Reversibly crosslinked temperature-responsive nano-sized polymersomes: synthesis and triggered drug release†

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Water-soluble temperature responsive triblock copolymers, poly(ethylene oxide)-*b*-poly(acrylic acid)-*b*-poly(*N*-isopropylacrylamide) (PEO-PAA-PNIPAM), were prepared in one pot by sequential reversible addition–fragmentation chain-transfer (RAFT) polymerization using a PEO–trithiocarbonate (PEO–*S*-1-dodecyl-*S*-(*R*,*R*-dimethyl-*R*-aceticacid) trithiocarbonate) as a macro chain transfer agent. The block copolymers with $M_{n\ PEO}$ of 5 kDa, $M_{n\ PAA}$ of 0.35–1.45 kDa, and $M_{n\ PNIPAM}$ varying from 11–39 kDa were freely soluble in water as unimers at room temperature, but quickly self-assembled into nano-sized vesicles (about 220 nm) when raising the solution temperature to 37 °C. The vesicular structure was confirmed by confocal scanning laser microscope (CSLM) and static light scattering (SLS) measurements. The size and size distribution of the polymersomes depended on the solution concentration, the molecular weight of PNIPAM, the equilibrium time and shaking. Interestingly, thus-formed vesicles could be readily cross-linked at the interface using cystamine *via* carbodiimide chemistry. The crosslinked polymersomes, while showing remarkable stability against dilution, organic solvent, high salt conditions and change of temperature in water, were otherwise rapidly dissociated under reductive conditions mimicking intracellular environment. Notably, FITC–dextran, used as a model protein, was shown to be encapsulated into the polymersomes with an unprecedently high loading efficiency (>85 wt%). The release studies showed that most FITC–dextran was retained within the polymersomes after lowering the temperature to 25 °C. However, in the presence of 10 mM dithiothreitol (DTT), fast release of FITC–dextran was achieved. These reversibly crosslinked temperature responsive nano-sized polymersomes are highly promising as smart carriers for triggered intracellular delivery of biopharmaceutics such as pDNA, siRNA, pharmaceutical proteins and peptides.

Introduction

In the past decade, polymersomes (also referred to as polymeric vesicles)[1–3] have attracted rapidly growing interest, motivated by their intriguing aggregation phenomena, cell and virus-mimicking dimensions and functions,[4,5] as well as tremendous potential applications in medicine, pharmacy and biotechnology.[6–9] Unlike liposomes, self-assembled from low molecular weight lipids, polymersomes are in general prepared from macromolecular amphiphiles of various architectures including amphiphilic diblock,[1,10,11] triblock,[12,13] graft[14,15] and dendritic[16] copolymers. Polymersomes exhibit very unique features, in particular, high stability,[1,17] tunable membrane properties, versatility and capacity to transporting hydrophilic as well as hydrophobic species, such as anticancer drugs, genes, proteins and diagnostic probes.[18–20]

Recently, much effort has been directed towards the development of intelligent polymersomes that respond to internal or external stimuli (pH, temperature, redox potential, light, and magnetic field *etc.*), either reversibly or non-reversibly.[9,12,21–25] The stimuli-sensitive polymersomes have emerged as novel programmable delivery systems in which the release of the encapsulated contents can be readily modulated by the stimulus.[26] The stimuli-responsive release may result in significantly enhanced therapeutic efficacy and minimal side effects. It is also feasible to form and/or deconstruct polymersomes in water simply by applying an appropriate stimulus.

Temperature is one of the most popular stimuli utilized for constructing smart nano-carriers due to the fact that there is a temperature difference naturally occurring in the body (like in tumor tissues) and that temperature can be conveniently tuned externally (*e.g.* hyperthermia). Though very attractive and promising, only a couple of studies were reported on the thermal sensitive polymersomes.[10,27,28] For instance, Discher *et al.* reported a temperature-dependent assembly and disassembly of polymersomes based on poly(ethylene glycol)-*b*-poly(*N*-isopropylacrylamide) (PEO-PNIPAAM).[10] McComick *et al.* recently developed thermal sensitive polymersomes based on diblock copolymer poly[*N*-(3-aminopropyl)-methacrylamide hydrochloride]-*b*-poly(*N*-isopropylacrylamide) (PAMPA-PNIPAM) upon increasing the temperature above its LCST (30–40 °C).[27]

The self assembled structures including polymersomes are often plagued by their limited stability upon i.v. administration,

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which leads to premature release of drugs. Robust polymersomes were reported by crosslinking of their membranes.[23,27,29–31] For example, polymersomes based on poly(2-cinnamoylethyl methacrylate)–poly(*N*-isopropylacrylamide) were cross-linked by UV irradiation.[29] Discher *et al.* used redox initiation to crosslink the polymersome membrane of poly(ethylene glycol)-*b*-polybutadiene.[30] However, for targeted drug delivery applications, it would be more desirable to introduce reversible crosslinks, which though stable in circulation are rapidly degradable at the site of action in response to an internal stimulus. Redox potential is one of the few promising internal stimuli interesting for biomedical applications, due to the existence of a high redox potential difference between the mildly oxidizing extracellular space and the reducing intracellular space.[32] Disulfide bonds, which are stable in the blood,[33,34] can respond to the reductive condition *via* reversible cleavage into free thiols, and have been utilized to instantaneously disrupt the polymer/DNA complexes,[35] crosslinked micelles,[36,37] and polymersomes.[22]

