pH- and Reductant-Responsive Polymeric Vesicles with Robust Membrane-Crosslinked Structures: In Situ Crosslinking in Polymerization-Induced Self-Assembly


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EXPERIMENT SECTION

Materials

Polyethylene glycol monomethyl ether (PEG-OH, $M_n = 4000$ g/mol, TCI) was dried via azeotropic distillation of a toluene solution. Triethylamine (99 %, Aladdin), 4-dimethylaminopyridine (DMAP, > 99.0 %, TCI), N,N-dicyclohexylcarbodiimide (DCC, > 99 %, MACKLIN), bis(2-hydroxyethyl)disulfide (> 98 %, Aladdin), cystamine dihydrochloride (99 %, Energy Chemical), methacryloyl chloride (95 %, Aladdin), rhodamine B (≥ 95 %, Aladdin), and DL-dithiothreitol (DTT, 99 %, Aladdin) were used as received. 2-(Diisopropylamino)ethyl methacrylate (DIPEMA, 97 %, Sigma-aldrich) was passed through a neutral alumina column before use. 2,2’-Azobis(2-methylpropionitrile) (AIBN, 98 %, Aladdin) was recrystallized from ethanol before use. 4-(4-Cyanopentanoic acid) dithiobenzoate (CPDB) was synthesized according to the previous literature. All solvents were of analytical grade and used as received without further purification.

Synthesis of the Macro RAFT Agent PEG-CPDB.

PEG-OH (4.0 g, 1.0 mmol), DMAP (61 mg, 0.5 mmol), and CPDB (1.12 g, 4 mmol) were dissolved into 60 mL of dichloromethane. After gently stirring in an ice-water bath under argon atmosphere for 30 min, a solution of DCC (824 mg, 4 mmol) in dichloromethane (15 mL) was added dropwise into the above solution. The reaction mixture was stirred for 48 h at room temperature, then the resultant mixture was filtered to remove the solid byproduct. After removing most of the solvent via rotary evaporation, the residual concentrated solution was added dropwise into a large excess of ice diethyl ether to obtain pink precipitate. The precipitate was collected by filtration, and the above procedure of precipitation was repeated for three times.
The precipitate was dried under vacuum at room temperature overnight, and the desired PEG-CPDB was obtained as pink powder.

**Synthesis of the Crosslinker N,N-Cystaminebismethacrylamide (CBMA).**

Cystamine dihydrochloride (4.5 g, 20 mmol) was added into a 100 mL round bottomed flask containing 50 mL of water. An aqueous solution (5 mL) of sodium hydroxide (3.2 g, 80 mmol) was added into above mixture, and then the flask was immersed in an ice-water bath. After gently stirring at 0°C for 30 min, a solution of methacryloyl chloride (4.0 g, 40 mmol) in 20 mL of dichloromethane was added dropwise into the flask in 30 min. Some white precipitate was obtained during the procedure of adding methacryloyl chloride. After stirring for another 3 h, the reaction mixture was filtered to collect the white precipitate. The obtained solid was washed with deionized water for three times. The crude product was recrystallized from ethyl acetate for two times to obtain the resultant CBMA in 43% yield.

**Synthesis of the Crosslinker Bis(2-methacryloyloxyethyl) disulfide (DSDMA).**

Bis(2-hydroxyethyl)disulfide (3.1 g, 20 mmol) and triethylamine (4.4 g, 44 mmol) were dissolved into 80 mL of dichloromethane, and then the mixture was stirred in an ice-water bath for 30 min. A solution of methacryloyl chloride (4.6 g, 44 mmol) in 20 mL of dichloromethane was added dropwise into the above mixture via a constant pressure funnel. After stirring at room temperature overnight, the resultant mixture was washed with saturated aqueous Na₂CO₃ for three times. The organic phase was dried over anhydrous Na₂SO₄ overnight, and then filtered to remove the solids. The resultant solution was concentrated by rotary evaporation. Then the crude product was purified by silica column chromatography using hexane/ethyl acetate (8/2, volume ratio) as eluent to obtain DSDMA as colorless oily liquid in 85% yield.
RAFT Dispersion Copolymerization of DIPEMA and the Crosslinker CBMA or DSDMA

