was dissolved in 100 μL of 10% SDS/0.1 M HCl overnight, and the absorbance at a wavelength of 570 nm of each well was measured using a microplate reader (Bio-rad, ELX808 IU). The cell viability (%) was determined by comparing the absorbance at 570 nm with control wells containing only cell culture medium. The experiments were performed four times each.

2.11. Loading of FITC-BSA, FITC-CC, FITC-Lys, FITC-OVA, and FITC-IgG into polymersomes

FITC-labeled proteins were loaded into polymersomes by the film hydration method. The polymersomes were prepared as previously described except that MES solutions (pH 5.3, 20 mM) of proteins at different protein concentrations were applied. Free protein was removed by dialysis (MWCO 500 kDa) against the same MES for 10 h at 20 °C with at least 4 times change of media. The control experiments on free proteins showed that this purification procedure is sufficient to remove free proteins if present. Protein loading efficiency and loading content were determined by fluorometry on protein-loaded polymersome suspensions supplemented with three time its volume of DMSO (to disrupt polymersomes and to release proteins). The calibration curve was established by fluorometry with known concentrations of FITC-labeled proteins in DMSO/water (3/1, v/v).

2.12. Protein release from polymersomes

The release of FITC-BSA or FITC-CC from polymersomes was investigated using a dialysis method (MWCO 500 kDa) at 37 °C with 0.5 mL of protein-loaded polymersome suspensions against 60 mL PB (pH 7.4, 20 mM) or MES (pH 5.4, 20 mM). At desired time intervals, 3 mL release media was taken out and replenished with an equal volume of fresh media. The amounts of released proteins as well as proteins remaining in the dialysis tube were determined by fluorescence measurements (FLS920, excitation at 492 nm, emission from 492 to 690 nm). The release experiments were conducted in triplicate.

2.13. Activity of released cytochrome C by ABTS assay

The electron transfer activity of cytochrome C (CC) was measured by examining the catalytic conversion of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). CC was loaded into polymersome **2** using the film hydration method as described previously. CC was released overnight from CC loaded polymersomes in MES buffer (pH 4.0, 20 mM). The amount of released CC was measured by UV–vis. The released CC was diluted 10 times by a MES buffer (pH 5.4, 20 mM) to a final concentration of 0.004 mg/mL. To this solution and the same concentration of native CC in MES buffer in quartz cuvettes, were simultaneously added 10 μL of hydrogen peroxide solution (0.045 M) and 100 μL of ABTS solution (1 mg/mL) both in the same MES buffer. The absorbance at 410 nm of the oxidized product was monitored every 15 s for 2.5 min.

2.14. Loading of doxorubicin (DOX) into polymersomes

Polymersomes were prepared using the film hydration method as described before. To the resulting polymersomes was added DOX solution in DMSO at a DOX to copolymer feed ratio of 20 wt.% and incubated for 5 h. Free DOX was removed by dialysis (MWCO 12 kDa) against distilled water for 12–24 h with at least 4 times change of media. The drug loading efficiency and loading content were determined

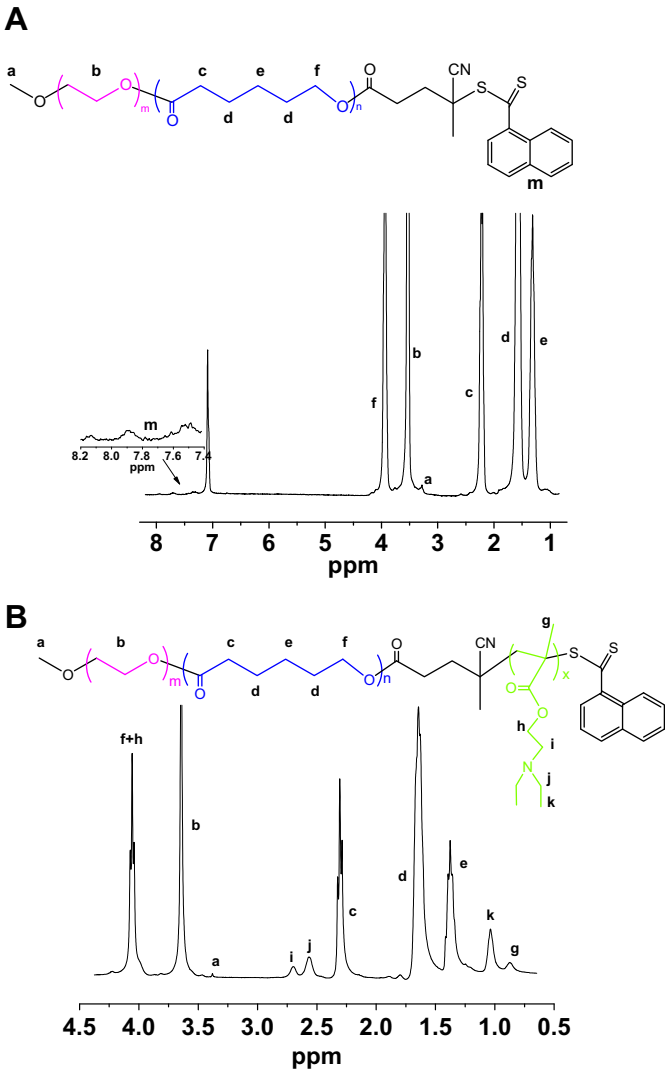


Fig. 1. ^{1}H NMR spectra (400 MHz, $CDCl_3$) of PEG-PCL-CPADN macro-RAFT agent (A) and PEG-PCL-PDEA (2.7 k) triblock copolymer (B).

