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Thermally Responsive Vesicles and Their Structural "Locking" through Polyelectrolyte Complex Formation**

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Vesicles composed of lipid molecules play an important role in several biological functions including the storage and transportation of small molecules.[1] Vesicles formed from the self-assembly of amphiphilic block copolymers are often more durable than conventional liposomes and have recently been the focus of research.[2–4] Among them, the chemically cross-linked vesicles reported by Du et al. are quite stable under extreme conditions.[5] Methods for vesicle formation from amphiphilic block copolymers typically involve the use of an organic solvent such as tetrahydrofuran, *N*,*N*-dimethylformamide (DMF), or dioxane[2,3,6] and require further purification, which can be time-consuming and problematic. Furthermore, the self-assembly is highly dependent on the rate of dialysis or the addition of another solvent, which is difficult to control. Recently, stimuli-responsive (pH-sensitive) block copolymers that self-assemble into vesicles without the addition of organic solvents have been reported.[7,8] Compared with pH-responsive materials, thermally responsive materials are advantageous for biological applications because of the stringent pH requirements in mammalian

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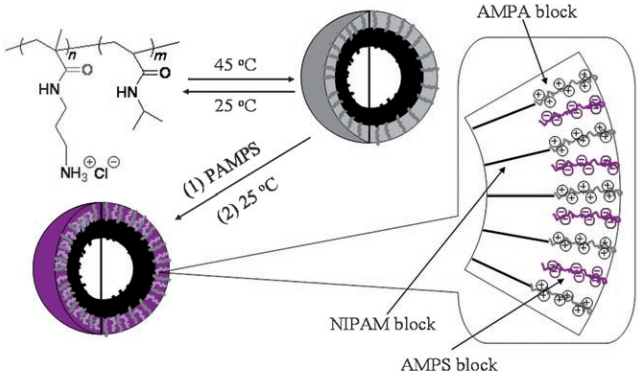
systems. Our group has a long-standing interest in the controlled polymerization of hydrophilic (meth)acrylamido monomers[9] and the self-assembly behavior of block copolymers in aqueous solutions.[10] Herein we report the first example of vesicle formation from the self-assembly of hydrophilic–hydrophilic block copolymers directly in water by variation of the solution temperature. The shells of the resultant vesicles were then cross-linked by polyelectrolyte complexation (Scheme 1).



Scheme 1. Schematic illustration of the formation of vesicles from PAMPA-PNIPAM diblock copolymers and their subsequent ionic cross-linking. PAMPS: poly(*N*-(3-aminopropyl)methacrylamide hydrochloride).

Table 1: Block copolymers composition, molecular weight, and molecular-weight distribution.

Entry	Structure	M_n	M_w/M_n
1	$AMPA_{40}$	7280[a]	1.04
2	$AMPA_{88}$	15700[a]	1.06
3	$AMPA_{40}$-$NIPAM_{34}$	11300[b]	1.11
4	$AMPA_{88}$-$NIPAM_{40}$	21200[b]	1.13
5	$AMPA_{88}$-$NIPAM_{50}$	22500[b]	1.16
6	$AMPA_{88}$-$NIPAM_{74}$	24100[b]	1.19

[a] Measured by SEC in aqueous solution. [b] Measured by SEC in DMF (the AMPA block was first converted to the primary amine form).

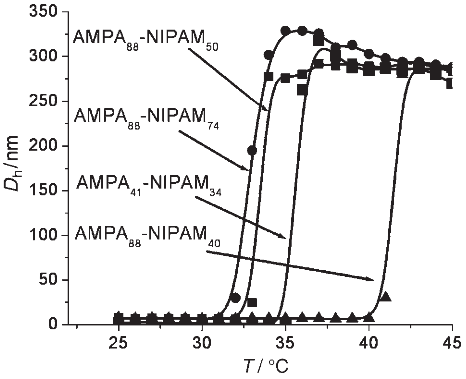


Figure 1. Variation of hydrodynamic diameter with temperature for a 0.5% (w/w) aqueous solution of the diblock copolymers (heating rate = 0.1 °C min^{-1}).