A typical procedure was carried out as follows. PEG-CPDB (43 mg, 10^{-2} mmol), AIBN (0.328 mg, 2\times10^{-3} mmol), CBMA (28 mg, 0.1 mmol), DIPEMA (192 mg, 0.9 mmol) and ethanol/water (mass ratio of 6/4, 1.05 g) were added into a glass tube. The tube was sealed under vacuum after three freeze-evacuate-thaw cycles, and then immersed in an oil bath at 70 °C while stirring for predetermined time. Then the reaction was quenched by rapid cooling to room temperature. For other RAFT dispersion copolymerization of DIPEMA and CBMA, the same procedures were conducted, and the feed molar ratio of (CBMA+DIPEMA)/PEG-CPDB/AIBN was kept at 100/1/0.2 with varying molar ratios of CBMA/(CBMA+DIPEMA) from 5% to 15%, and all the polymerizations were conducted for 18 h with almost complete reaction of the monomers. As contrast, RAFT dispersion copolymerization of DIPEMA and DSDMA was also conducted. The same procedure was conducted as above mentioned, and the feed molar ratio of (DIPEMA+DSDMA)/DSDMA/PEG-CPDB/AIBN = 100/10/1/0.2 with various feed molar ratio of DSDMA/(DIPEMA+DSDMA) was used in the RAFT dispersion copolymerization of DIPEMA and DSDMA.

Further Identification of the Hollow Structures of the Membrane Crosslinked Vesicles.

In order to further identifying the hollow structures of the membrane-crosslinked vesicles via TEM, silicon oxide was grafted in the membrane of the vesicles to enhance the contrast between the middle part and edge part of the vesicles. Polycondensation of tetraethyl orthosilicate (TEOS) catalyzed by the tertiary amine units of DIPEMA in the membrane-forming blocks was conducted via the following procedures. The obtained dispersion of vesicles were diluted with a mixture of ethanol and acidic aqueous solution (pH = 4.0) (v/v, 1/1), leading to a final
concentration of 0.5 mg/mL. The above diluted dispersion of vesicles (10 mL) were mixed with 0.25 g of tetraethyl orthosilicate (TEOS) and then stirred at room temperature for 24 h. Excess TEOS was removed by centrifugation, then the products were redispersed in ethanol/water (v/v, 8/2). The procedures of centrifugation and redispersion were repeated for another three times to remove the trace amount of unreacted TEOS. Then the resultant products were characterized via TEM.

**pH- and Reductant-Responsive Performances of the Crosslinked Vesicles**

The crosslinked vesicles (10 mg, D_{90}C_{10-18h}) were dispersed into 10 mL aqueous solution. The pH value of the above solution was adjusted to 8.0 via adding NaOH aqueous solution (0.1 M), and then adjusted to pH = 4.0 via adding HCl aqueous solution (0.1 M). The pH switching was repeated for three cycles. The size of the vesicles at each pH value was detected via DLS. The crosslinked vesicles (10 mg, D_{90}C_{10-18h}) were dispersed into 10 mL of PBS buffer (20 mM, pH 7.4) and acetate buffer (20 mM, pH 4.0) at room temperature, respectively. DTT was added into the above solution with the final concentration of DTT getting 10 mM. Then DLS was used to monitor the size changes at predetermined intervals.