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by fluorometry on DOX-loaded polymersome suspensions, supplemented with three times the volume of DMSO (to disrupt polymersomes and release DOX). The calibration curves were established by fluorometry with known concentrations of DOX in DMSO/water (3/1, v/v).

2.15. DOX release from polymersomes

The release of DOX from polymersomes was investigated using a dialysis method (MWCO 12 kDa) at 37 °C with 0.5 mL of DOX-loaded polymersome suspension against 30 mL PB (pH 7.4, 20 mM). At desired time intervals, 3 mL of release media was taken out and replenished with an equal volume of fresh media. The amounts of DOX released and DOX remaining in the dialysis tube were determined by using fluorometry (FLS920). The release experiments were conducted in triplicate.

2.16. Co-loading of proteins and DOX into polymersomes

Firstly, protein-loaded polymersomes were prepared as described before. Then, to the resulting polymersomes was added DOX solution in DMSO at a DOX to copolymer feed ratio of 20 wt.%. After incubation for 5 h, the suspension was dialyzed (MWCO 12 kDa) against distilled water for 12–24 h with at least 4 times change of media to remove free DOX.

2.17. CLSM of cells incubated with protein and/or DOX-loaded polymersomes

Murine RAW 264.7 macrophages or HeLa cells were plated on microscope slides in a 6-well plate (1×10^5 cells/well) using RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/ml). After 24 h, prescribed amounts of protein-loaded polymersome **2**, protein/DOX-loaded polymersome **2**, free protein, or free DOX were added. After incubation at 37 °C and 5% CO_2 for predetermined times, the culture medium was removed and the cells on microscope plates were washed three times with PBS. The cells were fixed with 4% formaldehyde for 20 min and washed with PBS for 3 times. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue) for 20 min and washed with PBS for 3 times. Fluorescence images of cells were obtained using confocal microscope (TCS SP2).

2.18. CLSM of subcellular localization of proteins loaded into polymersomes

RAW 264.7 cells were plated on microscope cover slides in a 24-well plate (5×10^4 cells/well) using RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/ml). After 24 h, FITC-CC loaded polymersomes **2** (0.13 mg/mL) were added. After incubation at 37 °C and 5% CO_2 for 24 h, the medium was removed and the cells on the microscope plates were washed three times with PBS. The cells were fixed with 4% formaldehyde for 20 min and washed twice with PBS. The late endosome and lysosome were stained with LysoTracker Red (Beyotime Institute of Biotechnology, China, 0.5 mL × 50 nM) for 30 min and washed with PBS for three times. The images were taken using the confocal microscope (TCS SP2).

3. Results and discussion

3.1. Synthesis of asymmetric PEG-PCL-PDEA triblock copolymers

PEG-PCL-PDEA triblock copolymers were synthesized by radical addition-fragmentation chain transfer (RAFT) polymerization of 2-(diethylamino)ethyl methacrylate (DEA) using PEG-PCL-CPADN ($M_{n\ PEG} = 5$ kg/mol, $M_{n\ PCL} = 18.2$ kg/mol) as a macro-RAFT agent and AIBN as the radical source at 60 °C (Scheme 2). The results of polymerization are shown in Table 1. ^{1}H NMR showed clearly signals characteristic of PEG (δ 3.65), PCL (δ 4.05, 2.28, 1.62 and 1.34) and PDEA (δ2.70, 2.56, 1.03 and 0.88) blocks, respectively (Fig. 1). The M_n values of PDEA could be estimated by comparing the intensities of signals at δ 1.03 and 3.65 which were attributable to the methyl protons of the ethyl group of PDEA and the methylene protons of PEG, respectively. Notably, results demonstrated that these block copolymers had $M_{n\ PDEA}$ varying from 1.1, 2.7, to 4.1 kg/mol (denoted as copolymer **1**, **2** and **3**, respectively), which correlated well with the design (Table 1). Gel permeation chromatography (GPC) further showed that these triblock copolymers had unimodal distributions with moderate polydispersities of 1.20–1.43 (Table 1). Using CPADN-PCL-CPADN as a macro-RAFT agent, we have previously obtained well-defined PDMAEMA-PCL-PDMAEMA triblock copolymers [35].

