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PAPER

Reduction and temperature dual-responsive crosslinked polymersomes for targeted intracellular protein delivery

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The study of biological functions of proteins in cells as well as therapeutic exploration of many protein drugs demands efficient and nontoxic intracellular protein delivery systems. Herein, reduction and temperature dual-responsive crosslinked polymersomes were developed for the facile encapsulation of various proteins under mild conditions as well as rapid release of proteins in cancer cells. Two thermosensitive triblock copolymers, PEG_{5k}-$PAA_{1.7k}$-$PNIPAM_{22k}$ and PEG_{5k}-$PAA_{0.7k}$-$PNIPAM_{12k}$ (denoted as polymer **1** and **2**, respectively), were prepared by controlled reversible addition–fragmentation chain-transfer (RAFT) polymerization. Interestingly, polymers **1** and **2** exhibited lower critical solution temperatures (LCST) of 39 and 38 °C in PBS (pH 7.4, 20 mM, 150 mM NaCl) and 34 and 32 °C in MES (pH 5.5, 20 mM), respectively. Increasing the temperature of polymer solutions in MES to 40 °C yielded robust polymersomes with average diameters of *ca.* 150∼170 nm following crosslinking the PAA segment with cystamine (Cys) *via* carbodiimide chemistry. These crosslinked polymersomes kept their structures in PBS at 37 °C but rapidly dissociated into unimers in response to 10 mM dithiothreitol (DTT). Remarkably, various proteins including bovine serum albumin (BSA), lysozyme (Lys), cytochrome C (CC), and ovalbumin (Ova) could be conveniently loaded into the polymersomes with markedly high protein loading efficiencies of 60∼100% at theoretical protein loading contents of 10∼50 wt%. The *in vitro* release studies using Cys-crosslinked polymersome **1** showed that release of BSA, Lys, and CC was minimal (*ca.* 20%) in 11 h in PBS at 37 °C, while fast protein release of over 70% was observed under an intracellular mimicking reductive environment. MTT assays revealed that these polymersomes were practically non-toxic to HeLa and MCF-7 cells up to a tested concentration of 200 μg mL^{-1}. Confocal laser scanning microscope (CLSM) observations showed that FITC-CC loaded Cys-crosslinked polymersomes efficiently delivered and released FITC-CC into the cytosol of MCF-7 cells after 12 h incubation. In contrast, little FITC-CC fluorescence was observed in MCF-7 cells treated with free FITC-CC as well as FITC-CC loaded 1,4-butadiamine crosslinked polymersomes (reduction-insensitive control). Flow cytometry studies showed that CC loaded Cys-crosslinked polymersomes induced markedly enhanced apoptosis of MCF-7 cells as compared to free CC and the reduction-insensitive controls. These novel reduction and temperature dual-responsive crosslinked polymersomes have opened a new avenue to targeted intracellular protein delivery.

Introduction

The past two decades have witnessed remarkable progress in the development of therapeutic proteins and peptides for the effective treatment of various types of human diseases including cancers.[1–4] To bring forth therapeutic effects, many proteins such as cytochrome C, BAX and caspase-3 have to be transported to specific intracellular compartments like the cytoplasm.[5,6] However, protein drugs usually suffer rapid degradation following injection, potential immune response, inferior cellular uptake, and poor intracellular trafficking.[7] The clinical success of many protein drugs is, therefore, intimately dependent on the advancement of safe, efficient and economically viable targeted intracellular protein delivery systems. In the past years, various types of nano-vehicles[8] such as liposomes,[9] polyion complex micelles,[10] nano- and micro-particles[11,12] and nanocapsules[13] have been explored for intracellular protein delivery. These delivery approaches, however, require the use of (toxic) organic

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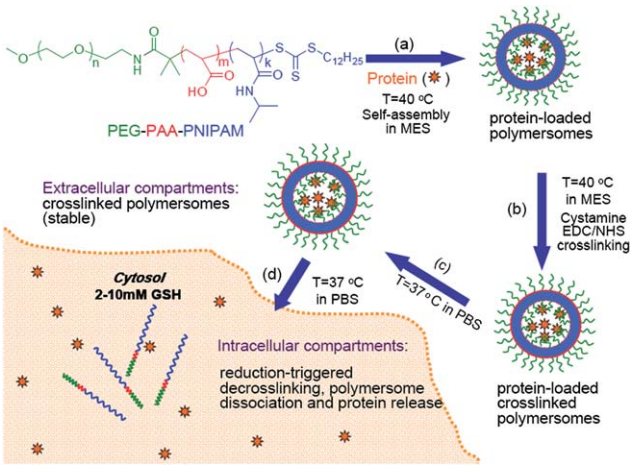
solvents possibly leading to protein denaturation, involve chemical modification of proteins, have low protein loading level, and/or display inadequate protein release inside cells.

In recent years, polymersomes with large aqueous compartments as well as robust hydrophobic membranes have emerged as ideal nano-carriers for encapsulation and controlled delivery of proteins and peptides.[14–16] In contrast to liposomes, most polymersomes are intrinsically stealthy since they are typically made from amphiphilic copolymers based on non-fouling polymers such as poly(ethylene glycol) (PEG), dextran and poly (acrylic acid) (PAA). Different kinds of proteins could be loaded into the aqueous lumen of polymersomes.[17,18] For example, Palmer and coworkers reported that hemoglobin loaded PEG-poly(ε-caprolactone) (PEG-PCL) polymersomes had similar oxygen affinities to human red blood cells.[19] Discher and coworkers explored encapsulation of insulin into PEG-PBD polymersomes.[20] Kokkoli and coworkers 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.[21] It should be noted, nevertheless, that polymersomes in general exhibit low protein loading contents and loading efficiency. We recently reported that biodegradable chimaeric polymersomes based on asymmetric PEG-PCL-PDEA (PDEA shorter than PEG) triblock copolymers could efficiently encapsulate a large amount of exogenous proteins, likely due to existing electrostatic interactions.[22] In a previous study, we found that freely water-soluble thermo-sensitive PEG-PAA-PNIPAM triblock copolymers can readily form robust nano-sized polymersomes through simply increasing the solution temperature to above their LCST (*ca.* 32 °C) followed by crosslinking with cystamine *via* carbodiimide chemistry.[23] Interestingly, FITC-dextran could be efficiently loaded into the polymersomes. Moreover, release studies showed that while most FITC-dextran was retained within the polymersomes after lowering the temperature to 25 °C (lower than LCST), fast release of FITC-dextran was achieved in the presence of 10 mM dithiothreitol (DTT) under otherwise the same conditions.[23]