In this paper, we report on novel reversibly crosslinked temperature-sensitive nano-sized polymersomes for triggered drug and protein release. These reversibly stabilized "smart" polymersomes have many superior properties, for example: (1) they are formed purely in water, with no need of using any organic solvents, which is particularly important for delivery of biopharmaceutics; (2) they have sizes of about 200 nm with narrow size distributions (~0.05); (3) they are stable in aqueous solution at a broad range of temperatures including room temperature; (4) they can be quickly de-crosslinked in a reductive environment analogous to that encountered inside the cells; and (5) drugs/proteins can be readily encapsulated and rate of drug/protein release can be elegantly controlled by temperature and redox potential.

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 used. α-Amino-ω-methoxy poly(ethylene glycol) 5000 (PEG-NH_2 5000)[38] was prepared from monomethoxy poly(ethylene glycol) (MW 5000 kDa, Fluka) and dried *via* azeotropic distillation from toluene under N_2. 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), FITC–dextrans (MW 4 and 40 kDa, Sigma) and doxorubicin hydrochloride (99%, Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd.) were used as-received.

Synthesis of PEG macro chain transfer agent (PEG-DMP)

In a 250 mL round-bottom flask equipped with a magnetic stir bar, *S*-1-dodecyl-*S*-(*R*,*R*-dimethyl-*R*-aceticacid)trithiocarbonate (DMP) (0.438 g, 1.20 mmol) synthesized according to ref. 39 and *N*-hydroxysuccinimide (NHS) (0.207 g, 1.80 mmol) were stirred to dissolve at 0–4 °C in 150 mL of anhydrous DCM. DCC (0.743 g, 3.60 mmol) in 10 mL DCM was then added drop-wise, and the reaction was stirred at r. t. for 20 h. Then 3.0 g of PEG-NH_2 5000 was added and allowed to react for another 8 h at room temperature. The opaque solution was filtered, and the filtrate was concentrated by rotary evaporator before precipitating in cold diethyl ether. The product was collected by filtration, and dried *in vacuo*. DMP functionality was over 95% (^{1}H NMR). Yield: 2.50 g, 80%.

One-pot two-step synthesis of PEG-PAA-PNIPAM by RAFT polymerization

Under an argon atmosphere, AIBN (0.62 mg, 3.77 μmol), PEG-DMP (0.10 g, 19 μmol), AA (0.0135 g, 185 μmol) and 4.0 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 (0.4260 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: 70–86%.

Formation and crosslinking of polymersomes

The PEG_{113}-PAA_{20}-$PNIPAM_{211}$ triblock copolymers (5.0 mg) were dissolved in MES buffer (0.5 ml, pH 5.5, 0.02 M) 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 (0.375 mg, 1.67 μmol) solution in 0.2 mL water was added and incubated for 1 h before the addition of preheated of NHS (0.575 mg, 5.0 μmol) in 0.1 mL water and EDC (3.84 mg, 20 μmol) 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.

Destabilization of the crosslinked polymersomes (CLPs) by dithiothreitol (DTT)

Under an N_2 flow, a predetermined amount of DTT was introduced to a cuvette containing 1.5 ml of CLP solution at different pHs, which resulted in a final DTT concentration of 10 or 100 mM. The cuvette was then sealed with a septum. The CLP solution was votexed at r.t. and the size of the solution was monitored with DLS in time.

Encapsulation and triggered release of FITC–dextran

Block copolymer (1.25 mg) and FITC–dextran (1.25 mg, 0.0125 mol mol^{-1} glucose) were dissolved in 0.125 mL MES buffer (pH 5.5, 0.02 M). The polymersomes were crosslinked as described above. Free FITC–dextran was removed from the loaded CLP by dialysis (MWCO 100 kDa for FICT-dextran 4 kDa and MWCO 500 kDa for FICT-dextran 40 kDa) under constant stirring at 40 °C for 48 h. The dialysis medium was refreshed at least 4 times.

After adjusting the pH to 7.4 using PB buffer (0.4 M), CLPs loaded with FITC–dextran were divided into two groups: one with 10 mM DTT at 20 °C, and one with only PB buffer at 20 °C (control). These solutions were then immediately transferred to

dialysis tubes, which were immersed into 30 mL of PB buffer with the same DTT concentration in 50 mL tubes. The tubes were under constant shaking at 20 °C. At desired time intervals, 5 mL of media were taken for fluorescence measurement and 5 mL of fresh media were replenished. The release experiments were conducted in triplicate, and the results presented are the average data with standard deviations. The amount of fluorescent probes loaded into polymersomes and the amount remained after the release study were determined by solubilizing the loaded polymersomes using 100 mM DTT for 1 h before dilution by 2000 times for fluorescence measurements based on a calibration curve of known concentrations of FITC–dextran.

