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Research paper

Biodegradable polymersomes with an ionizable membrane: Facile preparation, superior protein loading, and endosomal pH-responsive protein release

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ABSTRACT

Novel biodegradable polymersomes containing an ionizable membrane were developed for efficient loading and rapid intracellular release of proteins. The polymersomes were prepared from poly(ethylene glycol)-*b*-poly(trimethylene carbonate) (PEG–PTMC) block copolymer derivatives containing acrylate, carboxylic acid, and amine groups along PTMC block, which are denoted as PEG–PTMC(AC), PEG–PTMC(COOH), and PEG–PTMC(NH_2), respectively. Notably, nano-sized polymersomes (95.1–111.6 nm) were formed by directly dispersing these copolymers in phosphate buffer at room temperature. Both FITC-labeled bovine serum albumin (FITC-BSA) and cytochrome C (FITC-CC) were readily loaded into PEG–PTMC(COOH) and PEG–PTMC(NH_2) polymersomes with remarkably high loading levels. Interestingly, *in vitro* release studies showed that PEG–PTMC(COOH) and PEG–PTMC(NH_2) polymersomes had pH-responsive protein release behaviors in which significantly faster protein release was observed at endosomal pH than at physiological pH. MTT assays indicated that these polymersomes had low cytotoxicity. Furthermore, confocal laser scanning microscope (CLSM) observations revealed that FITC-CC loaded polymersomes efficiently delivered proteins into MCF-7 cells following 24 h incubation. Importantly, flow cytometry showed that CC-loaded polymersomes induced markedly enhanced apoptosis in MCF-7 cells as compared to free CC. These novel membrane ionizable biodegradable polymersomes have appeared as highly promising nanocarriers for efficient intracellular protein delivery.

1. Introduction

In the past decade, protein and peptide drugs have been developed for effective treatment of various human diseases including cancers [1–3]. Protein drugs with high specificity, high anti-cancer efficacy, and low side effects have significant advantages over chemotherapeutics. Nevertheless, there present also several challenges with applications of protein drugs such as rapid degradation *in vivo,* potential immune response, inferior cellular uptake, and poor intracellular trafficking [1]. The clinical success of many protein drugs is intimately dependent on the development of safe and efficient targeted protein delivery technologies. Recently, different nano delivery systems such as liposomes [4] and polyion complex micelles [5] have been investigated for intracellular protein delivery. However, most protein formulations involve (toxic) organic solvents possibly leading to protein denaturation and deactivation, display low protein loading, have inadequate stability, and/or exhibit sluggish protein release inside cells.

Polymersomes self-assembled from amphiphilic copolymers have attracted a lot of interests for controlled delivery of hydrophobic as well as hydrophilic drugs [6–9]. In comparison with liposomes, polymersomes are intrinsically stealthy and exhibit better stability [10–14]. In recent years, different proteins have been loaded into the large aqueous interior and/or the robust hydrophobic wall of polymersomes [15–20]. For example, Kataoka et al. reported that myoglobin encapsulated inside polyion complex vesicles could perform reversible oxygenation/deoxygenation reaction [21]. Palmer et al. reported that hemoglobin-loaded PEG–poly(ε-caprolactone) (PEG–PCL) polymersomes had similar oxygen affinities to human red blood cells [22]. Kokkoli et al. reported that PR_b-functionalized PEO–PBD polymersomes (PR_b is an effective $\alpha_5\beta_1$ targeting peptide) efficiently delivered tumor necrosis factor-α (TNFα) to LNCaP human prostate cancer cells resulting in dramatic enhancement of cytotoxic potential of TNFα [23]. Jiang et al. reported that tetrandrine and doxorubicin-loaded lactoferrin-conjugated polymersomes could pass blood–brain bar-

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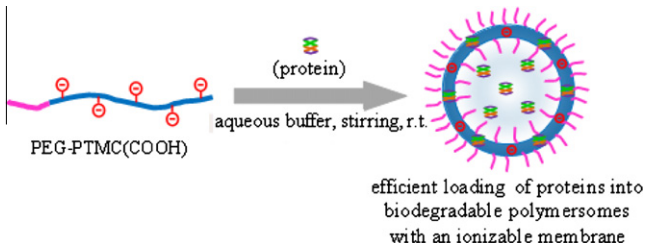


Scheme 1. Biodegradable polymersomes with an ionizable membrane allow efficient loading of proteins.

rier (BBB), accumulate at the tumor site, and shrink the tumor in glioma-bearing rats [24]. However, these polymersomes revealed low protein loading contents and loading efficiencies. Moreover, protein loading into polymersomes often involves organic solvents, which might result in protein denaturation and/or inactivation. We recently reported that biodegradable chimaeric polymersomes based on asymmetric PEG–PCL–PDEA triblock copolymers and reduction/temperature dual-sensitive polymersomes based on PEG–PAA–PNIPAM triblock copolymers could efficiently load exogenous proteins likely due to existing electrostatic interactions between proteins and ionizable PDEA or PAA blocks [18,19]. Jin et al. also reported that polymersomes with asymmetric membrane produced by phase-guided assembly method could encapsulate erythropoietin with high efficiency [25].

In this paper, we report on functional biodegradable polymersomes with an ionizable membrane obtained from poly(ethylene glycol)-*b*-poly(trimethylene carbonate) (PEG–PTMC) block copolymer derivatives containing carboxylic acid or amine groups along PTMC block for efficient loading and intracellular release of proteins (Scheme 1). These novel charged polymersomes were designed based on the following rationale: (i) PTMC is biocompatible and biodegradable to non-toxic products *in vivo* [26,27], and Feijen et al. reported formation of polymersomes from biodegradable PEG–PTMC block copolymer [28]. Lecommandoux et al. reported that polymersomes based on poly(trimethylene carbonate)–poly(L-glutamic acid) (PTMC–PGA) were prone to rapid enzymatic degradation [29,30]. (ii) unlike other biodegradable polymers such as polylactide and poly(ε-caprolactone), PTMC with molecular weights higher than 15.0 kg/mol is highly flexible at room temperature [26,27] that facilitates formation of polymersomes under mild conditions, minimizing protein denaturation and deactivation; (iii) charged membrane gives rise to efficient loading and stabilization of proteins through electrostatic interactions; and (iv) furthermore release of proteins might be accelerated under mildly acidic conditions due to weakened protein–membrane interactions, leading to efficient intracellular protein release. In this study, preparation of membrane-ionizable biodegradable polymersomes, loading of proteins, as well as *in vitro* and intracellular release of proteins was investigated.