The diblock copolymer poly(*N*-(3-aminopropyl)methacrylamide hydrochloride)-*block*-(*N*-isopropylacrylamide) (PAMPA-*b*-PNIPAM) was synthesized by the reversible addition–fragmentation chain-transfer (RAFT) polymerization technique (see the Supporting Information). AMPA was first polymerized in a dioxane/water mixture by employing 4-cyanopentanoic acid dithiobenzoate (CTP) as the chain-transfer agent (CTA) and 4,4′-azobis(4-cyanopentanoic acid) (V-501) as the free-radical source. The pH value of the solution was maintained between 4 and 5 to avoid hydrolysis and/or aminolysis of the CTA.[11] Under these conditions, the RAFT polymerization of AMPA is controlled (see the Supporting Information). The PAMPA homopolymer was then used as macroCTA for the block polymerization of NIPAM. Adjustment of the dioxane/water ratio allows the reaction to proceed under homogeneous conditions throughout the polymerization. Well-defined PAMPA-*b*-PNIPAM diblock copolymers were obtained (Table 1, see also the Supporting Information). The composition of the copolymer was calculated on the basis of size-exclusion chromatography (SEC) and ^{1}H NMR data (Table 1).

The diblock copolymers were dissolved in aqueous solution at room temperature. Increasing the solution temperature led to the formation of uniform aggregates with diameters of approximately 280 nm (Figure 1). Since the contour length of the diblock copolymers is approximately 35 nm, vesicular rather than micellar structures were found. The phase-transition temperature was shown to be dependent on the composition of the block copolymer; polymers with longer NIPAM block lengths had lower phase-transition temperatures. If the NIPAM block length is kept constant, increasing the AMPA block length results in an increase in the phase-transition temperature. This is in agreement with recent results by Stover and co-workers[12] which showed that the lower critical solution temperature of NIPAM homopolymers is dependent on both the molecular weight of the polymer and hydrophilicity of the end group.

The heating rate near the phase-transition temperature is an important factor for vesicle formation. To obtain a uniform size distribution, the heating rate should be kept around 0.1 °C min^{-1}. This was found to be especially important at higher solution concentrations (5 mg mL^{-1}). For example, at 5 mg mL^{-1}, an increase in the solution temperature from 25 to 45 °C over 5 min leads to a broad size distribution. If the solution concentration is kept relatively low ($<$ 0.5 mg mL^{-1}), faster heating rates can lead to uniform vesicles; however, the vesicles are smaller than those formed by a slow increase in the solution temperature (see the Supporting Information). The vesicles were shown to be stable, as their size remains constant above the phase-transition temperature. Thus, the vesicle self-assembly process seems to be kinetically controlled.

Figure 2 shows a TEM image of the vesicles formed from the $AMPA_{88}$-*b*-$NIPAM_{50}$ diblock copolymer. The number-average particle diameter of the vesicles in Figure 2 (left) is approximately 145 nm, which is slightly larger than the intensity-average diameter reported by dynamic light scattering (DLS; 118 nm) and suggests flattening of the vesicles adsorbed onto the TEM grid. The vesicle wall is estimated to

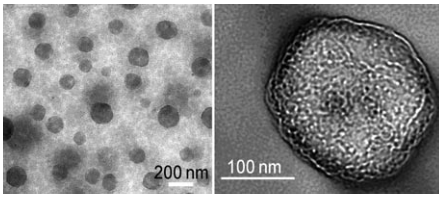


Figure 2. TEM images of vesicles prepared from PAMPA$_{88}$-PNIPAM$_{50}$ from the fast increase of the solution temperature from 25 to 45 °C (left) and a single vesicle (right).

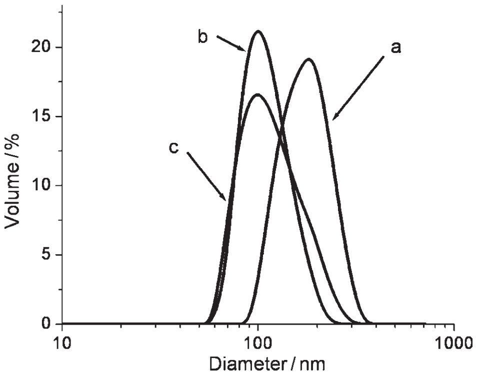


Figure 3. The size distribution (measured by dynamic light scattering) of the PAMPA$_{88}$-PNIPAM$_{50}$ diblock copolymer under specific conditions: a) 0.05 % aqueous solution at 45 °C; b) 0.05 % aqueous solution with PAMPS at 45 °C; c) 0.05 % aqueous solution with PAMPS at 25 °C.