In this paper, we report on the development of reduction and temperature dual-responsive crosslinked PEG-PAA-PNIPAM polymersomes, which have an elevated LCST of 38–39 °C under physiological conditions, for efficient loading of proteins under mild conditions as well as rapid release of proteins under an intracellular-mimicking reductive environment or in cancer cells (Scheme 1). In the past years, various types of stimuli-responsive polymersomes have been studied for programmed drug delivery.[24,25] In particular, reduction-sensitive nano-vehicles are promising for specific intracellular drug and gene delivery, due to their high stability in circulation while fast degradation inside cells.[26,27] Here, synthesis of dual-responsive polymersomes, loading and reduction-triggered release of various proteins, intracellular protein release of FITC-CC loaded dual-responsive polymersomes, and therapeutic activity of CC loaded dual-responsive polymersomes were investigated.



Scheme 1 Illustration of reduction and temperature dual-responsive crosslinked polymersomes based on PEG-PAA-PNIPAM triblock copolymers with an elevated LCST for triggered intracellular protein release. (a) Protein-loaded polymersomes are readily prepared in mild aqueous conditions (MES, pH 5.5, 20 mM) *via* simply increasing the solution temperature to 40 °C; (b) protein-loaded polymersomes can be stabilized *via* crosslinking with cystamine by carbodiimide chemistry; (c) protein-loaded Cys-crosslinked polymersomes are stable in physiological mimicking conditions (PBS, pH 7.4, 20 mM, 150 mM NaCl, 37 °C); and (d) protein-loaded Cys-crosslinked polymersomes are rapidly disassembled into unimers inside cells, resulting in highly efficient intracellular protein release.

Experimental

Materials

N-Isopropylacrylamide (NIPAM, 99%, J&K), and 2,2′-azobisisobutyronitrile (AIBN, 98%, J&K) were re-crystallized twice from hexane and methanol, respectively. Acrylic acid (AA, 99%, Alfa Aesar) was distilled before use. Dicyclohexyl carbodiimide (DCC) (99%, Alfa Aesar), N-hydroxysuccinimide (NHS, 98%, Alfa Aesar), cystamine dihydrochloride (98%, Alfa Aesar), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 98%, Alfa Aesar), 1,4-dithio-DL-threitol (DTT, 99%, Merck), 2-(*N*-morpholino)ethanesulphonic acid (MES, 98%, Beike Suzhou), lysozyme (Sigma), cytochrome C from equine heart (Sigma), bovine serum albumin V fraction (BSA, Roche), ovalbumin (Sigma), fluorescein isothiocyanate (FITC, >90%, Fluka) and Pierce® BCA protein assay (Thermo Scientific) were used as received.

Characterization

^{1}H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using deuterated chloroform ($CDCl_3$) or deuterated water (D_2O) as a solvent. The chemical shifts were calibrated against residual solvent signals. Particle sizing was achieved using a Zetasizer Nano ZS (Malvern, Worcestshire, United Kingdom) with a 633 nm helium–neon laser using back-scattering detection. This instrument uses dynamic light scattering to measure the average hydrodynamic size of particles in solution using the Stokes–Einstein equation. Data were analyzed using the associated Zetasizer software (Dispersion Technology Software v 5.00; Malvern). Scattering of the particle solutions was measured at a fixed 173° scattering angle in 3.5 mL plastic cuvettes giving the hydrodynamic diameter

($Z_{ave} = 2\ R_h$) of the polymersomes and their polydispersity index (PDI). Three measurements consisting of 10 runs with 5 s duration were performed at the desired temperatures. The instrument was standardized with 200 nm and 60 nm polystyrene beads and particle hydrodynamic size was reported as the average of the three measurements with standard deviation. Confocal laser scanning microscopy (CLSM) analyses were performed with a Nikon Digital Eclipse C1si Confocal Laser Scanning Microscope (Nikon) equipped with a heating stage. Fluorescence measurements were carried out on an FLS 920 spectrometer (Edinburgh Instruments, UK).

Synthesis of PEG-PAA-PNIPAM triblock copolymers

PEG-PAA-PNIPAM copolymers were synthesized according to our previous report.[23] Briefly, under an argon atmosphere, AIBN (0.62 mg, 3.77 μmol), PEG-DMP (0.10 g, 19 μmol), AA (0.0356 g, 0.564 mmol) and 5.0 mL of dioxane were charged into a 10 mL Schlenk flask. The mixture was degassed with argon for 30 min before placed in an oil bath thermostated at 70 °C. After 24 h, a sample was taken to determine monomer conversion, and to the rest, second monomer NIPAM (0.4294 g, 3.8 mmol) and additional AIBN (0.2 mg) in 0.2 mL of dioxane were added. The reaction was allowed to proceed at 70 °C for another 24 h. The copolymer was precipitated into cold diethyl ether and dried in vacuo for 48 h. Yields: *ca.* 85%. ^{1}H NMR analysis revealed that PEG-PAA-PNIPAM copolymers with molecular weights of 5k-1.7k-22k and 5k-0.7k-12k (denoted as polymer **1** and **2**, respectively) were prepared.