**Encapsulation of Rhodamine B with the Cross-linked Vesicles**

The crosslinked vesicles (D_{90}C_{10-18h}, 10 mg) and rhodamine B (200 mg) were dissolved into 0.5 mL of tetrahydrofuran (THF). After stirring at room temperature overnight, the above mixture was poured into 10 mL of deionized water. The resultant mixture was dialyzed against deionized water to remove the unloaded rhodamine B. The deionized water was renewed every 0.5 h until the fluorescence of rhodamine B outside the dialysis tube was negligible. The resultant rhodamine-loaded vesicles were used for the following experiments. 10% of the above dispersions were lyophilized to afford solid powder. The resultant solid was dispersed into THF.
to release the loaded rhodamine B, and then added into a large amount of deionized water. The concentration of rhodamine B was evaluated via fluorescence spectra according to the standard curve. The loading content (LC) of rhodamine B was calculated according to the following equation S1.

\[
LC = \frac{\text{Mass of the loaded rhodamine B}}{\text{Mass of the vesicles}} \times 100% = \frac{\text{Mass of the loaded rhodamine B}}{10\text{mg} \times 10\%} \times 100% \quad \text{Equation S1}
\]

**pH- and Reductive-Regulated Release**

The above rhodamine-loaded vesicles were divided into four dialysis bags (molecular weight cutoff: 3500 Da), and then immersed into 80 mL of phosphate buffer (10 mM) with different conditions, pH 7.4 without DTT, pH 4.0 without DTT, pH 7.4 with 10 mM DTT, and pH 4.0 with 10 mM DTT, respectively. The releasing procedures were conducted under gentle stirring. An aliquot of solution (2 mL) outside of the dialysis bag was removed and replaced with the same volume of fresh medium at the predetermined intervals. The release behavior of Rhodamine B was monitored based on the fluorescence emission at 577 nm against a standard calibration curve.

**Characterization**

\(^1\)H NMR spectra were recorded on a Bruker DMX300 spectrometer at room temperature. Dynamic Light Scattering (DLS) measurements were carried out on a dynamic laser light scattering spectrometer (Zetasizer Nano ZS90, Malvern Instruments Ltd., Malvern, UK) with a 4.0 mW, 633 nm He-Ne laser at 25 °C. Each sample was run three times to obtain average value of particles size. Transmission Electron Microscopy (TEM) observations were performed on a
Hitachi H-800 TEM at the accelerating voltage of 100 kV at room temperature. The samples of TEM except the hybrid vesicles shown in Figure 6 were stained with phosphotungstic acid. Cryo transmission electron microscopy (cryo-TEM) images were obtained on transmission electron microscope from FEI company, operating at 120 kV. The fluorescence spectra were measured on a Shimadzu RF-5301PC luminescence spectrometer at room temperature.

**Figure S1.** $^1$H NMR spectrum of N,N-cystaminebismethacrylamide (CBMA) in CDCl$_3$. 
Figure S2. (A) Number-average diameter and (B) scattered light intensity of the nano-objects obtained at different polymerization time. Green line: the data of the nano-objects dispersed in ethanol/water, red line: the data of the nano-objects dispersed in THF.
Figure S3. The $^1$H NMR spectra of the reaction media after polymerization of (A) 0 h, (B) 1 h, (C) 2 h, (D) 3 h, (E) 4 h, (F) 5 h, (G) 6 h, (H) 9 h, (I) 12 h, and (J) 18 h. 2,2'-Bipyridine was added into the polymerization system as an internal standard substance. 50 µL of the reaction medium was taken out for $^1$H NMR (DMSO-d$_6$) at scheduled time of polymerization. The conversions of DIPEMA and CBMA were calculated according to the following equations:

Conversion ($\%$)$_{DIPEMA} = (1 - a_t/a_0) \times 100\%$

Conversion ($\%$)$_{CBMA} = (1 - b_t/b_0) \times 100\%$

$a_0$ is the integral values of protons in DIPEMA (peak a) at 0 h of polymerization. $b_0$ is the integral values of protons in CBMA (peak b) at 0 h of polymerization. $a_t$ is the integral values of protons in DIPEMA (peak a) at t h of polymerization. $b_t$ is the integral values of protons in CBMA (peak b) at t h of polymerization.