2. Materials and methods

2.1. Materials

Methoxy poly(ethylene glycol) (PEG, M_n = 5.0 kg/mol, Fluka) was dried by azeotropic distillation from anhydrous toluene. Trimethylene carbonate (TMC) was recrystallized over dry toluene. Dichloromethane (DCM) was dried by refluxing over CaH_2 and distilled prior to use. Zinc bis[bis(trimethylsilyl)amide] (97%, Aldrich), cysteamine hydrochloride (>98%, Alfa Aesar), 3-mercaptopropionic acid (99%, Alfa Aesar), pyrene (97%, Fluka), doxorubicin hydrochloride (DOX·HCl) (99%, Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd.), cytochrome C (CC) from equine heart (Sigma), bovine serum albumin V fraction (>98%, Roche), fluorescein isothiocyanate (95%, Fluka), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)diammonium salt (ABTS, Amresco), and 4,6-diamidino-2-phenylindole (DAPI) were used without further purification. Annexin V-FITC/propidium iodide (PI, KeyGEN tech.) were purchased and used according to the supplier's instruction. Acryloyl carbonate (AC) monomer was synthesized according to a previous report [31]. Spectra/Pore® dialysis membranes (MWCO 500 kDa) were purchased from Spectrum Laboratories Inc., USA.

2.2. Characterization

The ^{1}H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using deuterated chloroform ($CDCl_3$) as a solvent. The chemical shifts were calibrated against residual solvent signal. The molecular weight and polydispersity of 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 DMF as an 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 thermal properties of the copolymers were determined by differential scanning calorimetry (DSC, Q20, TA). The samples were heated from −80 °C to 120 °C at a rate of 20 °C/min, kept at 120 °C for 2 min, cooled to −80 °C at a rate of 300 °C/min, kept at −80 °C for 2 min, and then a second heating scan from −80 °C to 120 °C at a rate of 20 °C/min was recorded. The second heating curves were used to determine the T_m and T_g. The size and size distribution of polymersomes were determined at 25 °C using dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern Instruments) equipped with a 633 nm He–Ne laser using back-scattering detection. Zeta potential measurements were carried out using Zetasizer Nano-ZS instrument (Malvern) equipped with a standard capillary electrophoresis cell. The measurements were performed in triplicate. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 120 kV. The samples were prepared by dropping 10 μL of 0.2 mg/mL polymersome suspension on the copper grid followed by staining with phosphotungstic acid (1 wt.%). The CLSM images of polymersomes and cellular uptake were taken on a confocal laser scanning microscope (TCS SP2).

2.3. Synthesis of PEG–PTMC(AC) diblock copolymer

Under a N_2 atmosphere, to a solution of PEG (0.51 g, 0.10 mmol), trimethylene carbonate (1.83 g, 18 mmol) and AC (0.20 g, 1.0 mmol) in DCM (25 mL) was added with stirring a solution of zinc bis[bis(trimethylsilyl)amide] (21 mg, 55 μmol). The reaction was allowed to proceed at 40 °C for 24 h before termination with acetic acid. The product was isolated by precipitation in cold diethyl ether, filtration, and drying *in vacuo* for 2 d. Yield: 94%. ^{1}H NMR (400 MHz, $CDCl_3$): PEG (—$\underline{CH_2}$—$\underline{CH_2}$—O—: δ 3.63; $\underline{CH_3}$—O—: δ 3.38), PTMC (—OCO—$\underline{CH_2}$—CH_2—$\underline{CH_2}$—OCO—: δ 4.16; —OCO—CH_2—$\underline{CH_2}$—CH_2—OCO—: δ 2.06) and PAC (—OCO—$\underline{CH_2{=}CH_2}$: δ 6.47, 6.15 and 5.85; —OCO—$\underline{CH_2}$—C—$\underline{CH_2}$—OCO—: δ 4.16; —C—$\underline{CH_2}$—OCO—: δ 4.13; $\underline{CH_3}$—C—: δ 1.07). M_n (^{1}H NMR) = 25.5 kg/mol, M_n (GPC) = 36.2 kg/mol, PDI (GPC) = 1.35.

2.4. Synthesis of PEG–PTMC(COOH) and PEG–PTMC(NH_2) Copolymers

PEG–PTMC(COOH) and PEG–PTMC(NH_2) copolymers were prepared by Michael-type conjugate addition reaction of PEG–PTMC(AC) with 3-mercaptopropionic acid or cysteamine hydrochloride. In a typical experiment, PEG–PTMC(AC) (0.21 g,

80 μmol acrylate group) and 3-mercaptopropionic acid (84.8 mg, 0.80 mmol) were dissolved in 2 mL of DMF. After blowing N_2 for 0.5 h, pyridine (62.2 mg, 0.80 mmol, at a mole ratio of AC/SH/pyridine = 1/10/10) was added. The reaction was allowed to proceed at room temperature for 2 d under a nitrogen atmosphere. The product, denoted as PEG–PTMC(COOH), was isolated by precipitation in cold diethyl ether/ethanol (4/1, v/v), filtration, and drying *in vacuo* for 2 d. Yield: 80%. ^{1}H NMR (400 MHz, $CDCl_3$): PEG (—$\underline{CH_2}$—$\underline{CH_2}$—O—: δ 3.63; $\underline{CH_3}$—O—: δ 3.38), PTMC (—OCO—$\underline{CH_2}$—CH_2—$\underline{CH_2}$—OCO—: δ 4.16; —OCO—CH_2—$\underline{CH_2}$—CH_2—OCO—: δ 2.06), PAC (—OCO—CH_2—C—$\underline{CH_2}$—OCO—: δ 4.16; —C—$\underline{CH_2}$—OCO—: δ 4.13; $\underline{CH_3}$—C—: δ 1.07) and methylene groups (—OCO—CH_2—$\underline{CH_2}$—S—$\underline{CH_2}$—CH_2—COOH: δ 2.81; —OCO—$\underline{CH_2}$—CH_2—S—CH_2—$\underline{CH_2}$—COOH: δ 2.68). M_n (^{1}H NMR) = 26.1 kg/mol, M_n (GPC) = 39.7 kg/mol, PDI (GPC) = 1.37.

PEG–PTMC(NH_2) was synthesized in a similar way. Yield: 89%. ^{1}H NMR (400 MHz, $CDCl_3$): PEG (—$\underline{CH_2}$—$\underline{CH_2}$—O—: δ 3.63; $\underline{CH_3}$—O—: δ 3.38), PTMC (—OCO—$\underline{CH_2}$—CH_2—$\underline{CH_2}$—OCO—: δ 4.16; —OCO—CH_2—$\underline{CH_2}$—CH_2—OCO—: δ 2.06), PAC (—OCO—$\underline{CH_2}$—C—$\underline{CH_2}$—OCO—: δ 4.16; —C—$\underline{CH_2}$—OCO—: δ 4.13; $\underline{CH_3}$—C—: δ 1.07), methylene groups (—OCO—CH_2—CH_2—S—CH_2—$\underline{CH_2}$—NH_2: δ 2.97; —OCO—CH_2—$\underline{CH_2}$—S—$\underline{CH_2}$—CH_2—NH_2: δ 2.81; —OCO—$\underline{CH_2}$—CH_2—S—CH_2—CH_2—NH_2: δ 2.68) and amino group(—S—CH_2—CH_2—$\underline{NH_2}$: δ 4.73). M_n (^{1}H NMR) = 26.2 kg/mol, M_n (GPC) = 39.8 kg/mol, PDI (GPC) = 1.41.