3.2. Formation of chimaeric polymersomes

Polymersomes were prepared with high yields from PEG-PCL-PDEA triblock copolymers by the film rehydration method using MES buffer (20 mM, pH 5.3) at 60 °C (Scheme 1). Dynamic light scattering (DLS) measurements of the polymersomes at pH 5.3 (Fig. 2A) showed that they had average sizes varying from 130 to 175 nm depending on the PDEA block lengths and narrow size distributions (PDI 0.1–0.2). In comparison, when prepared under the same conditions, the parent PEG-PCL yielded polymersomes with relatively larger sizes of 230 nm (Fig. 2A). The easy formation of polymersomes with asymmetric PEG-PCL-PDEA triblock copolymers at 60 °C could be attributed to great flexibility of PCL chains at this temperature and their favored formation of curvature structures. When adjusting the pH of the solution from 5.3 to 7.4, the sizes of PEG-PCL-PDEA polymersomes increased by 10–15 nm

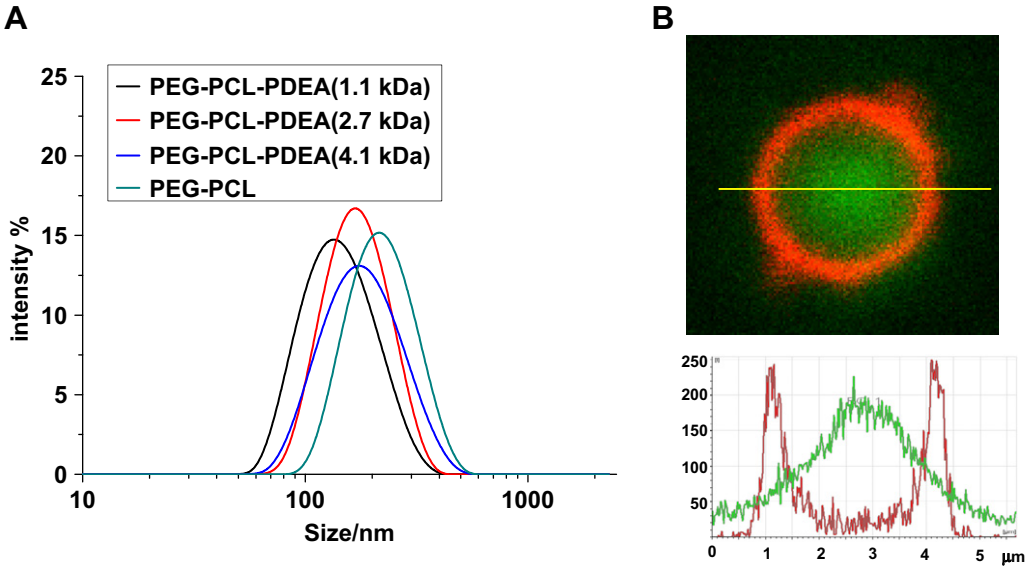

Fig. 2. Characterization of PEG-PCL-PDEA polymersomes. (A) size distributions of different polymersomes measured by DLS; (B) CLSM micrographs of a giant polymersome **2** loaded with nile red (red) and FITC-CC (green) and the fluorescence intensity profiles following the yellow lines.

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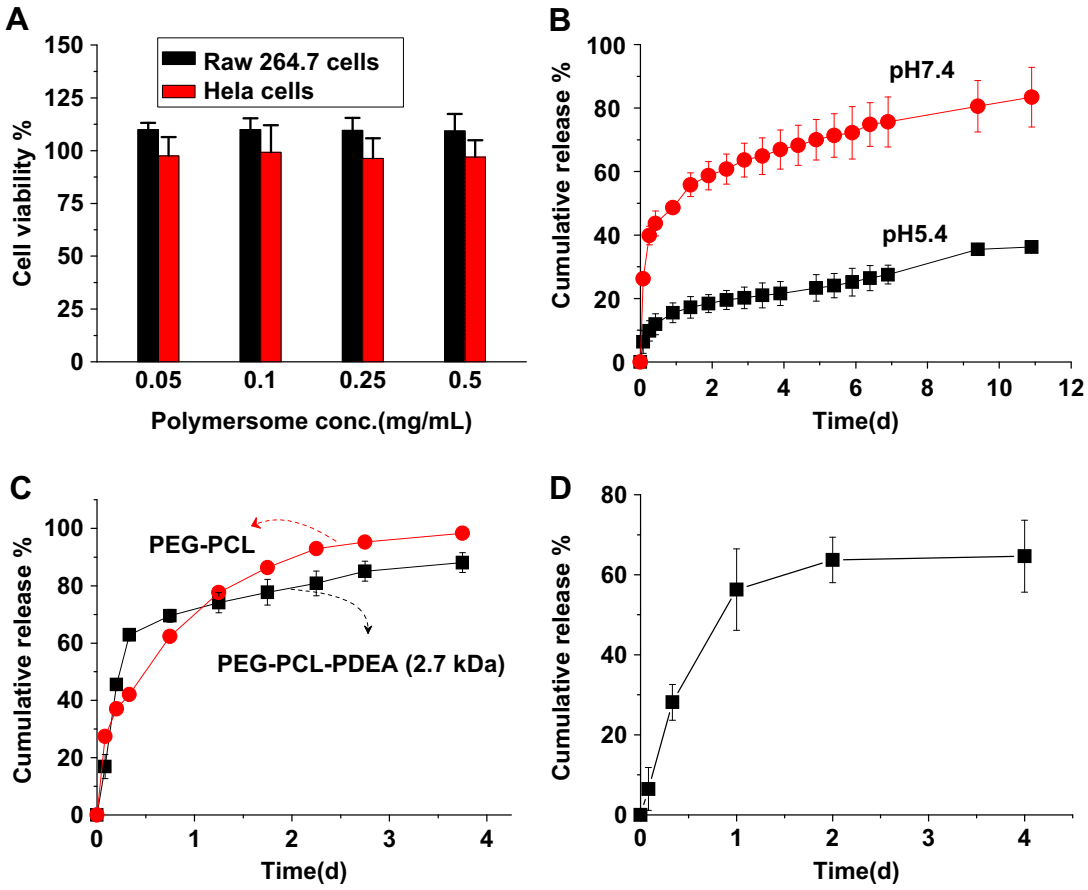