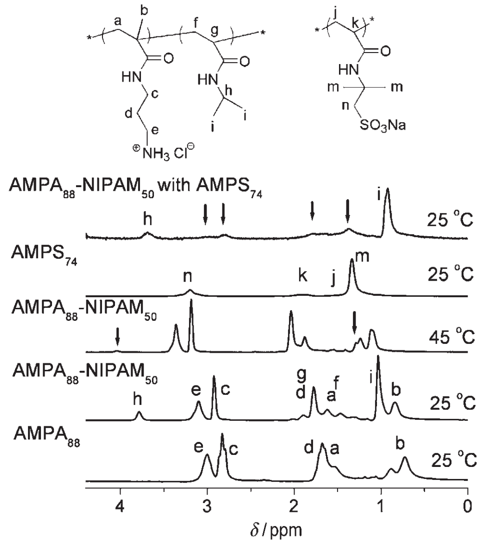


Figure 4. The ^{1}H NMR spectra of the homo and block copolymers at selected temperatures.

be 7 nm thick and is somewhat thinner than the contour length (12.5 nm) that was calculated for the hydrophobic PNIPAM$_{50}$ block.

The vesicles are stable between pH 0 and 11. However, the particle size was shown to vary with the pH of the solution (see the Supporting Information). At lower pH values, the vesicle was largest (310 nm at pH 3.0), and increasing the pH value of the solution decreased the size of the vesicles (e.g., 220 nm at pH 10.8). This is most likely caused by deprotonation of the AMPA block above its pK_a value. The solution concentration was also shown to influence the size distribution of the vesicles (see the Supporting Information). The vesicles were larger at higher solution concentrations (283 nm, 0.5 %, 50 °C) than at lower solution concentrations (192 nm, 0.005 %, 50 °C).

The complexation of oppositely charged polyelectrolytes has been used extensively for layer-by-layer deposition and DNA condensation. Recently, Armes and co-workers successfully applied this technology to form shell cross-linked micelles.[13] The group used a block polyelectrolyte (anionically charged) to cross-link a cationically charged micelle and concluded that a homopolyelectrolyte cross-linker led only to the formation of flocculated micelles. Herein, we found that the anionic homopolymer poly(sodium 2-acrylamido-2-methylpropanesulfonate) (PAMPS, $M_n = 17000$, $M_w/M_n = 1.18$), synthesized by RAFT, can be used efficiently to cross-link cationicaly charged vesicles. The rapid mixing of the vesicle solution with the PAMPS solution at 45 °C leads to the formation of ionically cross-linked vesicles owing to the inter-polyelectrolyte complexation of the PAMPA and PAMPS block (The AMPA/AMPS molar ratio was kept at 1:1). The vesicle size changes from 270 to 140 nm (Figure 3), which may be attributed to a decrease in the hydrophilicity of the APMA/AMPS block upon complexation; the vesicle morphologies remain the same.

After cross-linking, the solution was cooled to 25 °C. DLS indicated that the vesicles did not dissociate (Figure 3), which confirmed that the vesicle structure had been successfully "locked" by polyelectrolyte complexation. This conclusion was also confirmed by ^{1}H NMR analysis (Figure 4). At 25 °C, the diblock copolymers are fully solvated and signals associated with each block are visible. An increase in the solution temperature to 45 °C causes the NIPAM signal to become broadened, which indicates the formation of vesicles (this is also confirmed by DLS; Figure 1). After cross-linking at 45 °C, the solution was cooled to 25 °C upon which the NIPAM signal regained its intensity as the NIPAM block becomes hydrophilic whereas the AMPA and AMPS peaks broaden owing to their reduced mobility as a result of poleyelectrolyte complexation. The cross-linking process should be carried out at low concentrations ($< 0.5\,\mathrm{mg\,mL^{-1}}$) to prevent flocculation.

The ionically cross-linked vesicles are stable over a wide pH range (0–10.5). (DLS studies indicate these particles dissociate above pH 11, possibly because of hydrolytic instability.) Furthermore, the cross-linked vesicles retain their structural integrity in the presence of 0.8 M NaCl. Raising the salt concentration above 1.0 M causes the vesicles to dissociate.