2.5. Polymersome formation and determination of critical aggregation concentration (CAC)

The polymersomes were readily prepared by dispersing 0.5 mg of copolymer in 1.0 mL of PB buffer (10 mM, pH 7.4) with stirring at room temperature for 8 h, which yielded a homogenous dispersion. The CAC of copolymers was determined using pyrene as a fluorescence probe. The polymer concentration varied from 1.0×10^{-5} to 5×10^{-2} mg/mL, and pyrene concentration 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. The stability of polymersomes against extensive dilution or 150 mM NaCl was evaluated with DLS through monitoring polymersome sizes and size distributions.

2.6. MTT assays

MCF-7 or HeLa cells were grown on a 96-well plate for 24 h (1×10^4 cells/well) using Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 mg/mL). The prescribed amounts of PEG–PTMC(AC), PEG–PTMC(COOH), or PEG–PTMC(NH_2) polymersomes were added and incubated at 37 °C in an atmosphere containing 5% CO_2 for 24 h. Then, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) solution in PBS (5.0 mg/mL) was added and incubated for another 4 h. The medium was aspirated, the MTT-formazan generated by live cells was dissolved in 150 μL of 10% DMSO, and the absorbance at a wavelength of 490 nm of each well was measured using a microplate reader (Bio-Tek, ELX808 IU). The cell viability (%) was determined by comparing the absorbance at 490 nm with control wells containing only cell culture medium. The experiments were performed in tetraplets.

2.7. Loading of proteins into polymersomes

Protein-loaded polymersomes were readily prepared by dispersing 0.5 mg of copolymer in 1.0 mL of FITC-BSA or FITC-CC protein solution in PB buffer (10 mM, pH 7.4) at room temperature for 12 h followed by extensive dialysis (MWCO 500 kDa, Spectra/Pore®) against PB (10 mM, pH 7.4) to remove free proteins. The control experiments on free proteins showed that this purification procedure is sufficient to remove free proteins if present. The theoretical protein loading contents (PLC) were set at 5, 10, 20, and 50 wt.% (corresponding to protein concentrations of 0.05, 0.1, 0.25, and 0.5 mg/mL, respectively). In order to determine protein loading contents, protein-loaded polymersome dispersion was supplemented with three times its volume of DMSO. The amount of FITC-BSA and FITC-CC was determined by fluorometry (FLS920) with excitation 492 nm and emission from 500 to 600 nm on. The calibration curve was established by fluorometry with known concentrations of corresponding FITC-labeled proteins in DMSO/water (3/1, v/v). PLC and protein loading efficiency (PLE) were calculated according to the following formula:

$$\text{PLC (wt.\%)} = (\text{weight of loaded protein/weight of polymer}) \times 100\%$$

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

2.8. In vitro protein release

The release of FITC-BSA and FITC-CC from polymersomes was investigated in two different media, that is, PBS buffer (pH 7.4, 10 mM, 150 mM NaCl) and MES buffer (pH 5.4, 10 mM, 150 mM NaCl). Briefly, 500 μL of protein-loaded polymersomes in PB buffer (10 mM, pH 7.4, 0.5 mg/mL) was dialyzed (MWCO 500 kDa) against 30 mL of above release media. At desired time intervals, 5 mL of 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 510 to 600 nm). The release experiments were conducted in triplicate, and the results presented are the average data with standard deviations.

2.9. ABTS assays on enzymatic activity of released CC

The electron transfer activity of CC was measured by examining the catalytic conversion of 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). CC was released overnight from PEG–PTMC(COOH) polymersomes in MES buffer (pH 5.4, 10 mM, NaCl 150 mM), and the amount of released CC was determined using BCA protein assay (Pierce). The released CC was diluted by the same MES to a final concentration of 4.0 μg/mL. Then, 100 μL of hydrogen peroxide solution (45 mM) and 100 μL of ABTS solution (1.0 mg/mL) in MES buffer were added. The absorbance at 418 nm of the oxidized product was monitored every 15 s for 4 min. The native CC was used as a control.

2.10. Flow cytometry

MCF-7 cells were grown on a 24-well plate (5×10^4 cells/well) under 5% CO_2 atmosphere at 37 °C using DMEM medium supplemented with 10% fetal bovine serum, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/ml) for 24 h. The cells were treated with free CC, CC-loaded PEG–PTMC(COOH) polymersomes, or CC-loaded PEG–PTMC(NH_2) polymersomes (CC

dosage: 80 μg/mL) for 24 and 48 h under 5% CO_2 atmosphere at 37 °C. To quantify apoptotic cells, Annexin V-FITC kit was used as described by the manufacturer (KenGEN, China). Briefly, MCF-7 cells were digested with EDTA-free trypsin, washed twice with cold PBS, and re-suspended in binding buffer at a concentration of 1×10^5 cells/mL. Then, the cells were stained with 5 μL of Annexin V-FITC solution and 5 μL of propidium iodide (PI) solution for 15 min at room temperature in the dark. At the end of incubation, 400 μL of binding buffer was added, and the cells were analyzed immediately using flow cytometry (BD FACSCalibur, Mountain View, CA).

2.11. Cellular uptake observed by confocal laser scanning microscopy (CLSM)

MCF-7 cells were grown on microscope cover slides in a 24 well plate (5×10^4 cells/well) under 5% CO_2 atmosphere at 37 °C using DMEM medium supplemented with 10% fetal bovine serum, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL) for 24 h. 100 μL of FITC-CC loaded PEG–PTMC(COOH) polymersomes, FITC-CC loaded PEG–PTMC(NH_2) polymersomes, or free FITC-CC (FITC-CC dosage: 80 μg/mL) of each was added. After 12 and 24 h incubation at 37 °C, the culture 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 three times with PBS. The cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, 100 μL, 10 μg/mL) for 20 min and washed three times with PBS. The images were taken using the confocal microscope (TCS SP2).