Fig. 3. (A) Cytotoxicity of polymersome **2** determined by MTT assays using Hela cells and RAW cells ($n = 4$). (B) release profiles of FITC-BSA from polymersome **2** at pH 5.4 (MES, 20 mM) and 7.4 (PB, 20 mM); (C) release profiles of FITC-CC from polymersome **2** and PEG-PCL control at pH 7.4; (D) release profile of DOX from polymersome **2** in PB (20 mM, pH 7.4).

while maintaining a low PDI. TEM pictures showed that the particles had a hollow vesicular structure and no micellar structures were observed (Supporting information, Fig. S1). The formation of polymersomes is preferred due to a rather low weight fraction of hydrophilic blocks ($f_{phil} = 21–33$ wt.%) [19,20]. The critical aggregation concentration (CAC) determined using pyrene as a fluorescence probe revealed low CAC's of 2.73, 2.22, 2.24, and 3.57 mg/L for copolymer **1, 2, 3** and PEG-PCL in MES, respectively (Table 1). Confocal laser scanning microscopy (CLSM) of polymersome **2** loaded with FITC-cytochrome C (FITC-CC) and nile red clearly showed co-localization of protein and nile red (Supporting information, Fig. S2). To further verify the structure of the polymersomes, we also prepared larger polymersomes by applying a stirring time of 4 h instead of 12 h at 60 °C. The intensity profiles of red and green fluorescence in a captured giant polymersome following the yellow line in the image were in accordance with the location of nile red in the membrane and FITC-CC in the lumen (Fig. 2B), confirming their vesicular structure. The polymersome structure was further corroborated by the effective reduction of DTNB by using polymersomes loaded with 3,3′,3"-phosphinidyne tris(benzenesulfonic acid), which is hydrophilic and can not be loaded into hydrophobic domains [36] (Supporting information, Fig. S3).

It should be noted that the film rehydration method yielded PEG-PCL-PDEA polymersomes with a low PDI with excellent reproducibility. Unlike PEG-PLA polymersomes reported by Discher and coworkers [37], PEG-PCL-PDEA polymersomes were not readily hydrolyzed under the preparation conditions likely because PCL degrades much slower than PLA and furthermore the PEG-PCL-PDEA copolymers do not possess a hydroxyl terminal group. It is known that the presence of hydroxyl end group significantly accelerates the degradation of polyesters by a back-biting mechanism [38].

The alignment of short PDEA chains inside the polymersomes was investigated using NMR [39]. The ^{1}H NMR spectrum of polymersomes **2** in D_2O (0.6 wt.%, pH ca. 4) displayed significantly decreased intensities of signals attributable to PCL and PDEA relative to PEG (Supporting information, Fig. S4A), suggesting

Table 2
Characterization of protein-loaded polymersome **2**.[a]

Proteins	Protein feed ratio (wt.%)	Loading content (wt.%)	Loading efficiency (%)	Size (PDI)	ζ[b] (mV)
FITC-BSA	0	–	–	142 (0.20)	1.1
	10	9.7	96.6	132 (0.30)	−0.3
	25	19.6	78.5	146 (0.38)	0.2
	50	32.2	64.5	173 (0.25)	−4.5
	100	53.2	53.2	167 (0.24)	−4.8
FITC-CC	10	10.0	99.7	163 (0.15)	−2.1
	25	22.3	89.1	164 (0.24)	−3.0
	50	30.8	61.7	165 (0.22)	−0.6
	100	56.4	56.4	134 (0.23)	0.46
FITC-Lys	25	21.0	84.3	150 (0.27)	0.1
FITC-OVA	25	21.2	84.7	113 (0.31)	−0.2
FITC-IgG	25	22.4	89.6	158 (0.29)	−1.3

[a] Thin films were prepared at a copolymer **2** concentration of 0.5 mg/mL in DCM and the final polymersome concentration in MES buffer (pH 5.3) was 0.5 mg/mL.
[b] Determined using PB buffer (pH 7.4, 20 mM).