Characterization

The ^{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 solvents. The chemical shifts were calibrated against residual solvent signals.

The polydispersity of triblock copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with three ULTRAHYDROGEL columns (120, 250 and 1000 PKGD; M_w from 200 to 10^6 g mol^{-1}) following a INLINE precolumn and a differential refractive-index detector. The measurements were performed using Milli Q water with 0.05% NaN_3 as eluent at a flow rate of 0.5 mL min^{-1} at 30 °C and a series of polyethylene oxide as standard.

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 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.

The weight-average molecular weight (M_w) and radius of gyration (R_g) of CLP were measured by multiangle laser light scattering performed using a Wyatt Technology DAWN HELEOS 18 angle (from 15° to 165°) light scattering detector using a Ga As laser (658 nm, 50 mW). The concentration of CLP ranged from 0.01 to 0.08 mg mL^{-1} and concentrations were analyzed by the graphical method reported by Zimm.

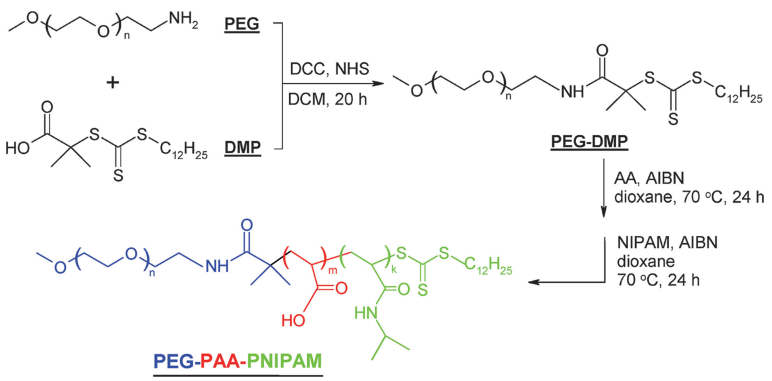
CLSM analyses were performed with a Nikon Digital Eclipse C1si Confocal Laser Scanning Microscope (Nikon) equipped with a heating stage. Fluorescence measurements were carried out on an FLS 920 spectrometer (Edinburgh Instruments, UK).

Results and discussion

Synthesis of PEO-PAA-PNIPAM triblock copolymers

Poly(ethylene oxide)-*b*-poly(acrylic acid)-*b*-poly(*N*-isopropylacrylamide) (PEG-PAA-PNIPAM) triblock copolymers, were synthesized in one pot by sequential reversible addition–fragmentation chain-transfer (RAFT) polymerization using PEO–trithiocarbonate macro chain transfer agent (PEO-DMP), which was conveniently prepared by reacting α-methoxy-ω-amino-PEO ($M_n = 5$ kDa) with *S*-1-dodecyl-*S*-(*R*,*R*-dimethyl-*R*-aceticacid) trithiocarbonate (DMP) (Scheme 1). DMP is a very versatile RAFT agent,[39–41] and has been found very useful in RAFT (co)polymerization of many monomers, including *N*-isopropylacrylamide (NIPAM)[42] and acrylic acid (AA).[43] DMP has also been made into macro RAFT agent by chemically linking to a polymer, which yields block copolymers.[44,45] In our studies, the polymerization of AA using PEO-DMP as macro RAFT agent was carried out in dioxane at 70 °C employing AIBN as the free-radical source. ^{1}H NMR showed complete monomer conversion after one day polymerization. Next, the second monomer, *N*-isopropylacrylamide (NIPAM), was added and polymerization was continued for another day (Scheme 1). The resulting triblock copolymers were isolated by precipitation in diethyl ether.

The compositions of the triblock copolymers were determined from ^{1}H NMR analysis (Fig. 1). Based on the ^{1}H NMR spectrum of PEG-PAA diblock copolymers (Fig. 1a), the PAA block length could be calculated by comparing integrals of signals at δ 3.63 (the methylene protons of PEG block) and 2.35 (the methine proton of the PAA block). From the ^{1}H NMR spectrum of PEG-PAA-PNIPAM triblock copolymers (Fig. 1b), the PNIPAM block length could be determined by comparing integrals of signals at δ 3.63 and 4.05 (the methine proton of the PNIPAM block). Notably, the results revealed that the block



Scheme 1 Synthesis of PEG-PAA-PNIPAM triblock copolymers.