3. Results and discussion

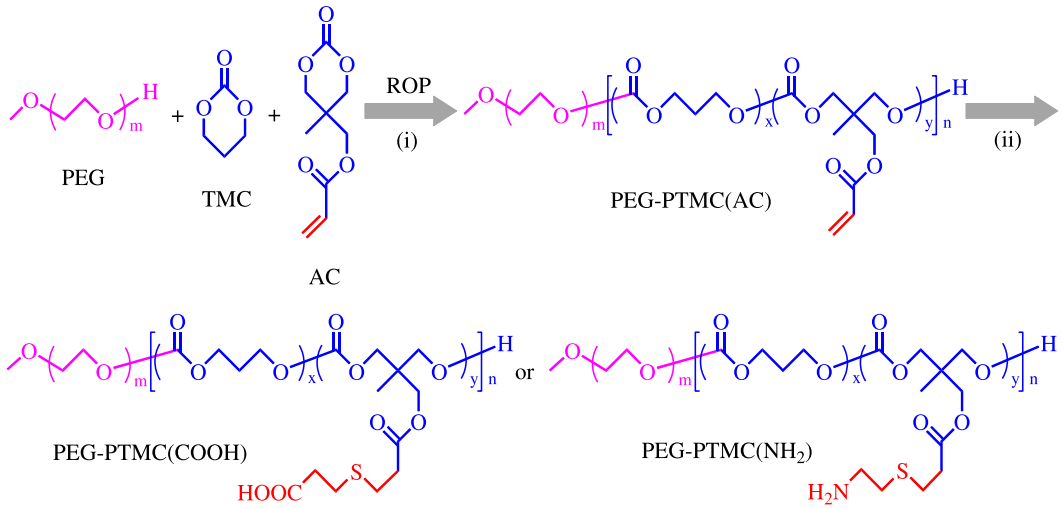
3.1. Synthesis of PEG–PTMC(AC), PEG–PTMC(COOH) and PEG–PTMC(NH2) copolymers

The aim of this study was to investigate the effects of ionizable membrane on protein loading and release behaviors of biodegradable polymersomes, for which poly(ethylene glycol)-*b*-poly(trimethylene carbonate) (PEG–PTMC) block copolymer derivatives containing acrylate, carboxylic acid, and amine groups along PTMC block, denoted as PEG–PTMC(AC), PEG–PTMC(COOH), and PEG–PTMC(NH_2), respectively, were designed. PEG–PTMC(AC) was readily synthesized by ring-opening copolymerization of trimethylene carbonate (TMC) and acryloyl carbonate (AC) using monomethoxyl PEG (M_n = 5.0 kg/mol) as an initiator and zinc bis[bis(trimethylsilyl)amide] as catalyst in dichloromethane at 40 °C (Scheme 2), as reported previously [31–33]. ^{1}H NMR showed signals at δ 3.63, 5.85–6.47, and 2.06, which were attributable to the methylene protons of PEG, acryloyl protons of AC units, and middle methylene protons of PTMC, respectively (Fig. 1A). The M_n of PEG–PTMC(AC) was determined to be approximately 5–19.5 kg/mol by comparing integrals of signals at δ 5.85–6.47 and 2.06 with 3.63, which was close to the design (5–20.0 kg/mol). The number of AC functionality per polymer chain (N_f) was calculated to be about 10. Gel permeation chromatography (GPC) showed that PEG–PTMC(AC) had a unimodal distribution with an M_n of 36.2 kg/mol and an M_w/M_n of 1.35 (Table 1, entry 1). The higher M_n value determined by GPC than that determined by ^{1}H NMR is most likely due to use of polystyrene standards for molecular weight calibration in our GPC measurements.

PEG–PTMC(COOH) and PEG–PTMC(NH_2) were prepared through post-polymerization modification of PEG–PTMC(AC) with 3-mercaptopropionic acid and cysteamine hydrochloride, respectively, via Michael-type addition at room temperature in DMF (Scheme 2). ^{1}H NMR spectra showed that signals assignable to the acryloyl protons (δ 5.85–6.47) completely disappeared while new peaks owing to methylene protons next to the carbonyl and thiol ether groups appeared at δ 2.68–2.97 (Fig. 1B and C). Moreover, the integral ratio between the signals attributable to the methylene protons next to the carbonyl (δ 2.68) or amino (δ 2.97) groups and that of the methyl protons of PAC block (δ 1.07) was close to the theoretical values within the experimental error, supporting quantitative functionalization. GPC measurements showed that PEG–PTMC(COOH) and PEG–PTMC(NH_2) maintained moderate molecular weight distribution (M_w/M_n = 1.37–1.41) and had slightly higher M_n than PEG–PTMC(AC) (Table 1, Entries 2 and 3). Differential scanning calorimetry (DSC) measurements demonstrated that PEG–PTMC(AC), PEG–PTMC(COOH) and PEG–PTMC(NH_2) were amorphous with glass transition temperatures (T_g) of −16.4, −23.1, and −17.8 °C, respectively (Table 1). Hence, these functional biodegradable copolymers, similar to PTMC, are highly flexible at physiological temperature.

3.2. Formation of biodegradable polymersomes

Interestingly, nano-sized polymersomes were obtained by directly dispersing PEG–PTMC(AC), PEG–PTMC(COOH), and PEG–PTMC(NH_2) copolymers in PB buffer at room temperature. Dy-



Scheme 2. Synthesis of PEG–PTMC(AC), PEG–PTMC(COOH), and PEG–PTMC(NH_2) copolymers. Conditions: (i) CH_2Cl_2, zinc bis[bis(trimethylsilyl)amide], 40 °C, 24 h; (ii) 3-mercaptopropionic acid or cysteamine hydrochloride, DMF, r.t., 48 h.

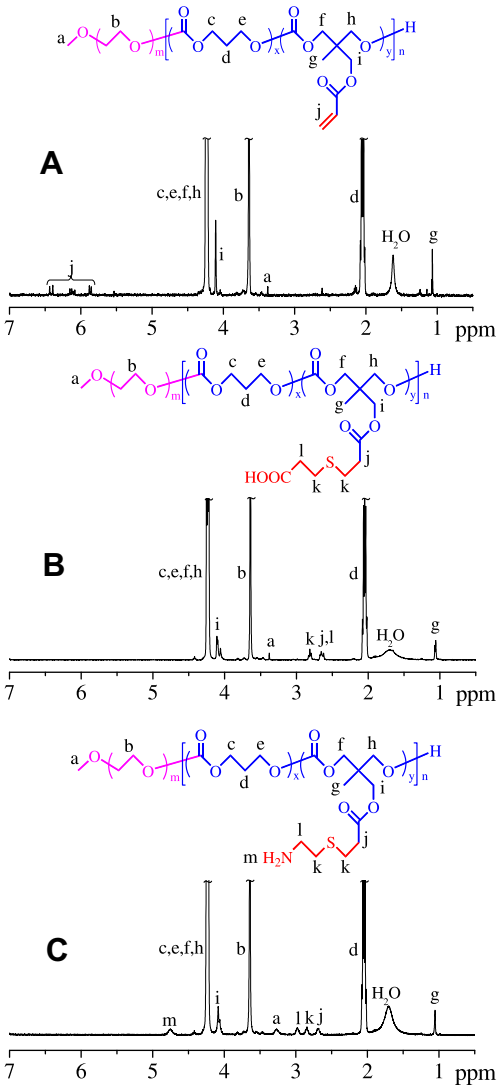


Fig. 1. ^{1}H NMR spectra (400 MHz, $CDCl_3$) of PEG–PTMC(AC) (A), PEG–PTMC(COOH) (B), and PEG–PTMC(NH_2) (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