Formation and crosslinking of polymersomes

5.0 mg of PEG-PAA-PNIPAM triblock copolymer was dissolved in 0.5 mL of MES buffer (pH 5.5, 20 mM) at 4 °C, and the solution was filtered through a 0.22 μm filter followed by incubating at 40 °C under constant shaking for 60 min to allow the formation of polymersomes. Preheated cystamine dihydrochloride (0.465 mg, 2.06 mmol) solution in 0.2 mL water was added and incubated for 1 h before the addition of preheated of NHS (0.715 mg, 6.21 mmol) in 0.1 mL water and EDC (4.755 mg, 24.8 mmol) in 0.2 mL water. The final copolymer concentration was 5 g L^{-1} and the cystamine/carboxyl molar ratio was kept constant at 1 : 2. This reaction solution was shaken overnight at 40 °C before measurement.

Preparation of protein-loaded crosslinked polymersomes

5 mg of PEG-PAA-PNIPAM triblock copolymer and varying amounts of proteins or FITC-labeled proteins (protein/polymer weight ratios = 0.1, 0.20, 0.5, and 1) were dissolved in 1 mL of MES (pH 5.5, 20 mM) at 4 °C. The solution was heated up to 40 °C under constant shaking for 60 min to form protein-loaded polymersomes. Free proteins were removed by dialysis (MWCO 500 kDa) for 20 h with at least 4 times medium (MES) exchange. The polymersomes were crosslinked as described above. Then, protein loaded crosslinked polymersomes were dialyzed (MWCO 500 kDa) for 8 h with 4 times medium (MES) exchange. To determine protein loading contents (PLC) and loading efficiency (PLE), polymersomes were destructed with 50 mM DTT for 1 h at 25 °C resulting in complete release of encapsulated proteins. The amount of FITC labeled proteins was then determined by fluorescence measurements. In case of native proteins, the proteins released from polymersomes following treating with 50 mM DTT were firstly dialyzed (MWCO 3.5 kDa) against phosphate buffer at 25 °C. The amount of loaded proteins was determined by BCA assays according to the supplier's protocols (Thermo Scientific). PLC and PLE were calculated according to the following formula:

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

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

MTT assays

HeLa and MCF-7 cells were plated in a 96-well plate (1×10^4 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^{-1}) for 24 h to reach 70% confluence. The cells were incubated with varying amounts of Cys-crosslinked PEG-PAA-PNIPAM polymersomes 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-diphenyltetrazolium bromide (MTT) solution in PBS (5 mg mL^{-1}) 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 DMSO, and the absorbance at a wavelength of 490 nm of each well was measured using a microplate reader (Bio-rad, ELX808IU). The cell viability (%) was determined by comparing the absorbance at 490 nm with control wells containing only cell culture medium. Data are presented as average ± SD ($n = 4$).

Protein release from polymersomes

Protein-loaded polymersomes were prepared as described above. The MES medium in crosslinked polymersome dispersions was exchanged into PBS (pH 7.4, 20 mM, 150 mM NaCl) by dialysis prior to the release experiments. The release of FITC-BSA, FITC-Lys or FITC-CC from Cys-crosslinked polymersomes was investigated using a dialysis method (MWCO 500 kDa) at 37 °C with 0.4 mL of protein-loaded crosslinked polymersome suspensions in PBS (pH 7.4, 20 mM, 150 mM NaCl) against 30 mL of PBS buffer containing 10 mM DTT (mimicking intracellular reductive condition) or PBS buffer without DTT (non-reductive condition). At desired time intervals, 5 mL release media was taken out and replenished with an equal volume of corresponding fresh media. The amounts of released proteins as well as proteins remained 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, and the results presented are the average data with standard deviations.

Cellular uptake of FITC-CC loaded polymersomes

MCF-7 cells were plated on microscope 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^{-1}) for 24 h. 100 μL of FITC-CC loaded Cys-crosslinked polymersome **1**, FITC-CC loaded BDA-crosslinked polymersome **1** and free FITC-CC (CC dosage: 80 μg mL^{-1}) were added. The cells were cultured at 37 °C for another 12 h, 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 3 times. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 20 min and washed with PBS 3 times. Fluorescence images of the cells were obtained using a confocal microscope (TCS SP2).

Apoptotic activity of CC-loaded crosslinked polymersomes

MCF-7 cells were plated 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^{-1}) for 24 h. The cells were treated with CC-loaded Cys-crosslinked polymersomes, CC-loaded BDA-crosslinked polymersomes or free CC (CC dosage: 80 μg mL^{-1}) for 12 h under 5% CO_2 atmosphere at 37 °C. To quantify the percentage of 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^{-1}. Then, the cells were stained with 5 μL of Annexin V-FITC solution and 5 μL of PI solution for 15 min at room temperature in the dark. At the end of the incubation, 400 μL of binding buffer was added, and the cells were analyzed immediately by flow cytometry (BD FACSCalibur, Mountain View, CA).

Results and discussion

Synthesis of reduction and temperature dual-responsive crosslinked polymersomes with an elevated LCST

We reported previously that thermo-sensitive poly(ethylene glycol)-*b*-poly(acrylic acid)-*b*-poly(*N*-isopropylacrylamide) (PEG-PAA-PNIPAM) triblock copolymers were freely water-soluble at room temperature while readily formed nano-sized polymersomes through increasing the solution temperature to above their LCST (*ca.* 32 °C) in MES.[23] Interestingly, these thermo-sensitive polymersomes following crosslinking with cystamine (Cys) *via* carbodiimide chemistry became stable and robust at 25 °C (lower than their LCST), but were rapidly disassembled into unimers under a reductive environment. In this study, we were set to develop PEG-PAA-PNIPAM polymersomes with an elevated LCST (slightly higher than 37 °C) for efficient loading and rapid intracellular release of proteins.