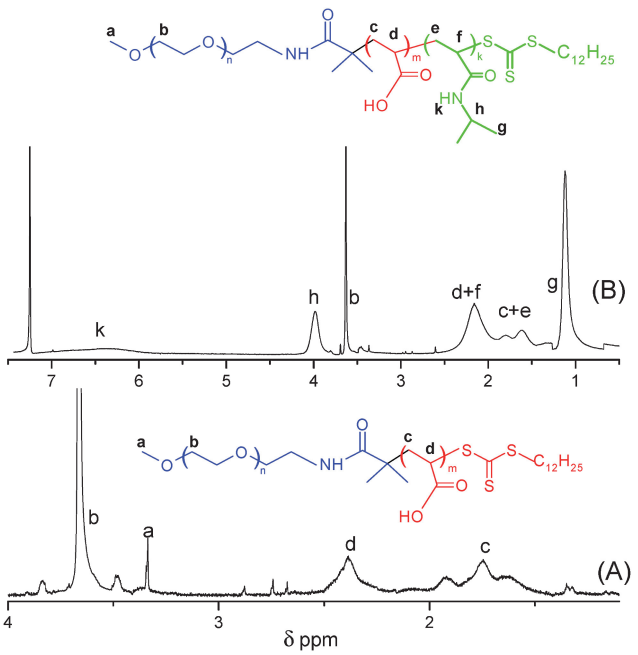


Fig. 1 ^{1}H NMR spectra (400 MHz) of PEG_{113}-PAA_{20}-DMP diblock copolymer (D_2O, A) and PEG_{113}-PAA_{20}-$PNIPAM_{210}$ triblock copolymer ($CDCl_3$, B, Table 1 entry 4).

lengths are close to the prescribed values, wherein PEG-PAA-PNIPAM triblock copolymers with $M_{n\ PEO}$ of 5 kDa, $M_{n\ PAA}$ of 0.35 to 1.45 kDa, and $M_{n\ PNIPAM}$ varying from 11 to 39 kDa were obtained (Table 1). The gel-permeation chromatography (GPC) using water (0.05% NaN_3) as an eluent showed a unimodal distribution with polydispersity index ranging from 1.4 to 1.8 (Table 1), corroborating successful synthesis of PEG-PAA-PNIPAM triblock copolymers. The M_n values obtained by GPC were higher than those calculated by ^{1}H NMR, which was probably due to the fact that PEG homopolymers were employed as GPC standards. This represents the first example on sequential RAFT copolymerization of AA and NIPAM using macro RAFT agent.

Temperature-responsive self-assembly of PEO-PAA-PNIPAM in water

PEO-PAA-PNIPAM triblock copolymers dissolved readily in aqueous solution at room temperature with a size of 7–8 nm,

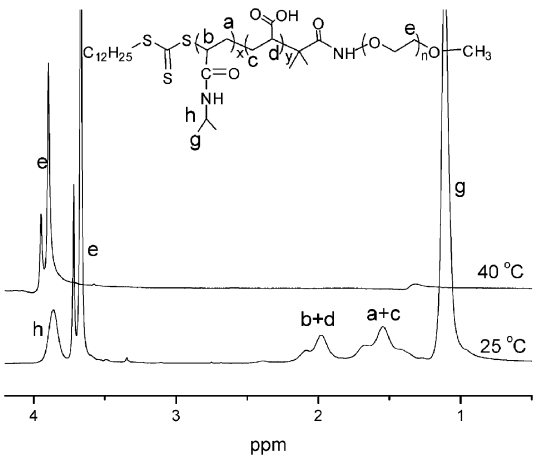


Fig. 2 ^{1}H NMR spectra (400 MHz, D_2O) of PEG_{113}-PAA_{20}-$PNIPAM_{210}$ triblock copolymer at temperature 25 °C and 40 °C.

indicating that the polymers exist as a unimer in water. The clear solution, however, turned turbid upon raising the temperature to 37 °C. Dynamic light scattering (DLS) measurements revealed the formation of supramolecular structures with sizes varying from 170 to 250 nm depending on the compositions of the triblock copolymers. Remarkably, all aggregates showed a particularly narrow size distribution of 0.03–0.1. The ^{1}H NMR of the triblock copolymers in D_2O at 25 °C (Fig. 2) showed that resonances attributed to PNIPAM block were clearly detected, and the ratio of signals assignable to PNIPAM to those of PEG complied well with that determined using $CDCl_3$, pointing to that triblock copolymers are fully solvated. An increase in the temperature to 40 °C caused the PNIPAM signal to become broadened with diminished intensity, confirming temperature-induced self-assembly.

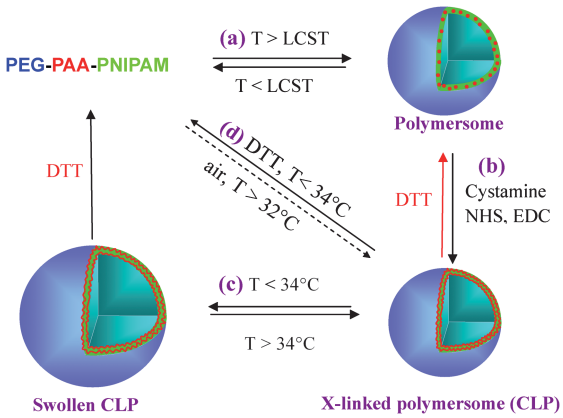
Temperature induced formation of polymersomes (Scheme 2a) was observed by confocal scanning laser microscope (CSLM) using hydrophilic FITC–dextran and hydrophobic Nile Red simultaneously. The results revealed that almost all the red spots and green spots were co-localized (data not shown), indicating formation of vesicular structures. CSLM and static light scattering (SLS) measurements of crosslinked particles have further corroborated formation of polymersomes (see later).