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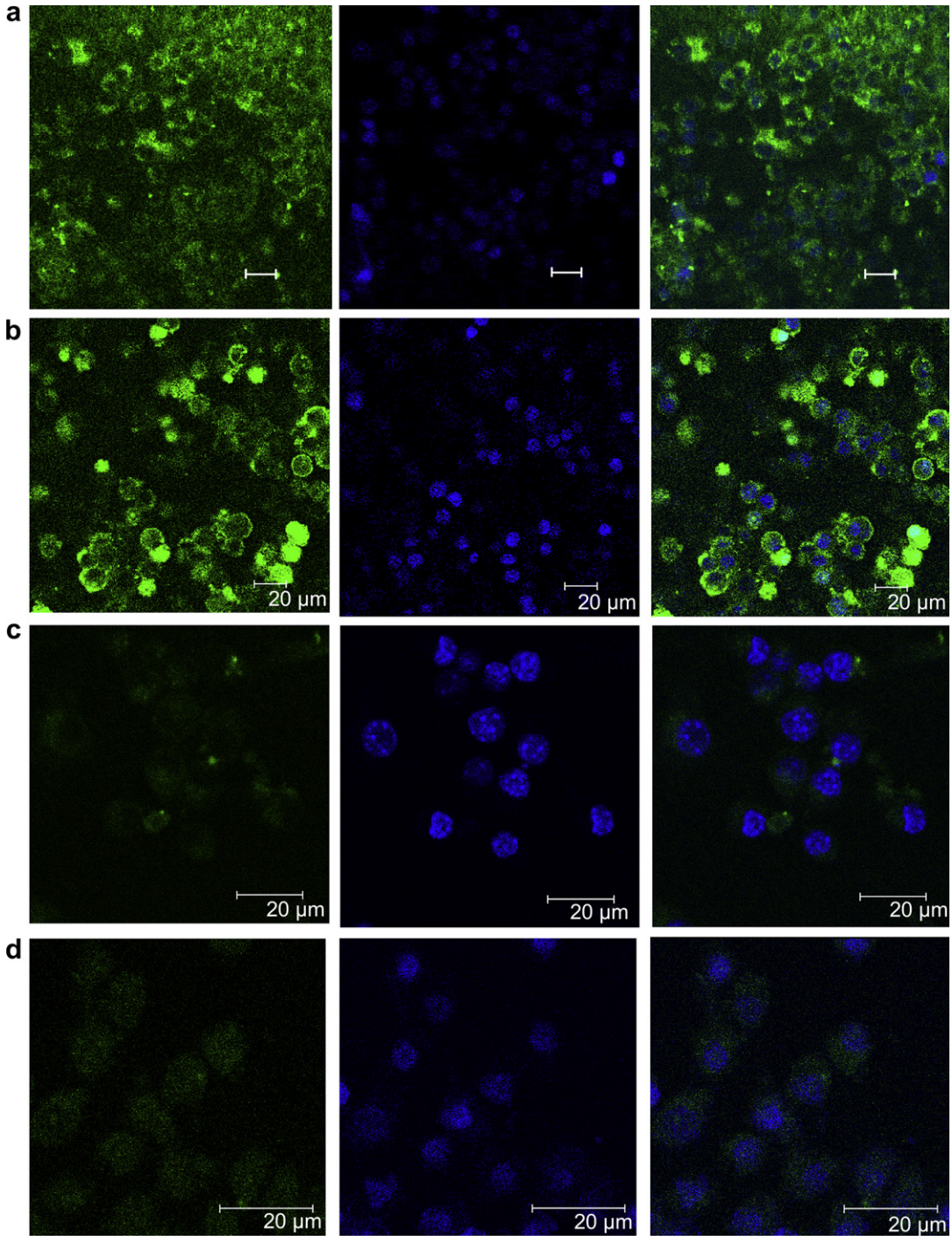

Fig. 4. CLSM images of RAW 264.7 cells incubated with FITC-CC loaded polymersome **2** and free FITC-CC (0.13 mg/mL). For each panel, images from left to right show FITC-CC fluorescence in cells (green), cell nuclei stained by DAPI (blue), and overlays of two images. The scale bars correspond to 20 μm in all the images. a) FITC-CC loaded polymersome **2**, 4 h incubation; b) FITC-CC loaded polymersome **2**, 24 h incubation; c) free FITC-CC, 2 h; d) free FITC-CC, 24 h incubation.

that PEG is located at the outside of polymersomes while both PCL and PDEA are shielded. The 2D-^{1}H-NOESY-NMR spectrum (Supporting information, Fig. S4B) revealed only cross signals among methylene protons of the PCL block (δ 3.95, 2.23, 1.53, and 1.27), and none between PDEA (δ 0.97) and PEG (δ 3.64) or between PDEA and PCL, likely implying the absence of 3D spatial correlations between PDEA and PEG or PCL. These results are in accordance with the formation of chimaeric polymersomes (Scheme 1). The preferential alignment of longer hydrophilic chains towards the outside and shorter ones towards the inside of the polymersomes has been reported for mixed polystyrene based diblock copolymers [40,41] and asymmetric triblock copolymers such as PEG_{45}-$PDPA_{50}$-$PDMA_{14}$ and PEG_{45}-PS_{130}-$PDEA_{120}$ [39,42–44].

Zeta potential measurements demonstrated that PEG-PCL-PDEA polymersomes, similar to PEG-PCL polymersomes, had close to neutral surface charges (−2 ∼ +2.7 mV) in PB buffer at pH 7.4 (Table 1), further supporting a chimaeric structure with PDEA situated inside the polymersomes. MTT assays using HeLa and RAW 264.7 cells revealed that PEG-PCL-PDEA polymersomes are non-

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toxic up to a tested concentration of 0.5 mg/mL (Fig. 3A and Supporting information, Fig. S5).