Two thermo-sensitive triblock copolymers, PEG_{5k}-$PAA_{1.7k}$-$PNIPAM_{22k}$ and PEG_{5k}-$PAA_{0.7k}$-$PNIPAM_{12k}$ (denoted as polymer **1** and **2**, respectively), were prepared as our previous report *via* controlled reversible addition–fragmentation chain-transfer (RAFT) polymerization (Table 1). Notably, dynamic light scattering (DLS) studies showed that polymers **1** and **2** had LCST of 39 and 38 °C in PBS (pH 7.4, 20 mM, 150 mM NaCl) and 34 and 32 °C in MES (pH 5.5, 20 mM), respectively (Fig. 1). The effect of the medium on LCST is most likely due to different extents of PAA ionization. It was reported that the LCST of PNIPAM could be increased or decreased by conjugating with a (charged) hydrophilic or hydrophobic polymer, respectively.[28,29] Upon increasing the pH from 5.5 to 7.4, PAA block became more hydrophilic as a result of increased PAA deprotonation, leading to elevated LCST. In addition, the medium had also significant influence on the sizes of polymersomes, of which average hydrodynamic sizes of *ca.* 162.4 and 170.2 nm were observed for polymer **1** and **2** in MES and 40.7 and 46.9 nm for polymer **1** and **2** in PBS, respectively (Table 1). Interestingly, all formed polymersomes had low polydispersities (PDI) of 0.15∼0.18 (Table 1). The vesicular structure was confirmed as our previous report by confocal laser scanning microscopy (CLSM).[23] It should further be noted that these thermo-sensitive polymersomes would quickly disassociate into unimers with

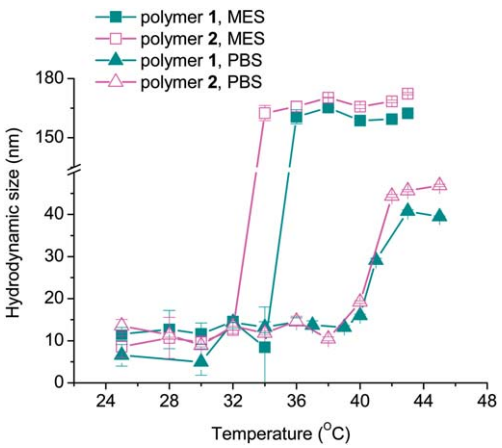


Fig. 1 Hydrodynamic sizes of PEG-PAA-PNIPAM polymersomes (measured by DLS at an angle of 173°) as a function of temperature in MES (pH 5.5, 20 mM) or PBS (pH 7.4, 20 mM, 150 mM NaCl) (copolymer concentration: 1 mg mL^{-1}).

Table 1 Characteristics of PEG-PAA-PNIPAM triblock copolymers

	M_n (kg mol^{-1})		LCST (°C)[b]		Size (nm)/PDI[c]	
Copolymer	Design	^{1}H NMR[a]	MES	PBS	MES	PBS
PEG_{5k}-$PAA_{1.7k}$-$PNIPAM_{22k}$	5.0-1.87-23	5.0-1.72-22	34	39	162.4/0.16	40.7/0.15
PEG_{5k}-$PAA_{0.7k}$-$PNIPAM_{12k}$	5.0-0.72-12	5.0-0.71-12	32	38	170.2/0.18	46.9/0.16

[a] Determined by ^{1}H NMR. [b] LCST, defined as the onset of transition from a plot of hydrodynamic size *versus* temperature, was determined by DLS (1.0 mg mL^{-1}) in MES (pH 5.5, 20 mM) or PBS (pH 7.4, 20 mM, 150 mM NaCl). [c] Measured by DLS at 37 °C in MES or PBS.

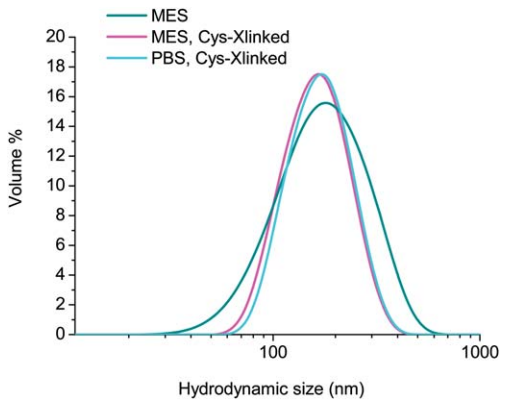


Fig. 2 Size distribution profiles of non-crosslinked or Cys-crosslinked polymersome **1** in MES or PBS measured by DLS at an angle of 173° with a concentration of 1.0 mg mL^{-1} at 37 °C.

average sizes of *ca.* 7∼8 nm upon decreasing the temperature to below their LCST.

In the following studies, robust polymersomes were prepared by increasing the temperature of polymer solutions in MES to 40 °C followed by crosslinking with Cys *via* carbodiimide chemistry. The size of polymersomes was reduced by *ca.* 10∼20 nm after crosslinking in MES (Fig. 2). Importantly, crosslinked polymersomes became stable also in PBS at 37 °C (lower than LCST) with similar sizes to those in MES (Fig. 2), corroborating successful locking of polymersomal structures. The crosslinked polymersomes were rapidly disassembled into unimers (*ca.* 7∼8 nm) in the presence of 10 mM dithiothreitol (DTT) in PBS at 37 °C (Fig. 3). In contrast, no change in polymersome sizes was observed in MES under otherwise the same conditions (Fig. 3), in accordance with the fact that polymersome **1** has an LCST of 34 °C (lower than 37 °C) in MES. Therefore, reduction and temperature dual-responsive Cys-crosslinked polymersomes with an elevated LCST over 37 °C are likely stable under the physiological conditions but will rapidly dissociate into unimers in an intracellular reductive environment.