The preparation conditions had a great influence on the size and quality (PDI) of the polymersomes obtained. When the equilibrating time was increased from 5 min to 60 min, the PDI of the polymersomes decreased from 0.5 to 0.03 and the size

Table 1 Characteristics of PEG-PAA-PNIPAM triblock copolymers

Entry	PEG-PAA-PNIPAM triblock copolymers	M_n (designed)/kDa	M_n[a] (^{1}H NMR)/kDa	M_n[b] (GPC)/kDa	PDI[b] GPC	LCST[c]/°C	Size[d] /nm
1	PEG_{113}-PAA_5-$PNIPAM_{100}$	5-0.36-10	5-0.35-11	—	—	28	250
2	PEG_{113}-PAA_{10}-$PNIPAM_{180}$	5-0.72-20	5-0.71-20	—	—	29	230
3	PEG_{113}-PAA_{20}-$PNIPAM_{106}$	5-1.44-10	5-1.45-12	26.7	1.78	33	185
4	PEG_{113}-PAA_{20}-$PNIPAM_{210}$	5-1.44-20	5-1.45-24	55.0	1.41	32	180
5	PEG_{113}-PAA_{20}-$PNIPAM_{350}$	5-1.44-32	5-1.45-39	—	—	31	175

[a] Determined by ^{1}H NMR. [b] Aqueous GPC with 0.05% NaN_3 at 30 °C. [c] Determined from a plot of hydrodynamic size (DLS) *versus* temperature, LCST is defined as the onset of transition. [d] Hydrodynamic size was measured by DLS with a concentration of 10 mg mL^{-1} at 40 °C.



Scheme 2 Illustration of reversibly crosslinked temperature-responsive nano-sized polymersomes. (a) Formation of polymersomes by simply increasing temperature; (b) stabilization of the vesicular structure *via* crosslinking at the interface between the membrane and hydrophilic PEG layer; (c) formation of swollen polymersomes by decreasing temperature below its LCST; (d) destabilization of polymersomes by decreasing temperature below its LCST in the presence of 10 mM DTT.

decreased from a fluctuating value to a constant value. Further increase in equilibrating time had no effect on the PDI and size. In addition, during equilibration shaking (200 rpm) the solution produced slightly smaller particles, but a similar PDI, comparable to still conditions. Therefore, to obtain uniform thermal sensitive polymersomes, we prepared the polymersomes by shaking at 200 rpm at certain temperatures for at least 60 min. The vesicles were stable at 37–45 °C for at least two weeks, judging from their constant size over time. Thus, the vesicle self-assembly process appears to be kinetically controlled.

The lower critical solution temperature (LCST) and the size of the polymersomes were dependent on the concentration and the composition of the triblock copolymers (Fig. 3). The copolymers with longer PNIPAM block lengths had lower LCST, in which LCST of 31, 32, and 33 °C were observed for PEG_{113}-PAA_{20}-$PNIPAM_{350}$, PEG_{113}-PAA_{20}-$PNIPAM_{210}$ and PEG_{113}-PAA_{20}-$PNIPAM_{106}$, respectively (Fig. 3A). This is in agreement with results by Stover who reported that the LCST of PNIPAM homopolymers was dependent on both the molecular weight of the polymer and hydrophilicity of the end group.[46,47] Increase in the concentration of the block copolymers in water from 0.2, 1 to 10 mg mL^{-1} brought about a decrease in the LCST from 32 to 28 °C (Fig. 3B), while no change in size and PDI was observed. Furthermore, it appeared that longer PAA led to higher LCST and larger particle size. It should be noted that in general the transitions of these triblock copolymers were very sharp (within 2–3 °C), which is similar to PNIPAM homopolymers. In contrast, polymersomes based on PEO_{45}-$PNIPAM_{215}$ were reported to display a broad transition of *ca.* 10 °C,[10] and PLA_{188}-$PNIPAM_{100}$ 4–10 °C,[31] which were attributed to incomplete phase separation. It is noticeable that the vesicles dissociated quickly to a size of 8 nm when the temperature was lowered below its LCST, indicating that the self assembly process of the polymersomes was completely reversible.

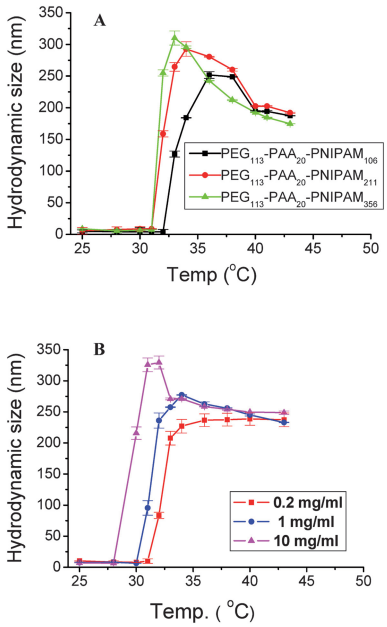


Fig. 3 Hydrodynamic size of polymersomes of PEG-PAA-PNIPAM triblock copolymers (measured by DLS at an angle of 173°) with different lengths of PNIPAM at 10 mg mL^{-1} (A), and of PEG_{113}-PAA_{10}-$PNIPAM_{180}$ at different concentrations (B).