3.3. Loading and in vitro release of proteins

We studied encapsulation of five FITC-labelled proteins, *i.e.* bovine serum albumin (BSA), CC, lysozyme (Lys), ovalbumin (OVA) and immunoglobulin G (IgG), into PEG-PCL-PDEA polymersomes. The proteins were conveniently loaded into polymersomes by direct rehydration of copolymer thin films in protein-containing MES buffer (20 mM, pH 5.3). Interestingly, polymersome **2** demonstrated remarkably high protein loading efficiencies of 96.6, 78.8, 64.5 and 53.2% at initial FITC-BSA/copolymer feed ratios of 10, 25, 50 and 100 wt.%, respectively, resulting in a high FITC-BSA payload of up to 53.2 wt.% (Table 2). In contrast, PEG-PCL control polymersomes demonstrated a protein loading content of 3.3 wt.% at a FITC-BSA/copolymer feed ratio of 50 wt.% (Table 2), a loading efficiency 10 times lower than for polymersome **2**. Similar results were observed for FITC-CC, wherein a protein loading efficiency of up to 100% and a protein loading content of 56 wt.% were obtained for polymersome **2** (Table 2). Notably, polymersome **2** showed also high protein loading efficiencies of 84.3–89.6% for FITC-Lys, FITC-OVA, and FITC-IgG at an initial protein loading content of 25 wt.% (Table 2). The encapsulation of proteins did not significantly alter the polymersome size distribution and zeta potentials (Table 2). It should be noted that polymersome **2** revealed higher protein loading levels as compared to polymersomes **1** and **3** (Supporting information, Table S1). The relatively lower protein loading efficiency of polymersome **1** is most likely due to its reduced interactions with proteins as a result of the shorter PDEA block. For polymersome **3**, the longer PDEA block (which has a block length close to PEG) renders it unlikely forming defined chimaeric polymersomes.

It is remarkable that these chimaeric polymersomes achieved such a high protein loading efficiency and content, since low protein loading levels have been an issue for many current protein carriers. For example, liposomes were reported to load proteins with efficiencies of 40–50% through freeze–thaw cycles and of 24% for BSA with emulsification [45]. For polymersomes, the encapsulation efficiency of BSA and haemoglobin was 5% [46] and 2.7–12.2% [47], respectively, and that of glucose oxidase in a THF/water system was 25% [48]. Hubbell et al. recently reported that polymersomes could be loaded with efficiencies of 15–37% via direct hydration [27]. The high protein loading level of chimaeric polymersomes obtained in this study is likely due to effective electrostatic and/or hydrogen bonding interaction between exogenous proteins and PDEA blocks during the formation of the polymersomes at pH 5.3.

The *in vitro* release of proteins from chimaeric polymersomes at pH 7.4 and 5.4 was investigated. Interestingly, FITC-BSA was released from polymersome **2** in a sustained manner at pH 7.4, in which approximately 85% protein was released in 11 days (Fig. 3B).