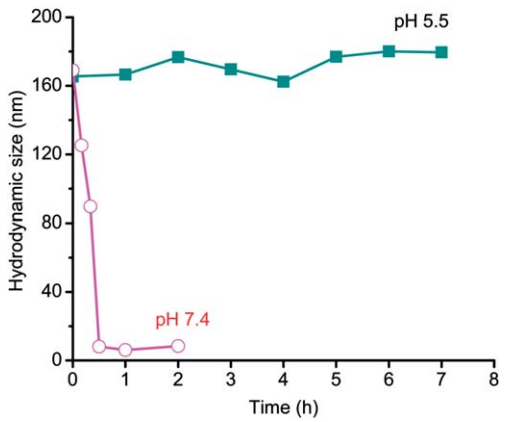


Fig. 3 Hydrodynamic size change of Cys-crosslinked polymersome **1** over time in response to 10 mM DTT at 37 °C in MES (pH 5.5, 20 mM) or PBS (pH 7.4, 20 mM, 150 mM NaCl). The sizes were measured by DLS at an angle of 173°.

Loading of proteins into dual-responsive crosslinked polymersomes

Protein-loaded polymersomes were prepared by increasing the temperature of protein-containing polymer solutions in MES to 40 °C followed by extensive dialysis to remove free proteins and crosslinking with Cys *via* the carbodiimide chemistry. The results of protein loading are summarized in Table 2. Remarkably, various types of proteins including bovine serum albumin (BSA), lysozyme (Lys), cytochrome C (CC), and ovalbumin (Ova) could be loaded into PEG-PAA-PNIPAM polymersomes with high protein loading efficiencies of approximately 60∼100% at theoretical protein loading contents of 10∼50 wt%, corresponding to protein loading contents of *ca.* 10∼40 wt% (Table 2). For example, protein loading efficiencies of *ca.* 96.8, 94.5, and 81.0% were obtained for BSA and ∼100, 95.5, and 80% for CC at initial protein/polymer **1** feed ratios of 10, 20 and 50 wt%, respectively. Similar protein loading levels were also achieved for polymersome **2** (Table 2).

These results are remarkable given the fact that low protein loading levels are a bottleneck for many current protein carriers. For example, liposomes were reported to load proteins with efficiencies of 40–50% through freeze-thaw cycles and 24% for BSA with emulsification.[30] For polymersomes, the encapsulation efficiency of BSA and haemoglobin *via* film hydration was 5%[31] and 2.7–12.2%,[32] respectively, and that of glucose oxidase in THF/water system was 25%.[33] Recently, Hubbell *et al.* explored direct hydration method to increase protein loading efficiency of polymersomes to 15–37%.[34] The high protein loading level of PEG-PAA-PNIPAM polymersomes is most likely due to the effective electrostatic and/or hydrogen bonding interaction between exogenous proteins and PAA blocks at pH 5.5 during the polymersome formation as well as to the large aqueous interior of polymersomes. The average sizes of protein-loaded crosslinked polymersomes ranged from 134.7 to 167.9 nm in PBS at 37 °C (Table 2), which was similar to or slightly smaller than the parent empty polymersomes. Notably, these protein-loaded crosslinked polymersomes maintained low PDIs of 0.11∼0.16 and 0.20∼0.22 for polymersomes **1** and **2**, respectively (Table 2). Zeta potential measurements displayed that they had slightly negative surface charges of *ca.* −1 ∼ −5 mV (data not shown).

In vitro and intracellular protein release

To investigate reduction-triggered protein release behaviors, protein release studies were performed in PBS (pH 7.4, 20 mM, 150 mM NaCl) at 37 °C in the presence or absence of 10 mM DTT. We selected polymersome **1** for the following studies due to its optimal LCST and better size distributions. In line with our expectation, the protein release from protein-loaded Cys-crosslinked polymersomes was minimal (*ca.* 20%) within 11 h under a non-reductive environment, signifying superb stability of crosslinked polymersomes (Fig. 4). However, fast release of FITC-CC, FITC-BSA and FITC-Lys was observed in the presence of 10 mM DTT under otherwise the same conditions (mimicking the intracellular reductive environment) (Fig. 4). For instance, approximately 80% of FITC-BSA and FITC-CC were released from FITC-BSA loaded crosslinked polymersomes in

Table 2 Characterization of protein-loaded Cys-crosslinked polymersomes

Polymersome	Protein	Protein loading content (*wt.*%)		Protein loading efficiency (%)[a]	Size[b](nm)	PDI[b]
		Theory	Result[a]			
1	FITC-BSA	10	9.7	96.8	165.8	0.16
		20	18.9	94.5	156.8	0.11
		50	40.5	81.0	150.1	0.17
		100	76.8	76.8	158.5	0.13
	FITC-CC	10	10.0	~100	146.3	0.11
		20	19.1	95.5	150.6	0.14
		50	40.0	80.0	154.6	0.15
	FITC-Lys	10	10.0	~100	140.2	0.12
		20	12.3	61.3	157.7	0.14
		50	32.3	64.6	149.0	0.14
	FITC-Ova	10	10.0	~100	158.2	0.15
		20	15.3	76.4	154.3	0.16
		50	44.2	88.3	147.5	0.16
2	FITC-BSA	10	9.9	99.0	146.6	0.21
		20	20	~100	134.7	0.20
	FITC-CC	10	9.8	98.0	167.9	0.21
		20	17.7	88.5	164.7	0.22

[a] Determined by fluorescence measurements (excitation 492 nm, emission 527 nm). [b] Measured by DLS in PBS (pH 7.4, 20 mM, 150 mM NaCl) at 37 °C at a polymersome concentration of *ca.* 1.0 mg mL^{-1}.

7.5 h and from FITC-CC loaded crosslinked polymersomes in 11 h, respectively.