Reversibly crosslinked temperature-responsive polymersomes

The biomedical application of the self assembled structures including polymersomes is currently limited because of the instability and/or premature release of drugs upon i.v. administration. In this study, polymersomes were crosslinked using cystamine to yield reversibly crosslinked polymersomes, which were stable in circulation but rapidly de-crosslinked in a reductive environment analogous to the cytoplasm and the cell nucleus. The crosslinking took place in the interface of the shell and the membrane by the reaction between carboxyl groups of PAA block in the triblock copolymers with a bioreducible crosslinker, cystamine, *via* carbodiimide method using EDC/NHS in aqueous solution. Crosslinking the polymersomes *via* the PAA block in the middle of the triblock copolymers can minimize the inter-particle crosslinking.[36] After cross-linking at 40 °C, the size of the polymersomes changed from *ca.* 210 nm to 185

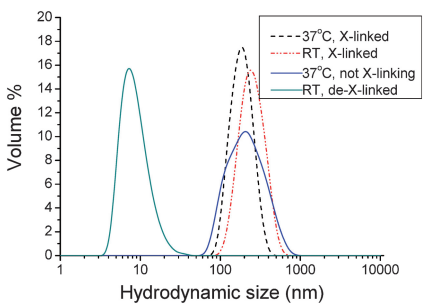


Fig. 4 Hydrodynamic size of non-crosslinked polymersomes and crosslinked polymersomes of PEG_{113}-PAA_{20}-$PNIPAM_{210}$ measured by DLS at an angle of 173° with a concentration of 1.0 mg mL^{-1} at room temperature and 37 °C.

nm, while the size distribution remained narrow (Fig. 4). The crosslinked polymersomes showed a larger particle size of 245 nm after lowering the temperature to 25 °C, whereas the non-crosslinked polymersomes were rapidly dissociated to unimers with sizes of 7–8 nm at 25 °C (Fig. 4). These results confirmed that polymersome structure has been successfully "locked" by crosslinking (Scheme 2b).

The crosslinked polymersomes (CLP) prepared from PEG_{113}-PAA_{20}-$PNIPAM_{106}$ and PEG_{113}-PAA_{20}-$PNIPAM_{350}$ were studied using static light scattering (SLS). It has been established that the ratio of radius of gyration to hydrodynamic radius (R_g/R_h) could be used to characterize the morphology of particles, *i.e.* 0.774 for nanoparticles and hard sphere micelles, 1.0 for vesicles, 1.5 for linear random coil chains in good solvents and 2.0 for wormlike or rod like chains.[48] The results showed at 20 °C R_g/R_h ratios of 1.00 and 1.05 for PEG_{113}-PAA_{20}-$PNIPAM_{106}$ and PEG_{113}-PAA_{20}-$PNIPAM_{350}$, respectively (Zimm plots were presented in the ESI†). These data were very close to unity, in line with a vesicular structure. In addition, the weight -average molecular weights of the corresponding polymersomes were 1.094×10^8 and 3.540×10^7 g mol^{-1}. The density, calculated based on an equation ($\rho = M_w/N_a V$, where N_a is Avogadro's number and V is the average volume of particles calculated *via* R_h) as reported earlier,[49] was 0.0925 and 2.94×10^{-3} g cm^{-3} for polymersomes of PEG_{113}-PAA_{20}-$PNIPAM_{106}$ and PEG_{113}-PAA_{20}-$PNIPAM_{350}$, respectively. This low density is characteristic of a vesicular structure.

Moreover, a giant crosslinked polymersome based on block copolymer PEG_{113}-PAA_{20}-$PNIPAM_{350}$ was captured with Nile Red encapsulated in the membrane by CSLM (Fig. 5). Hydrophobic Nile Red distributed uniformly in the polymersome membrane yielding a red fluorescent ring. The fluorescence intensity profile of the line marked in the micrograph displayed that the fluorescence intensity of the membrane was much higher than that of the interior. All these results pointed to formation of polymersomes from these PEG-PAA-PNIPAM triblock copolymers.

Stability of the crosslinked polymersomes (CLP)

The stability of the CLPs against change of temperature, large volume dilution, concentrated salt conditions, and organic solvents were investigated. We have shown previously that the CLPs maintained their vesicular structures after lowering the temperature below their LCST. The size of the polymersomes, however, became larger upon cooling to 25 °C as a result of swelling of the PNIPAM block in the CLP membrane (Fig. 6A). Interestingly, the transition temperature of the CLPs of PEG_{113}-PAA_{20}-$PNIPAM_{210}$ appeared to be *ca.* 34 °C, which is slightly higher than the non-crosslinked polymersomes (*ca.* 32 °C) and is close to body-temperature. The extent of swelling (Scheme 2c) and thus permeability of the polymersome membranes is temperature dependent, which could be utilized for tuning the rate of drug and protein delivery.