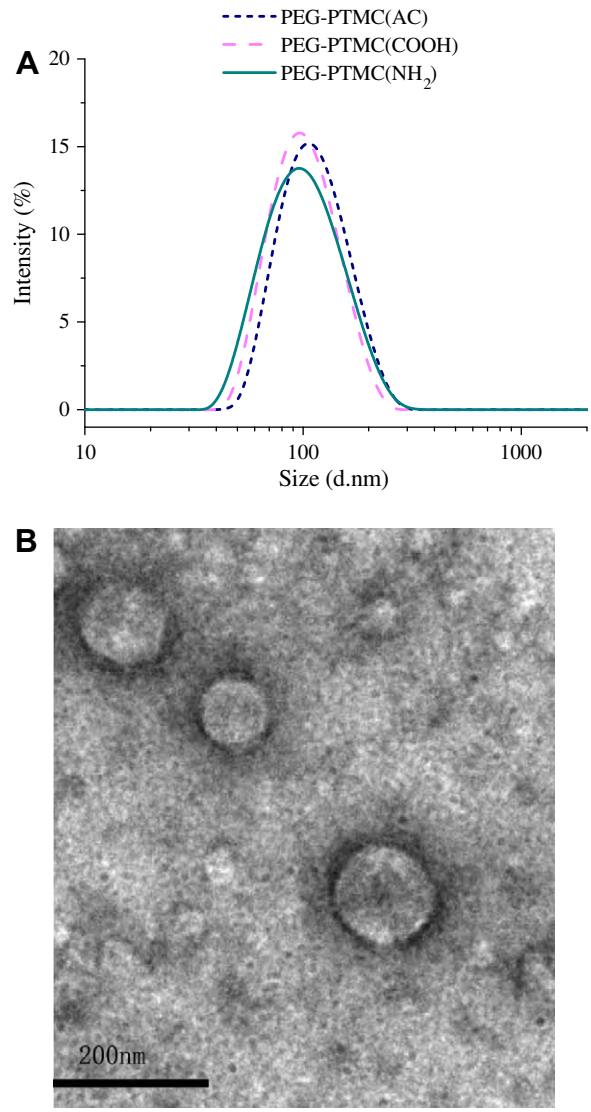


Fig. 2. The size distribution profiles of PEG–PTMC(AC), PEG–PTMC(COOH), and PEG–PTMC(NH_2) polymersomes determined by DLS (A) and TEM micrograph of PEG–PTMC(COOH) polymersomes (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Synthesis of PEG–PTMC(AC), PEG–PTMC(COOH), and PEG–PTMC(NH_2) copolymers.

Entry	Copolymer	M_n (kg/mol)		M_w/M_n[b] (GPC)	N_f[c]	T_g[d] (°C)
		^{1}H NMR[a]	GPC[b]			
1	PEG–PTMC(AC)	5.0–19.5	36.2	1.35	10	−16.4
2	PEG–PTMC(COOH)	5.0–20.3	39.7	1.37	10	−23.1
3	PEG–PTMC(NH_2)	5.0–20.4	39.8	1.41	10	−17.8

[a] Calculated from ^{1}H NMR spectra (400 MHz, $CDCl_3$).
[b] Determined by GPC (eluent: DMF, flow rate: 1.0 mL/min, standards: polystyrene, 30 °C).
[c] Number of functional groups per polymer chain determined by ^{1}H NMR.
[d] Glass transition temperature determined by DSC.

namic light scattering (DLS) showed that all polymersomes had low polydispersity indexes (PDI) in the range of 0.14–0.18 and polymersomes of PEG–PTMC(COOH) and PEG–PTMC(NH_2) had smaller sizes than PEG–PTMC(AC) polymersomes (96.7 and 95.1 nm *versus* 110.6 nm) (Fig. 2A). It should be noted that polymersome sizes could be tailored to certain extent by external preparation conditions (copolymer concentration, stirring rate, *etc.*). For example, the sizes of polymersomes with ionizable membranes decreased from ca. 110 nm to 80 nm with increasing polymer concentrations from 0.4 to 2 mg/mL at pH 6.4, while little size change was observed for PEG–PTMC(AC) polymersomes over the same concentration range. In addition, higher stirring rate caused faster formation of homogenous polymersome dispersions and somewhat smaller sizes. Notably, all polymersomes displayed negative zeta potentials (−1.8 to −6.1 mV), in which PEG–PTMC(COOH) polymersomes showed lower zeta potentials than PEG–PTMC(NH_2) counterparts (Table 2). These three copolymers had low PEG contents of 19.7–20.4 wt.%, which favors formation of vesicular structures [10]. TEM micrograph confirmed that these nanoparticles had vesicular structures (Fig. 2B). In addition, confocal laser scanning microscopy (CLSM) observations clearly showed co-localization of DOX·HCl (hydrophilic) and nile red (hydrophobic) (Fig. S1), in accordance with the polymersomal structure. The critical aggregation concentration (CAC) determined using pyrene as a fluorescence probe revealed that PEG–PTMC(COOH) and PEG–PTMC(NH_2) copolymers had slightly higher CAC than PEG–

Table 2
Polymersomes formed from PEG–PTMC(AC), PEG–PTMC(COOH), and PEG–PTMC(NH_2) copolymers in phosphate buffer (pH 7.4, 10 mM) at a polymer concentration of 0.5 mg/mL.

Entry	Copolymer	Size (nm)[a]	PDI [a]	ζ (mV)[b]	CAC (mg/L)[c]
1	PEG–PTMC(AC)	110.6 ± 2.2	0.18	−3.7 ± 0.6	6.7
2	PEG–PTMC(COOH)	96.7 ± 1.9	0.14	−6.1 ± 0.9	10.1
3	PEG–PTMC(NH_2)	95.1 ± 2.2	0.15	−1.8 ± 0.5	16.6

[a] Determined by DLS in PB (pH 7.4, 10 mM).
[b] Determined by zeta potential measurements in PB (pH 7.4, 10 mM).
[c] Critical aggregation concentration (CAC) determined using pyrene as a fluorescence probe.