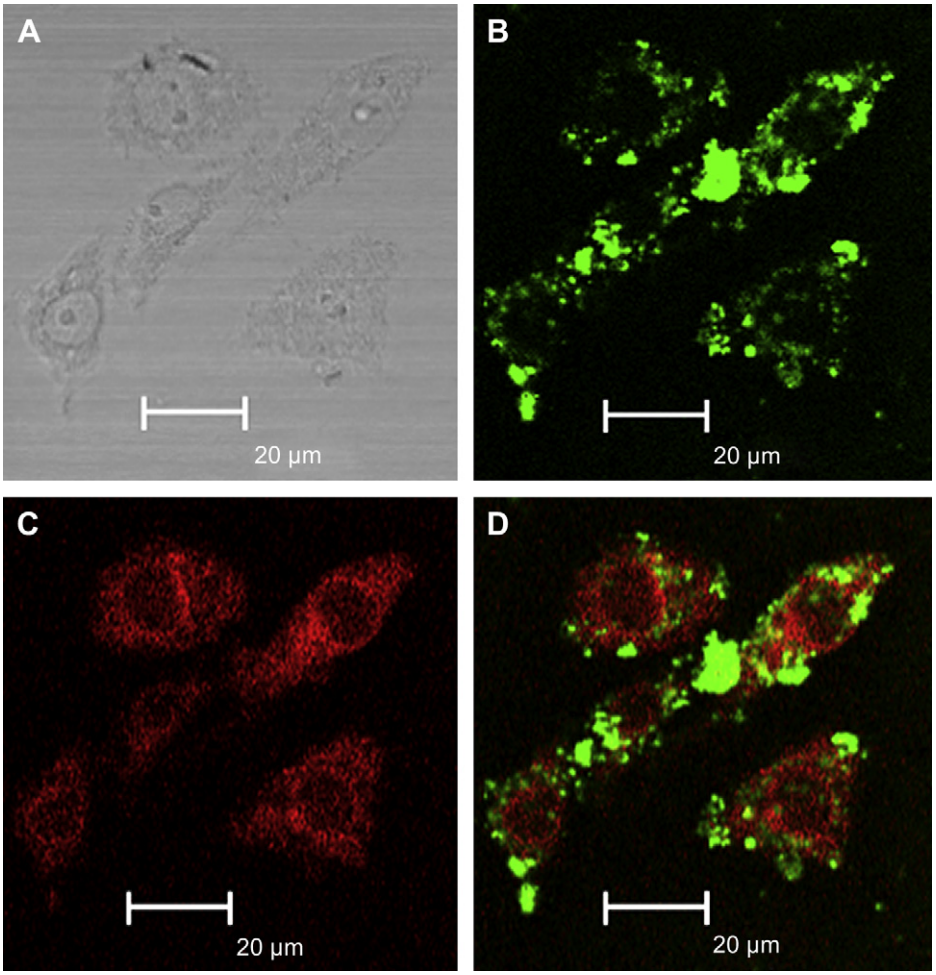

Fig. 5. CLSM images of RAW 264.7 cells incubated for 24 h with FITC-CC loaded polymersome **2**. The late endosome and lysosome were stained with LysoTracker Red. (A) transmittance image, (B) FITC-BSA, (C) LysoTracker Red, and (D) overlay of B and C.

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Table 3
Characterization of DOX-loaded polymersomes.[a]

Polymersomes	Loading content (wt.%)	Loading efficiency (%)	Size (nm)	PDI	ζ[b] (mV)
Polymersome 1	9.6	48.1	151.0	0.12	−1.20
Polymersome 2	14.4	72.2	108.7	0.20	−1.69
Polymersome 3	12.7	63.6	144.5	0.26	−0.18
PEG-PCL	11.0	55.2	253.0	0.18	−6.32

[a] DOX/copolymer feed ratio was kept at 20 wt.%.
[b] Measured in PB buffer (pH 7.4, 20 mM).

In comparison, only 30% protein was released in 11 days at pH 5.4 under otherwise the same conditions (Fig. 3B). This comparably slow protein release at mildly acidic pH is most probably related to protonation of PDEA which gives rise to a strong interaction between PDEA and BSA. The electrostatic interaction between PDEA and BSA is weakened at pH 7.4 since PDEA is de-protonated at neutral pH. The release of FITC-CC was faster as compared to that of FITC-BSA (Fig. 3C), most likely due to the smaller size of CC. Notably, polymersome **2** showed a biphasic release behaviour in which a fast zero-order release of CC up to ca. 62% in 8 h was followed by a sustained slow release of CC (Fig. 3C). In comparison, CC was released from PEG-PCL polymersomes in a likely diffusion controlled manner. Usually, only non-charged species can be transported through the hydrophobic membrane. The controlled release of proteins from polymersome **2** is most probably due to incorporation of some proteins and/or PDEA segments in the hydrophobic membrane during protein loading providing channels for subsequent protein release.

The enzymatic activity of CC released from the polymersomes was examined using the ABTS assay [13,49]. The results showed that there is no significant difference in UV absorption of oxidized ABTS catalyzed either by CC released from polymersome **2** or by native CC (Supporting information, Fig. S6), implying that released proteins maintain their bioactivity. An initial Annexin V-FITC/propidium iodide (PI) flow cytometry experiment showed that about 7% Hela cells and 40% MCF-7 cells were apoptotic/dead after 12 h incubation with CC loaded polymersomes (Supporting information, Fig. S7). In comparison, about 5.4% Hela cells and 29% MCF-7 cells were apoptotic/dead after 12 h incubation with the native CC. These results further confirmed that CC released from the polymersomes maintains its apoptotic activity.

3.4. Intracellular release of proteins

The intracellular uptake and release of proteins was studied using RAW 264.7 cells using CLSM. Remarkably, FITC-CC loaded polymersomes efficiently delivered and released the protein into the cells. A strong fluorescence inside the cells was observed after 4 h incubation, and the FITC-CC fluorescence was intensified and distributed to the whole cell at a longer incubation time of 24 h (Fig. 4a and b). In contrast, minimal fluorescence in the cells was observed when the cells were exposed to free FITC-CC (Fig. 4c and d). The efficient cellular uptake and cytoplasmic protein delivery were also observed