The cellular uptake and intracellular protein release of protein-loaded crosslinked polymersomes were studied in MCF-7 cells using confocal laser scanning microscope (CLSM). Notably, strong FITC-CC fluorescence was observed in the perinuclear region of MCF-7 cells incubated with FITC-CC loaded Cys-crosslinked polymersomes (Fig. 5A). While practically no FITC-CC fluorescence was detected for cells treated with free FITC-CC under otherwise the same conditions (Fig. 5B), probably due to low permeation of FITC-CC across the cellular membrane. Moreover, MCF-7 cells incubated with FITC-CC loaded 1,4-butadiamine (BDA) crosslinked polymersomes (reduction-insensitive control) revealed only modest FITC-CC fluorescence (Fig. 5C), signifying the role of the cleavable disulfide bond (*i.e.* reduction-sensitivity) in the crosslinker for the intracellular protein release. It is evident, therefore, that reduction and temperature dual-responsive polymersomes are able to efficiently deliver and release proteins into cancer cells. These reduction and temperature dual-responsive crosslinked polymersomes have appeared to be ideal protein carriers as (i) they are prepared in all aqueous conditions with high protein loading efficiency and content, and (ii) they exhibit superior stability with minimal protein release under physiological conditions while rapidly release proteins under the intracellular reductive conditions.

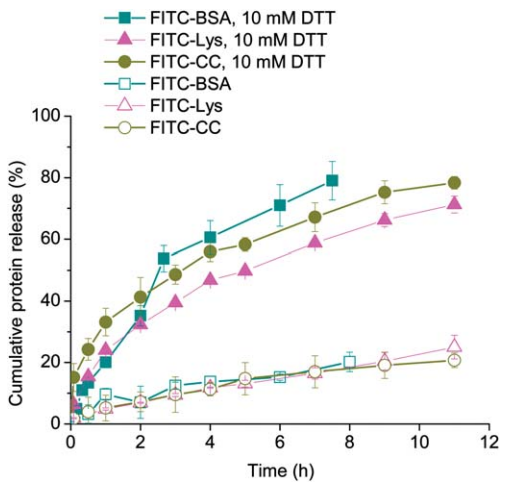


Fig. 4 DTT induced release of FITC-BSA, FITC-Lys and FITC-CC from Cys-crosslinked polymersome **1** in PBS at 37 °C. The dialysis tubes were under constant shaking.

Apoptotic activity of CC-loaded crosslinked polymersomes

It is of great importance for protein delivery systems that released proteins maintain their biological activity. PEG and PAA are among a few polymers approved by the U.S. FDA for use in biomedical devices while thermo-sensitive PNIPAM-based biomaterials have been widely applied for different biomedical applications including protein separation and cell sheets. Hence, PEG-PAA-PNIPAM copolymers are expected to display excellent biocompatibility. Indeed, MTT assays demonstrated that Cys-crosslinked polymersome **1** was essentially non-toxic to both HeLa and MCF-7 cells (cell viability ≥ 90%) up to a tested concentration of 200 μg mL^{-1} (Fig. 6). Notably, in our studies, preparation of polymersomes, loading of proteins and cross-linking reactions all were carried out under extremely mild conditions, effectively minimizing protein denaturation. Herewith, horse heart cytochrome C (CC) was selected as a model therapeutic protein. CC is known to play a role in programmed cell death,[35–37] and the intracellularly injected CC was reported to induce effective cell apoptosis.[6,38,39] Recently, Perez *et al.* reported that intracellular CC delivery with nanoparticles caused appreciable apoptosis in MCF-7 cells.[40]

The apoptotic activity of CC loaded Cys-crosslinked polymersomes was studied in MCF-7 cells using flow cytometry. The level of apoptosis was determined by annexin V-FITC/propidium iodide (PI) staining. Free CC and CC loaded