PTMC(AC) (Table 2), likely due to their more hydrophilic nature. The stability tests showed that both PEG–PTMC(AC) and PEG–PTMC(COOH) polymersomes were sufficiently stable against dilution (a concentration lower than CAC) as well as physiological salt (150 mM NaCl) conditions (Fig. S2). These results demonstrated that PEG–PTMC(AC), PEG–PTMC(COOH), and PEG–PTMC(NH_2) copolymers can self-assemble after 8 h stirring into well-defined polymersomes in aqueous solutions, possibly due to their low T_g and excellent flexibility. Albertsson and Feijen reported that PTMC with molecular weight higher than 15.0 kg/mol is amorphous [26,27]. PTMC with an M_n of 19.5 kg/mol is highly flexible at room temperature that would facilitate polymersome formation under mild conditions. Lecommandoux et al. prepared polymersomes based on PTMC–poly(L-glutamic acid) ($PTMC_{24}$–PGA_{12}), in which PTMC with an M_n of 2.75 kg/mol had a T_m of ca. 37 °C, by direct dissolution at 50 °C or nano-precipitation using organic solvents [29,30]. Good polymersomes could be prepared from PEG–PCL based copolymers only after 12 h stirring at 60 °C, or long time stirring (e.g. 1–2 days) at room temperature, or with assistance of an organic solvent [11,18,28]. MTT assays indicated that PEG–PTMC(AC), PEG–PTMC(COOH), and PEG–PTMC(NH_2) polymersomes were practically non-toxic to MCF-7 and HeLa cells at a concentration of up to 0.75 mg/mL and 1.0 mg/mL, respectively (Fig. S3).

3.3. Loading of proteins into polymersomes

To investigate how ionizable membrane will influence protein loading into polymersomes, two model proteins, FITC-labeled bovine serum albumin (FITC-BSA) and cytochrome C (FITC-CC), were encapsulated into PEG–PTMC(AC), PEG–PTMC(COOH), and PEG–PTMC(NH_2) polymersomes. The theoretical protein loading contents (PLC) were set at 10, 20, and 50 wt.% for FITC-BSA while 5, 10, and 20 wt.% for FITC-CC. Interestingly, the results showed that PEG–PTMC(COOH) and PEG–PTMC(NH_2) polymersomes had remarkably high protein loading efficiencies (PLE) of 86.4–92.1% and 94.5–97.8%, respectively, for FITC-BSA, whereas PEG–PTMC(AC) polymersomes with a neutral membrane displayed much lower loading efficiencies of 41.6–51.7% under otherwise the same conditions (Table 3). In case of FITC-CC, PEG–PTMC(COOH), and PEG–PTMC(NH_2) polymersomes showed over four times higher PLE than PEG–PTMC(AC) polymersomes at the same theoretical PLC, in which loading efficiencies of 51.0–80.9% and 50.1–83.2% were observed for PEG–PTMC(COOH) and PEG–PTMC(NH_2) polymersomes, respectively (Table 4). In contrast, PEG–PTMC(AC) polymersomes had low loading efficiencies of 8.0–18.8% for FITC-CC. In a control experiment, preformed PEG–PTMC(AC), PEG–PTMC(COOH), and PEG–PTMC(NH_2) polymersomes were incubated with FITC-BSA at BSA/polymer ratio of 20 wt.%. The same work-up procedure led to PLE of 3.1–4.6% (Table S1), which were significantly lower than that observed for protein-loaded PEG–PTMC(COOH) and PEG–PTMC(NH_2) polymersomes (PLE 87.1–95.9%). These results indicate that proteins are mostly encapsulated inside the polymersomes with minimal protein association with the outer surface of polymersomes, possibly due to the nonfouling nature of PEG shell. Furthermore, polymersomes with positively and negatively charged membranes had similar protein loading efficacies. It is surprising to note that high loading efficiencies were obtained even for proteins with the same net charge as the polymersome membrane. For example, PEG–PTMC(COOH) polymersomes with negatively charged membranes could efficiently encapsulate BSA of the same net charge (PI 4.9) at pH 7.4. It is likely that the positively charged patches on BSA molecule surfaces might serve as multivalent counter-ions of the charged carboxyl groups in the polymersome membranes, as reported previously for BSA interaction with PAA (PMPA theory) [34]. Similar phenomenon was also observed for PEG–PAA–PNIPAM, PEG–PCL–PDEA, and PEG–SS–PDEA polymersomes [18,19,35].

Notably, the average sizes of polymersomes slightly decreased following loading with low amounts of FITC-BSA (10 wt.%), while increased with further increasing protein loading levels (Table 3). For example, sizes of FITC-BSA loaded PEG–PTMC(COOH) polymersomes increased from 83.3, 104.1, to 110.9 nm when increasing PLC from 9.2, 17.4, to 43.2 wt.%. However, no significant size change was observed for FITC-CC loaded polymersomes (Table 4). It should be noted that protein-loaded polymersomes based on PEG–PTMC(COOH) and PEG–PTMC(NH_2) maintained a low PDI (in between 0.08–0.26) as well as slightly negative zeta potentials (Tables 3 and 4).

3.4. In vitro release of proteins

The *in vitro* release of proteins was studied at 37 °C in two different pH media, that is, phosphate buffered saline (PBS, 10 mM, pH 7.4, 150 mM NaCl) and MES buffered saline (10 mM, pH 5.4,

Table 3
Characteristics of FITC-BSA-loaded polymersomes in PB (pH 7.4, 10 mM) at a polymer concentration of 0.5 mg/mL at different FITC-BSA weight ratio of 10–50 wt.% (n = 5).

Copolymer	FITC-BSA ratio (wt.%)	Size[a] (nm)	PDI	ζ[b] (mV)	PLC[c] (wt.%)	PLE[c] (%)
PEG–PTMC(AC)	10	101.4 ± 0.9	0.20	−3.3 ± 0.6	5.2	51.7
	20	112.3 ± 2.3	0.23	−3.7 ± 0.5	9.5	47.6
	50	118.9 ± 1.1	0.21	−3.2 ± 0.9	20.8	41.6
PEG–PTMC(COOH)	10	83.3 ± 1.5	0.17	−7.4 ± 0.8	9.2	92.1
	20	104.1 ± 0.3	0.13	−7.3 ± 0.3	17.4	87.1
	50	110.9 ± 0.8	0.24	−6.9 ± 0.9	43.2	86.4
PEG–PTMC(NH_2)	10	90.8 ± 1.6	0.25	−2.0 ± 0.6	9.8	97.8
	20	94.1 ± 1.0	0.08	−1.5 ± 0.4	19.2	95.9
	50	104.6 ± 4.1	0.19	−2.9 ± 0.4	47.3	94.5

[a] Determined by DLS in PB (pH 7.4, 10 mM).
[b] Determined by zeta potential measurements in PB (pH 7.4, 10 mM).
[c] PLE and PLC were determined by using fluorometry.