The CLPs were robust against large volume dilution, wherein dilutions with 10, 100 and 1000-fold water were found to hardly change the size and PDI of the polymersomes (Fig. 6B). Moreover, the CLPs retained their structural integrity even in the presence of 2 M NaCl. However, 4 M NaCl caused the vesicles to flocculate and precipitate out soon (Fig. 6C). Furthermore, these

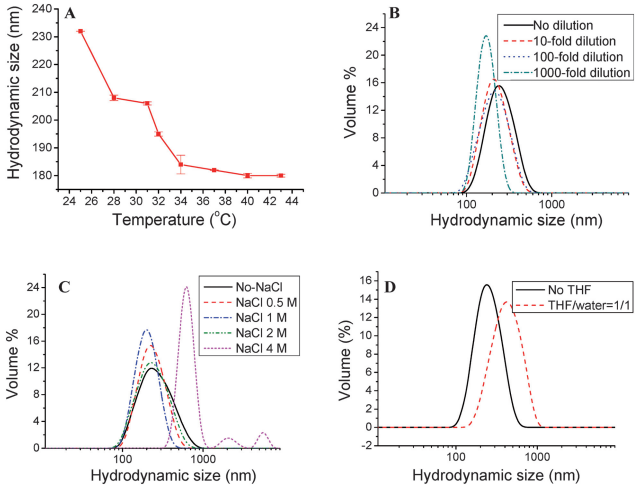


Fig. 6 Stability (in terms of hydrodynamic size and PDI) of the crosslinked polymersomes of PEG_{113}-PAA_{20}-$PNIPAM_{210}$ measured by DLS at an angle of 173° with a concentration of 1.0 mg mL^{-1} at room temperature for B–D. (A) Against temperature; (B) against dilution; (C) against NaCl concentration; (D) against organic solvent.

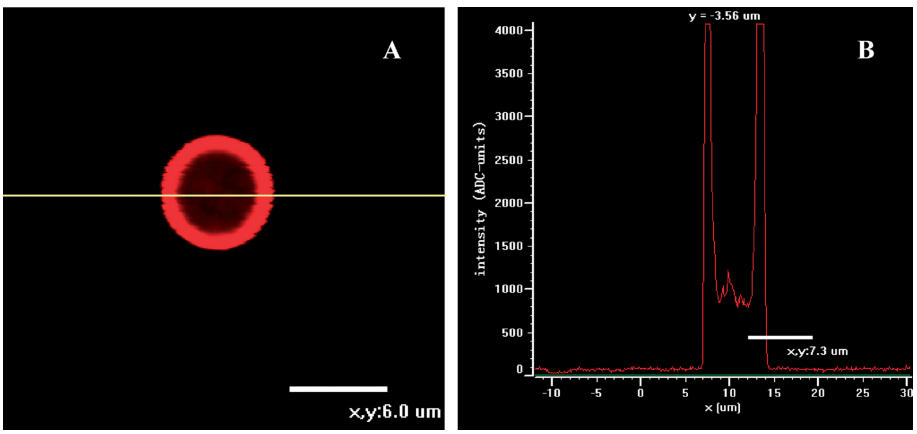


Fig. 5 CSLM micrograph of crosslinked polymersomes based on PEG_{113}-PAA_{20}-$PNIPAM_{350}$ with encapsulated Nile Red (A) and fluorescence intensity profile of the yellow line marked (B).

CLPs though swelled to 350 nm maintained their integrity when equal volume of THF was added (Fig. 6D). These results have further demonstrated successful "locking" of the vesicular structure by cross-linking with cystamine.

Triggered destabilization of CLPs with DTT

Despite their excellent stability against the harsh conditions described above, these CLPs could be rapidly destabilized in the presence of a reducing agent such as DTT by cleaving the disulfide bonds of the crosslinker cystamine in CLP into free thiol groups *via* a thiol–disulfide exchange reaction. The DLS studies of the CLPs in response to 10 and 100 mM DTT at 20 °C at different pHs revealed a pH and DTT dependent dis-assembly of CLPs (Fig. 7). In the presence of 100 mM DTT, at pH 5.5 dramatic decrease of size to *ca.* 8 nm was observed after 7 h incubation, whereas at pH 6.5 and 7.4, the CLPs were disrupted within 1.5 h and 2 min, respectively. In the presence of 10 mM DTT, at both pH 5.5 and 6.5, no change of size and PDI was observed within 7 h. However, at pH 7.4, 10 mM DTT caused complete disruption of the polymersomes within 30 min (Scheme 2d). It is evident that the rate of CLP disassembly increased with increasing pH. The results are in line with thiol–disulfide exchange, wherein the higher the pH the faster the reductive degradation. This rapid reduction by DTT at pH 7.4 contrasts to the previously reported reversibly crosslinked systems. For example, shell crosslinked micelles of PEG-(DMA-s-NAS)-PNIPAM by cystamine required a much more harsh conditions (0.7 M DTT at 45 °C for 10 h) to accomplish dis-assembly.[37] The fast degradation of CLPs is most probably due to the fact that the crosslinks are located in the interface of the hydrophilic and hydrophobic region, which made it easier for DTT to gain access to the disulfide bonds. It is worth noting that de-crosslinked polymersomes can be readily re-crosslinked by oxidation (exposure to air) at 40 °C (Scheme 2).