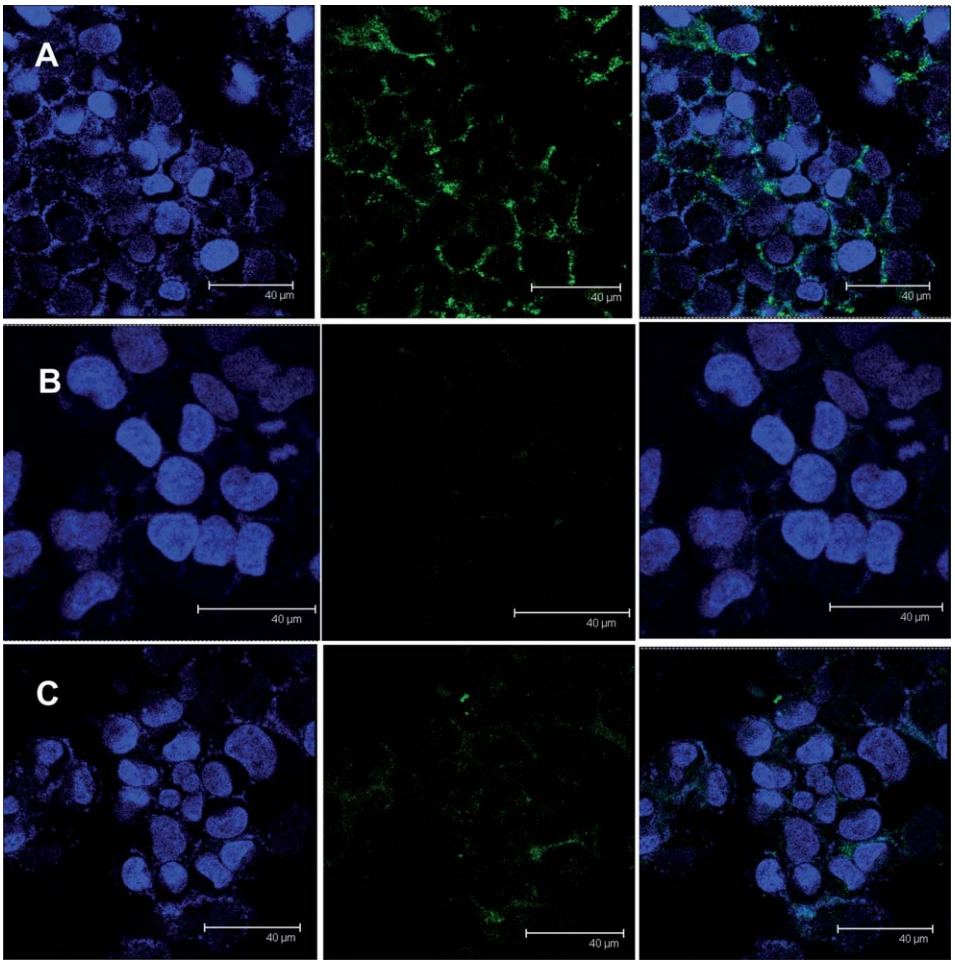


Fig. 5 CLSM images of MCF-7 cells incubated for 12 h with FITC-CC loaded Cys-crosslinked polymersome **1** (A), free FITC-CC (B) and FITC-CC loaded BDA-crosslinked polymersome **1** (C). For each panel, images from left to right show cell nuclei stained by DAPI (blue), FITC-CC fluorescence in cells (green), and overlays of two images. The scale bars correspond to 40 μm. CC dosage was set at 80 μg mL^{-1}.

BDA-crosslinked polymersomes were used as controls. The results showed that both free CC and CC loaded BDA-crosslinked polymersomes (reduction-insensitive control) provoked low levels of apoptosis of MCF-7 cells (Fig. 7A,B), in accordance

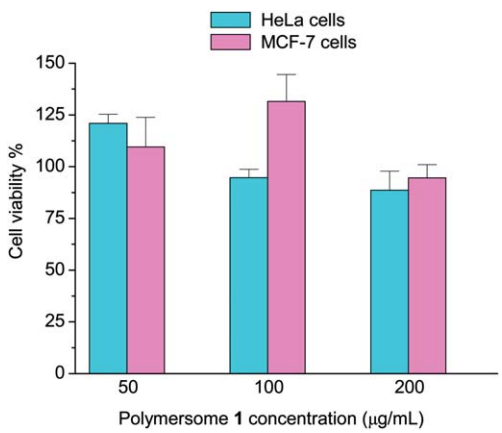


Fig. 6 Cytotoxicity of polymersome **1** determined by MTT assays using HeLa cells and MCF-7 cells (n = 6).

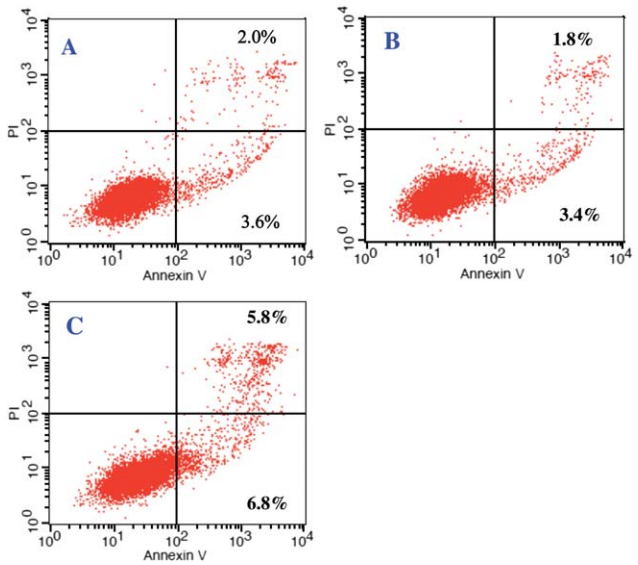


Fig. 7 Contour diagram of Annexin V-FITC/PI flow cytometry of MCF-7 cells following 24 h incubation with free CC (A), CC-loaded BDA-crosslinked polymersome **1** (B), and CC-loaded Cys-crosslinked polymersome **1** (C). CC dosage was set at 80 μg mL^{-1}.

with low cellular uptake of free CC and minimal intracellular release of CC from reduction-insensitive crosslinked polymersomes. In comparison, notable cell death (*ca.* 12.5%) was observed for CC loaded Cys-crosslinked polymersomes under otherwise the same conditions (Fig. 7C). These results confirm that dual-responsive crosslinked polymersomes are able to deliver and release CC into cancer cells, and importantly released CC maintains its apoptotic activity.

Conclusions

We have demonstrated that reduction and temperature dual-responsive crosslinked polymersomes based on PEG-PAA-PNIPAM triblock copolymers with an elevated LCST are superb nano-carriers for intracellular protein release. These dual-responsive polymersomes as novel protein delivery vehicles offer several unique features: (i) they are readily prepared in mild aqueous conditions *via* simply increasing solution temperature, preventing use of (toxic) organic solvent as well as protein denaturation; (ii) they exhibit excellent protein loading efficiency (up to 100%) as well as protein loading content (up to 76.8 wt%); (iii) they are sufficiently stable with restrained protein release under physiological conditions due to chemical crosslinking of the polymersome shells; (iv) they are rapidly disassembled into unimers under intracellular-mimicking reductive environments due to reduction-triggered de-crosslinking, resulting in highly efficient intracellular protein release; and (v) the released proteins preserve their bioactivity. These reduction and temperature dual-responsive polymersomes have opened a new avenue to targeted intracellular delivery of protein and peptide drugs.