Note: The formed polymers at each time may have some residual vinyl units of CBMA, so a little portion of the peaks b may be due to the unreacted vinyl units of CBMA in the resultant polymers. For simplification, we hypothesized the peaks b is only attributed to the unreacted CBMA monomer in the solution herein.
Figure S4. GPC trace of the polymer obtained via RAFT dispersion copolymerization of DIPEMA and CBMA with feed molar ratio of CBMA/(DIPEMA+CBMA) = 10% after 1 h of polymerization (sample D_{90}C_{10-1h}).
Figure S5. AFM image of the polymeric vesicles D_{90}C_{10}-6h.
Figure S6. $^1$H NMR spectrum of bis(2-methacryloyloxyethyl) disulfide (DSDMA) in CDCl$_3$. 

Chemical shift (ppm)
Figure S7. The images of the gels obtained via RAFT dispersion copolymerization of DIPEMA and DSDMA at various feed molar ratios of DSDMA/(DIPEMA+DSDMA) and then immersed in THF for 24 h, (A) DSDMA/(DIPEMA+DSDMA) = 7%, (B) DSDMA/(DIPEMA+DSDMA) = 8%, and (C) DSDMA/(DIPEMA+DSDMA) = 10%.
Figure S8. The $^1$H NMR spectra of the reaction media after polymerization of (A) 0 h, (B) 0.5 h, and (C) 1 h. 2,2'-Bipyridine was added into the polymerization system as an internal standard substance. 50 µL of the reaction medium was taken out for $^1$H NMR (DMSO-d$_6$) at scheduled time of polymerization.

The conversions of DIPEMA and DSDMA were calculated according to the following equations:

Conversion (%)$_{\text{DIPEMA}} = (1 - a_t/a_0) \times 100$

Conversion (%)$_{\text{DSDMA}} = (1 - b_t/b_0) \times 100$

$a_0$ is the integral values of protons in DIPEMA (peak a) at 0 h of polymerization. $b_0$ is the integral values of protons in DSDMA (peak b) at 0 h of polymerization. $a_t$ is the integral values of protons in DIPEMA (peak a) at $t$ h of polymerization. $b_t$ is the integral values of protons in DSDMA (peak b) at $t$ h of polymerization.

Note: The formed polymers at each time may have some residual vinyl units of DSDMA, so a little portion of the peaks b may be due to the unreacted vinyl units of DSDMA in the resultant polymers. For simplification, we hypothesized the peaks b is only attributed to the unreacted DSDMA monomer in the solution herein.
Figure S9. The molar ratios of DIPEMA/DSDMA in the reaction media at different polymerization time for the RAFT dispersion copolymerization of DIPEMA and DSDMA with feed molar ratio of DSDMA/(DIPEMA+DSDMA) = 10%.
**Figure S10.** TEM images of particles fabricated via RAFT dispersion copolymerization of DIPEMA and DSDMA with feed molar ratio of DSDMA/(DIPEMA+DSDMA) = 10% at different polymerization time, A) 0.5 h and B) 1 h.
Calculation of the extent of protonation (PE) of PDIPEMA (pKa~6.3) at pH 4.0 and pH 8.0 as follows, wherein B represents PDIPEMA.

\[
BH^+ \leftrightarrow B + H^+
\]

\[
K_a = \frac{[B][H^+]}{[BH^+]}
\]

\[
-\log K_a = -\log \frac{[B][H^+]}{[BH^+]}
\]

\[
pK_a = pH - \log \frac{[B]}{[BH^+]}
\]

\[
\frac{[B]}{[BH^+]} = 10^{(pH-pK_a)}
\]

\[
PE = \frac{[BH^+]}{[B] + [BH^+]} \times 100\% = \frac{1}{[B] + [BH^+]} \times 100\% = \frac{1}{10^{(pH-pK_a)+1}} \times 100\% \quad \text{Equation S2}
\]

At pH 4.0, \[PE = \frac{1}{10^{(4.0-6.3)+1}} \times 100\% = 99.50\%
\]

At pH 8.0, \[PE = \frac{1}{10^{(8.0-6.3)+1}} \times 100\% = 1.96\%
\]

REFERENCES