Ionically cross-linked systems have advantages over chemically cross-linked vesicles in that the cross-linking reaction is facile (the process can be completed within a few minutes) and reversible (the cross-links can be removed by adding electrolyte), which will facilitate the removal of the vesicles after bioapplication.

In summary, thermally responsive AB diblock copolymers were successfully prepared by RAFT polymerization. At room temperature, these block copolymers exist as unimers in aqueous solution and self-assemble into vesicles when the solution temperature is increased. It has been shown that both the solution concentration and the heating rate influence the size and size distribution of the vesicles. These vesicles can be cross-linked (structurally "locked") by adding an oppositely charged polyelectrolyte.

Experimental Section

All chemicals were purchased from Aldrich at the highest available purity and used as received unless otherwise noted. *N*-(3-Aminopropyl)methacrylamide hydrochloride (AMPA) was purchased from Polysciences Inc. and used directly. *N*-Isopropylacrylamide (NIPAM; 97%, Aldrich) was recrystallized twice from a benzene/hexane mixture. 4,4′-Azobis(4-cyanopentanoic acid) (V-501) was donated by Wako Chemicals and used directly. 4-Cyanopentanoic acid dithiobenzoate (CTP) was synthesized and purified as previously reported.[14] ^{1}H NMR spectra were recorded in D_2O on a temperature-controlled Mercury Innova 500 MHz spectrometer. Size-exclusion chromatography (SEC) of aqueous solutions was performed with SynChropak CATSEC columns (100, 300, and 1000 Å; Eichrom Technologies Inc.), a Knauer K-2301 RI detector ($\lambda = 950$ nm), a Wyatt DAWN DSP multiangle laser light scattering detector ($\lambda = 633$ nm), and 1 wt% acetic acid/0.1 M Na_2SO_4(aq) as the eluent at a flow rate of 0.3 mL min^{-1}. The d*n*/d*c* of poly(AMPA) (0.181 mL g^{-1}) in the above eluent was determined at 25 °C with a Knauer K-2301 RI detector ($\lambda = 950$ nm). SEC in *N*,*N*-dimethylformamide was performed with a Viscotek-TDA (302 RI (633 nm), 7-mW 90° and 7° true low-angle light scattering detectors, $\lambda = 670$ nm) equipped with two Polymer Labs PLgel Mixed "C" columns (5 µm (30 cm), 200–2 000 000 g mol^{-1}) at 60 °C (flow rate 0.5 mL min^{-1}). Dynamic light scattering (DLS) studies of the block copolymer vesicles in aqueous solution were conducted with a Malvern Instruments Zetasizer Nano series instrument equipped with a 4-mW He–Ne laser operating at $\lambda = 632.8$ nm, an avalanche photodiode detector with high quantum efficiency, and an ALV/LSE-5003 multiple tau digital correlator electronics system. Transmission electron microscopy (TEM) measurements were conducted on a JEOL JEM-2100 electron microscope at an acceleration voltage of 200 kV. The specimens were prepared by placing a drop of the vesicle solution on a carbon-coated copper grid followed by water evaporation at 45 °C.

General procedure for the RAFT polymerization of AMPA: CTP (0.0078 g, 0.028 mmol) and AMPA (1.00 g, 5.6 mmol) were added along with deionized (DI) water (2.0 mL) to an ampoule. V-501 (0.00156 g, 0.0056 mmol) dissolved in dioxane (1.0 mL) was then added. The solution was stirred until all the CTP was dissolved. The ampoule was sparged with nitrogen for approximately 30 min and then placed in a preheated oil bath at 70 °C. The reaction was terminated after a specified time by cooling the reaction tube in an ice bath followed by exposure to air. The product was purified by dialysis against water (pH 4–5) and isolated by lyophilization.

Block copolymer synthesis: NIPAM (0.272 g, 2.4 mmol), $PAMPA_{88}$ (0.20 g), and V-501 (0.8 mg, 0.0024 mmol, dissolved in 0.6 g dioxane) were added along with DI water (0.8 mL) to an ampoule. After sparging with nitrogen for 30 min, the reaction was allowed to proceed at 70 °C for 2 h. The reaction was quenched by cooling the reaction vessel in an ice bath and exposure to air. The product was purified by dialysis against deionized water and isolated by lyophilization.

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