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References

1 R. Kerbel and J. Folkman, *Nat. Rev. Cancer*, 2002, **2**, 727.
2 M. Harries and I. Smith, *Endocr. Relat. Cancer*, 2002, **9**, 75.
3 H. Marshall, *Trends Immunol.*, 2001, **4**, 183.
4 D. Schrama, R. A. Reisfeld and J. C. Becker, *Nat. Rev. Drug Discovery*, 2006, **5**, 147.
5 B. Guo, D. Y. Zhai, E. Cabezas, K. Welsh, S. Nouraini, A. C. Satterthwait and J. C. Reed, *Nature*, 2003, **423**, 456.
6 B. Zhivotovsky, S. Orrenius, O. T. Brustugun and S. O. Doskeland, *Nature*, 1998, **391**, 449.
7 V. P. Torchilin and A. N. Lukyanov, *Drug Discovery Today*, 2003, **8**, 259.
8 Z. Gu, A. Biswas, M. Zhao and Y. Tang, *Chem. Soc. Rev.*, 2011, **40**, 3638.
9 D. J. A. Crommelin, T. Daemen, G. L. Scherphof, M. H. Vingerhoeds, J. L. M. Heeremans, C. Kluft and G. Storm, *J. Controlled Release*, 1997, **46**, 165.
10 Y. Lee, T. Ishii, H. Cabral, H. Kim, J.-H. Seo, N. Nishiyama, H. Oshima, K. Osada and K. Kataoka, *Angew. Chem., Int. Ed.*, 2009, **48**, 5309.
11 S. R. Van Tomme and W. E. Hennink, *Expert Rev. Med. Devices*, 2007, **4**, 147.
12 A. Taluja, Y. S. Youn and Y. H. Bae, *J. Mater. Chem.*, 2007, **17**, 4002.
13 M. Yan, J. J. Du, Z. Gu, M. Liang, Y. F. Hu, W. J. Zhang, S. Priceman, L. L. Wu, Z. H. Zhou, Z. Liu, T. Segura, Y. Tang and Y. F. Lu, *Nat. Nanotechnol.*, 2010, **5**, 48.
14 D. E. Discher and F. Ahmed, *Annu. Rev. Biomed. Eng.*, 2006, **8**, 323.
15 C. LoPresti, H. Lomas, M. Massignani, T. Smart and G. Battaglia, *J. Mater. Chem.*, 2009, **19**, 3576.
16 F. H. Meng and Z. Y. Zhong, *J. Phys. Chem. Lett.*, 2011, **2**, 1533.
17 A. Kishimura, A. Koide, K. Osada, Y. Yamasaki and K. Kataoka, *Angew. Chem., Int. Ed.*, 2007, **46**, 6085.
18 S. F. M. van Dongen, W. P. R. Verdurmen, R. Peters, R. J. M. Nolte, R. Brock and J. C. M. van Hest, *Angew. Chem., Int. Ed.*, 2010, **49**, 7213.
19 S. Rameez, H. Alosta and A. F. Palmer, *Bioconjugate Chem.*, 2008, **19**, 1025.
20 D. A. Christian, S. Cai, D. M. Bowen, Y. Kim, J. D. Pajerowski and D. E. Discher, *Eur. J. Pharm. Biopharm.*, 2009, **71**, 463.
21 D. Demirgoz, T. O. Pangburn, K. P. Davis, S. Lee, F. S. Bates and E. Kokkoli, *Soft Matter*, 2009, **5**, 2011.
22 G. J. Liu, S. B. Ma, S. K. Li, R. Cheng, F. H. Meng, H. Y. Liu and Z. Y. Zhong, *Biomaterials*, 2010, **31**, 7575.
23 H. F. Xu, F. H. Meng and Z. Y. Zhong, *J. Mater. Chem.*, 2009, **19**, 4183.
24 F. H. Meng, Z. Y. Zhong and J. Feijen, *Biomacromolecules*, 2009, **10**, 197.
25 M. H. Li and P. Keller, *Soft Matter*, 2009, **5**, 927.
26 R. Cheng, F. Feng, F. H. Meng, C. Deng, J. Feijen and Z. Y. Zhong, *J. Controlled Release*, 2011, **152**, 2.
27 F. H. Meng, W. E. Hennink and Z. Y. Zhong, *Biomaterials*, 2009, **30**, 2180.
28 H. Zhang, L. Y. Chu, Y. K. Li and Y. M. Lee, *Polymer*, 2007, **48**, 1718.
29 Z. M. O. Rzaev, S. Dincer and E. Piskin, *Prog. Polym. Sci.*, 2007, **32**, 534.
30 C. Kirby and G. Gregoriadis, *Biol/Technology*, 1984, **2**, 979.
31 J. C. M. Lee, H. Bermudez, B. M. Discher, M. A. Sheehan, Y. Y. Won, F. S. Bates and D. E. Discher, *Biotechnol. Bioeng.*, 2001, **73**, 135.
32 D. R. Arifin and A. F. Palmer, *Biomacromolecules*, 2005, **6**, 2172.
33 S. F. M. van Dongen, M. Nallani, J. J. L. M. Cornelissen, R. J. M. Nolte and J. C. M. van Hest, *Chem.–Eur. J.*, 2009, **15**, 1107.
34 C. P. O'Neil, T. Suzuki, D. Demurtas, A. Finka and J. A. Hubbell, *Langmuir*, 2009, **25**, 9025.
35 R. Kim, K. Tanabe, Y. Uchida, M. Emi, H. Inoue and T. Toge, *Cancer Chemother. Pharmacol.*, 2002, **50**, 343.
36 K. Li, Y. C. Li, J. M. Shelton, J. A. Richardson, E. Spencer, Z. J. Chen, X. D. Wang and R. S. Williams, *Cell*, 2000, **101**, 389.
37 X. S. Liu, C. N. Kim, J. Yang, R. Jemmerson and X. D. Wang, *Cell*, 1996, **86**, 147.
38 Y. L. P. Ow, D. R. Green, Z. Hao and T. W. Mak, *Nat. Rev. Mol. Cell Biol.*, 2008, **9**, 532.
39 O. T. Brustugun, K. E. Fladmark, S. O. Doskeland, S. Orrenius and B. Zhivotovsky, *Cell Death Differ.*, 1998, **5**, 660.
40 S. Santra, C. Kaittanis and J. M. Perez, *Mol. Pharmaceutics*, 2010, **7**, 1209.