Table 4
Characteristics of FITC-CC loaded polymersomes in phosphate buffer (pH 7.4, 10 mM) at a polymer concentration of 0.5 mg/mL at different FITC-CC weight ratio of 5–20 wt.% ($n = 5$).

Copolymer	FITC-CC ratio (wt.%)	Size[a] (nm)	PDI	ζ[b] (mV)	PLC[c] (wt.%)	PLE[c] (%)
PEG–PTMC(AC)	5	113.3 ± 3.4	0.21	−2.4 ± 0.8	0.9	18.8
	10	106.8 ± 1.5	0.24	−1.9 ± 0.2	1.2	11.5
	20	111.1 ± 2.2	0.31	−3.3 ± 0.9	1.6	8.0
PEG–PTMC(COOH)	5	94.3 ± 0.9	0.14	−4.1 ± 0.5	4.1	80.9
	10	94.9 ± 2.1	0.20	−5.3 ± 0.9	6.7	66.7
	20	98.9 ± 2.8	0.26	−3.8 ± 0.6	10.2	51.0
PEG–PTMC(NH_2)	5	93.5 ± 1.5	0.19	−0.7 ± 0.4	4.1	83.2
	10	100.3 ± 1.4	0.20	−0.8 ± 0.6	5.7	56.9
	20	102.0 ± 1.0	0.24	−0.6 ± 0.8	10.0	50.1

[a] Determined by DLS in PB (pH 7.4, 10 mM).
[b] Determined by zeta potential measurements in PB (pH 7.4, 10 mM).
[c] PLE and PLC were determined by using fluorometry.

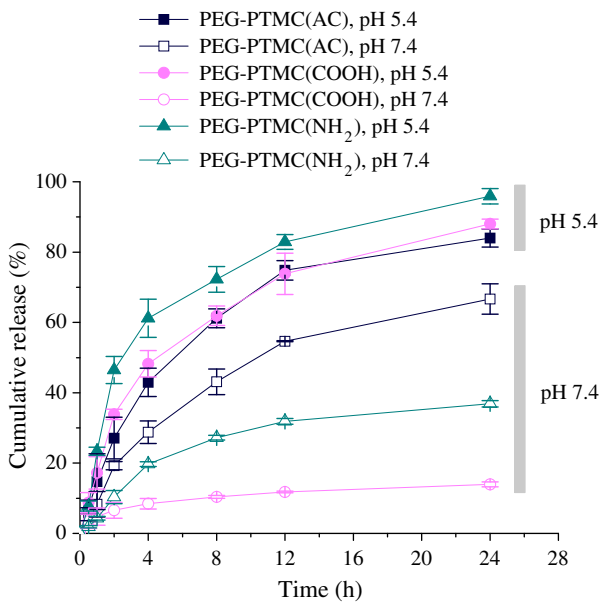


Fig. 3. *In vitro* protein release from FITC-CC-loaded polymersomes at pH 5.4 (MES, 10 mM, 150 mM NaCl) and pH 7.4 (PB, 10 mM, 150 mM NaCl). The initial polymersome concentration was set at 0.5 mg/mL. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

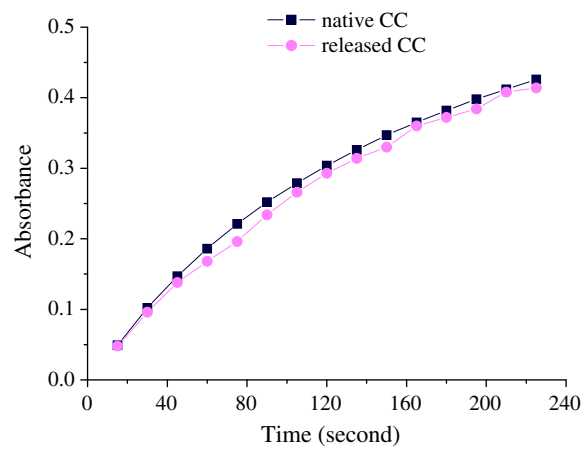


Fig. 4. Oxidation of ABTS catalyzed by cytochrome C released from PEG–PTMC(COOH) polymersomes and native cytochrome C at pH 5.4 (MES, 10 mM, 150 mM NaCl). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

150 mM NaCl). Interestingly, the results showed that FITC-CC-loaded PEG–PTMC(COOH) and PEG–PTMC(NH_2) polymersomes released FITC-CC in a pH-dependant manner, in which significantly enhanced protein release was observed at pH 5.4 (Fig. 3). For example, FITC-CC-loaded PEG–PTMC(COOH) polymersomes released 88.0% FITC-CC in 24 h at pH 5.4 while only 13.9% FITC-CC was released in 24 h at pH 7.4 under otherwise the same conditions. In case of FITC-CC-loaded PEG–PTMC(NH_2) polymersomes, 36.9% and 95.9% release of FITC-CC was observed at pH 7.4 and 5.4, respectively. In contrast, FITC-CC-loaded PEG–PTMC(AC) polymersomes displayed only small difference in release rate, in which 66.7% and 84.0% FITC-CC was released at pH 7.4 and 5.4, respectively. Very similar release results were also observed for corresponding FITC-BSA-loaded polymersomes (Fig. S4). It should be noted that in a control experiment on release of free proteins, no significant diffusional barrier of the dialysis membrane was observed at both pH 5.4 and 7.4 (Fig. S5). This pH-dependant release behavior of "charged" polymersomes is likely due to the weakened interactions between protein and polymersome membrane at mildly acidic pH. It is likely that the release of proteins embedded in the polymersome membranes could form channels facilitating protein penetration through the membranes. These biodegradable polymersomes with low protein release under physiological pH and fast release at endosomal pH are highly interesting for intracellular protein delivery.

It is important that released proteins maintain their biological activity. Here, cytochrome C (CC) from horse heart was selected as a model protein. The enzymatic activity of CC released from the polymersomes was examined using ABTS assay. The results showed that CC released from polymersomes had similar enzymatic activity toward oxidation of ABTS as the native CC (Fig. 4), implying that released CC maintains its bioactivity [5,36].