Triggered release of FITC–dextran from crosslinked polymersomes

As compared to other carrier systems such as micelles and nanoparticles, polymersomes are particularly attractive for encapsulation and delivery of hydrophilic compounds such as

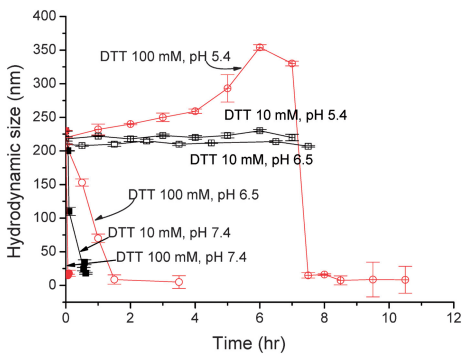


Fig. 7 Destabilization of CLPs of PEG_{113}-PAA_{20}-$PNIPAM_{210}$ by DTT (10 or 100 mM) at different pH values at 20 °C. Hydrodynamic size was measured by DLS at an angle of 173° with a concentration of 0.5 mg mL^{-1}.

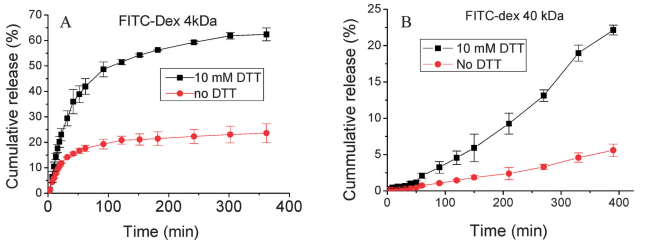


Fig. 8 DTT induced release of FITC–dextran (A) 4 kDa and (B) 40 kDa from crosslinked polymersomes of PEG_{113}-PAA_{20}-$PNIPAM_{210}$ at 20 °C. The dialysis tubes were under constant shaking.

anticancer drugs, peptides, genes and proteins. In this study, loading and triggered release of FITC–dextran (as a model protein) with CLPs were investigated. As the polymersomes were prepared purely in water *via* increasing the temperature, FITC–dextran could be readily encapsulated into the polymersomes during the self-assembling process. The polymersomes were subsequently crosslinked and dialyzed to remove free FITC–dextran. The results showed that FITC–dextran had little influence on formation of polymersomes, as revealed by their sizes of *ca.* 180 nm (37 °C) and low PDI of 0.1. Remarkably, a high loading content of FITC–dextran of *ca.* 85–140 wt% was achieved.

The release of FITC–dextran from the CLPs with and without 10 mM DTT were followed using a dialysis method. The results showed that most FITC–dextran 4 kDa was retained within the CLPs after lowering the temperature to room temperature (20 °C), in which approximately 20% FITC–dextran 4 kDa was released in 6 h. However, in the presence of 10 mM DTT, fast release of FITC–dextran 4 kDa from the polymersomes was observed (Fig. 8A), in which 45% FITC–dextran 4 kDa was released within the first 1 h and 61% after 6 h ($\tau_{1/2}$ = 100 min). FITC–dextran 40 kDa, was released more slowly (Fig. 8B). However, an enhanced release of dextran was observed at 10 mM DTT at 20 °C compared to the control without DTT (22% *versus* 5% in 6.5 h).

The facile and high loading of hydrophilic macromolecules and their triggered release in response to a reductive environment analogous to that encountered inside the cells render these reversibly crosslinked polymersomes particularly interesting as smart carriers for triggered intracellular delivery of biopharmaceutics such as pDNA, siRNA, pharmaceutical proteins and peptides.

Conclusion

We have developed a novel class of reversibly crosslinked temperature responsive nano-sized polymersomes for triggered drug release applications. The triblock copolymers PEG-PAA-PNIPAM have been prepared in one pot *via* sequential RAFT polymerization. These triblock copolymers are molecularly soluble in water at room temperature, but quickly self-assembled into nano-sized vesicles when the solution temperature was raised to above 32 °C. CSLM and SLS results confirmed the vesicular structure. Interestingly, thus-formed vesicles could be readily cross-linked at the interface using cysteamine *via* carbodiimide chemistry. The crosslinked polymersomes, while

showing remarkable stability against high salt conditions and change of temperature, are rapidly dissociated in the presence of 10 mM DTT, mimicking the intracellular reducing potential. It is particularly worthy to note that these polymersomes could efficiently encapsulate FITC–dextran, which was used as a model protein, with an unprecedently high loading efficiency (85%). Furthermore, rapid release of FITC–dextran from crosslinked polymersomes could be achieved by applying DTT. These reversibly crosslinked temperature responsive nano-sized polymersomes are highly promising as smart carriers for triggered intracellular delivery of biopharmaceutics such as DNA, siRNA, peptides, and proteins.

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