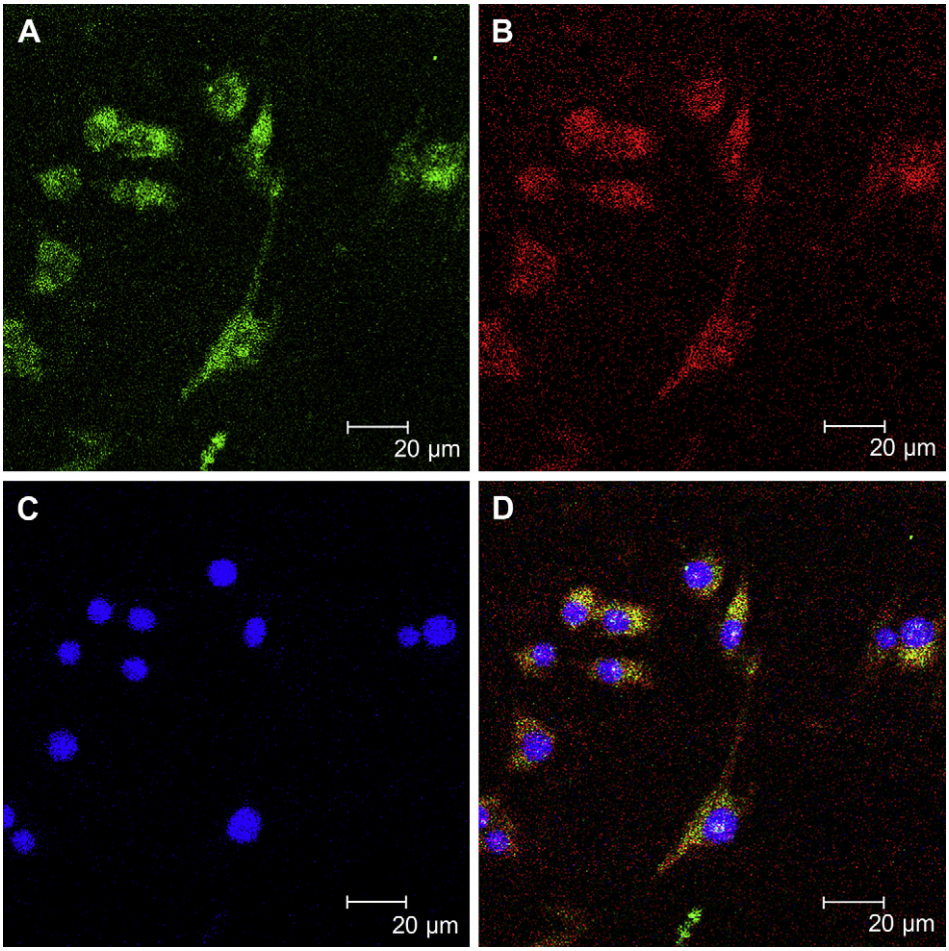

Fig. 6. CLSM images of RAW 264.7 cells incubated for 4 h with FITC-BSA/DOX-loaded polymersome **2**. The scale bars correspond to 20 μm in all the images. (A) FITC-BSA (green), (B) DOX (red), (C) cell nuclei stained with DAPI (blue), and (D) overlays of A, B and C.

for FITC-BSA loaded polymersomes (Supporting information, Fig. S8). To investigate whether proteins had escaped from endosomes, the late endosome and lysosome were stained by LysoTracker Red (red) as described by Kataoka and coworkers [13]. The CLSM images of RAW 264.7 cells following incubation for 24 h with FITC-CC loaded polymersomes showed a strong green fluorescence as well as red fluorescence (Fig. 5), indicating efficient protein release from endosomes. The effective endosomal escape is most likely due to a high buffer capacity of PDEA at endosomal pH, which results in the so called "proton sponge effect" effectively disrupting the endosomal membrane [28]. These results are exceptional in that few protein carriers can efficiently load and deliver exogenous proteins into the cytoplasm of living cells, which is the location for many proteins to carry out their biological functions [5,6].

Polymersomes are of special interest because they are one of the few nano-carriers ideally suited to simultaneously deliver hydrophilic and hydrophobic drugs. It has been reported that several types of cancers may benefit from combination therapy in which co-delivery of two kinds of therapeutics, e.g. one is a hydrophobic drug and the other is a protein, DNA or siRNA, is required [50,51]. Herewith, we firstly studied the encapsulation and *in vitro* delivery of doxorubicin using chimaeric polymersomes. DOX was loaded into polymersomes via the film rehydration method. In our initial experiments, the theoretical drug loading content was set at 20 wt.%. The results showed that polymersome **2** yielded a high drug loading efficiency of 72.2% and a drug loading content of 14.4 wt.% (Table 3). Under otherwise the same conditions, a drug loading efficiency of 55.2% was obtained for PEG-PCL polymersomes. Interestingly, the *in vitro* release studies performed at pH 7.4 showed that DOX was released from polymersome **2** in a controlled manner, in which a cumulative release of ca. 65% DOX was observed in 2 days (Fig. 3D). In the following, we loaded both FITC-BSA and DOX into polymersome **2**. Remarkably, CLSM studies revealed that both FITC-BSA (green) and DOX (red) were effectively transported into cells and released into the cytoplasm as well as the cell nucleus after 4 h incubation (Fig. 6). Due to the self-quenching effect, DOX fluorescence is observed only when DOX has been released from nanoparticles [52–55].

4. Conclusions

We have demonstrated that biodegradable chimaeric polymersomes based on PEG-PCL-PDEA triblock copolymers are a fascinating type of multifunctional nano-carriers that efficiently deliver and release exogenous proteins into cancer cells. These chimaeric polymersomes display unusually high protein loading efficiencies and release proteins in a controlled manner under physiological conditions. Importantly, the released proteins fully retain their bioactivity. Moreover, these chimaeric polymersomes are able to simultaneously deliver and release hydrophobic anticancer drugs and proteins into cells, which render them particular interesting for combination cancer therapy. These biodegradable chimaeric polymersomes have opened a new avenue to intracellular delivery of protein and peptide drugs and may further be employed as a powerful tool for understanding protein functions in cells.

Acknowledgement

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Appendix. Supplementary data

The supplementary data associated with this article can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.06.021.

Appendix

Figures with essential color discrimination. Figs. 1–6 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.2010.06.021

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