3.5. Cellular uptake of FITC-CC-loaded polymersomes and apoptotic activity of CC-loaded polymersomes

The cellular uptake and release of proteins was studied in MCF-7 cells using confocal laser scanning microscopy (CLSM). The results showed that FITC-CC loaded polymersomes efficiently delivered and released proteins into cells, in which strong FITC-CC fluorescence was observed inside cells following 24 h incubation with FITC-CC-loaded PEG–PTMC(COOH) and PEG–PTMC(NH_2) polymersomes (Fig. S6A and B). In contrast, little fluorescence was detected in MCF-7 cells following 24 h treatment with free FITC-CC under otherwise the same conditions (Fig. S6C), probably

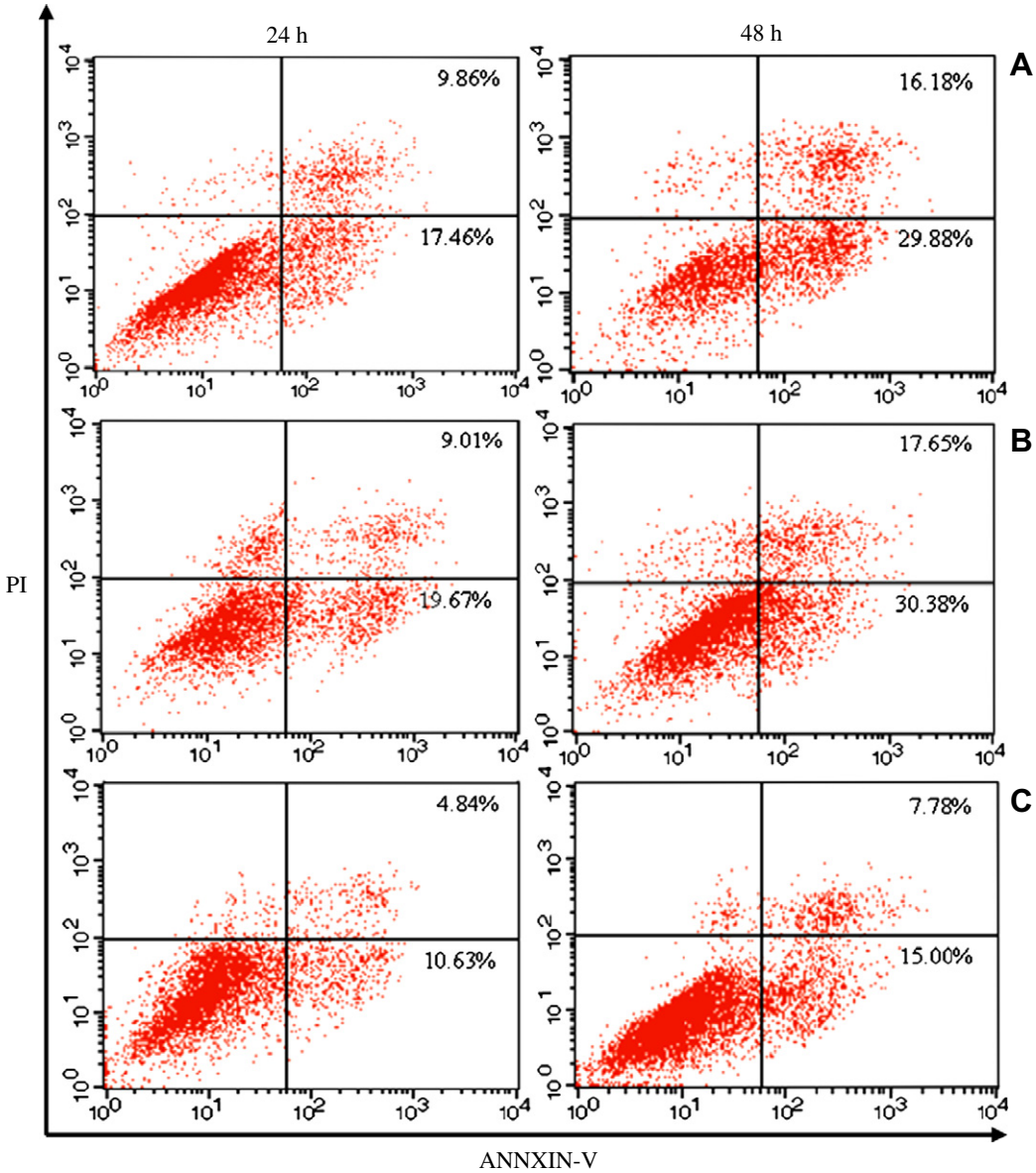


Fig. 5. Contour diagram of Annexin V-FITC/PI flow cytometry of MCF-7 cells after 48 h incubation with CC-loaded PEG–PTMC(COOH) polymersomes (A), CC loaded PEG–PTMC(NH_2) polymersomes (B) and free CC (C) (CC dosage: 80 μg/mL). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

due to their low permeability to cellular membranes and fast proteolytic degradation.

CC is known to play a significant role in programmed cell death [37,38]. The intracellularly injected CC was reported to cause effective cell apoptosis [39,40]. Recently Perez et al. reported that CC-loaded nanoparticles delivered CC into MCF-7 cells resulting in appreciable apoptosis [41]. In this study, the apoptotic activity of CC-loaded polymersomes was investigated in MCF-7 cells with flow cytometry using annexin V-FITC/propidium iodide (PI) staining, similar to our previous reports [18,19]. The CC dosage was set at 80 μg/mL. The results showed that CC-loaded polymersomes brought about significantly more pronounced apoptosis of MCF-7 cells as compared to the native CC (Fig. 5). For example, CC-loaded PEG–PTMC(COOH) and PEG–PTMC(NH_2) polymersomes induced apoptosis rates of 46.1% and 48.0%, respectively, after 48 h (Fig. 5A and B). In comparison, native CC brought about a much lower level of apoptosis (22.8%) under otherwise the same conditions (Fig. 5C). These initial results confirm that PEG–PTMC(COOH) and PEG–PTMC(NH_2) polymersomes are able to efficiently deliver and release CC into cancer cells, resulting in enhanced apoptotic activity as compared to native CC.

4. Conclusions

We have demonstrated that novel functional biodegradable polymersomes with an ionizable membrane can be facilely prepared by directly dispersing poly(ethylene glycol)-*b*-poly(trimethylene carbonate) (PEG–PTMC) block copolymer derivatives containing carboxylic acid or amine groups along PTMC block in aqueous solutions. In contrast with common biodegradable polymersomes, these "charged" polymersomes display remarkably high protein loading contents and loading efficiencies and furthermore exhibit a pH-dependant protein release behavior, that is, inhibited protein release at physiological pH while fast release at endosomal pH. The cellular studies have shown that these membrane ionizable biodegradable polymersomes are able to efficiently deliver and release cytochrome C into cancer cells, resulting in markedly enhanced apoptotic activity as compared to native

cytochrome C. The many unique features of membrane ionizable polymersomes including easy synthesis, superior protein loading, and endosomal pH-responsive protein release behavior render them highly appealing for targeted "active" intracellular protein release. We are currently developing ligand-decorated membrane ionizable biodegradable polymersomes for tumor-targeting delivery of various therapeutic proteins.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejpb.2012.05.009.

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