

Feasible Fabrication of Hollow Micro-vesicles by Non-amphiphilic Macromolecules Based on Interfacial Cononsolvency

Jing-Hong Wang^{a,b}, Rui Chen^{a*}, Zi-Qing Zhao^a, Jie Shen^a, He Yang^a, Yan Luo^a, Gao-Jian Chen^{a,b}, Hong Chen^{a*}, and John L. Brash^c

^a State and Local Joint Engineering Laboratory for Novel Functional Polymeric Materials, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou 215123, China

^b Center for Soft Condensed Matter Physics and Interdisciplinary Research & School of Physical Science and Technology, Soochow University, Suzhou 215006, China

^c Department of Chemical Engineering and School of Biomedical Engineering, McMaster University, Hamilton, Ontario, Canada

Electronic Supplementary Information

Abstract Herein we present a new perspective showing that water-soluble liquids, when added to water, undergo transient emulsification before complete dissolution. Thus, non-amphiphilic macromolecules can self-assemble at the two-miscible-phase interface when cononsolvent effect appears. A representative case shown here is that when poly(*N*-isopropylacrylamide) (PNIPAm), prepared by aqueous radical polymerization, in methanol solution is added into water, the polymer chains rapidly self-assemble into hollow micro-vesicles based on the cononsolvency at water/methanol interface. This finding provides a subtle strategy to prepare hollow micro-vesicles by non-amphiphilic polymers without template participating. We proposed a new concept “interfacial cononsolvency” to describe the formation process. Due to the easy modification process, sugar-contained PNIPAm chains are synthesized by copolymerization. As an application example, it is shown that these sugar-contained PNIPAm chains can afford “sweet” micro-vesicles (containing glucose residues). And the “sweet” micro-vesicles can well mimic the protocells which are involved in the recognition of bacteria.

Keywords Non-amphiphilic; Interfacial cononsolvency; Two-miscible-phase interface; Hollow micro-vesicles; Protocells

Citation: Wang, J. H.; Chen, R.; Zhao, Z. Q.; Shen, J.; Yang, H.; Luo, Y.; Chen, G. J.; Chen, H.; Brash, J. L. Feasible fabrication of hollow micro-vesicles by non-amphiphilic macromolecules based on interfacial cononsolvency. *Chinese J. Polym. Sci.* 2021, 39, 856–864.

INTRODUCTION

Emulsification is a common phenomenon in which one liquid disperses in another immiscible one in the form of tiny droplets. The addition of surfactant reduces the interfacial tension and makes the droplets thermodynamically stable by forming a molecular layer or by electrostatic repulsion.^[1] Inspired by this principle, chemists have prepared delicate, soft structures by self-assembly of amphiphilic lipids,^[2–4] block copolymers^[5–7] or supramolecular complexes at the oil/water interface.^[8–10] These bottom-up methods are widely used in micro-nano printing,^[11,12] membrane science,^[13,14] biomedical materials,^[15,16] controlled drug release,^[17,18] biosensing^[19] and catalysis.^[20,21]

As a typical example, water and methanol are naturally miscible. The essence of the mixing process is the formation of hydrogen bonds between the two molecules, seemingly quite different from the traditional emulsification process.

Herein we present a new perspective showing that the water-methanol miscibility includes a time-dependent emulsion forming process. Upon mixing water and methanol, numerous methanol droplets are formed simultaneously. In this process, the methanol acts as the oil phase and the water as the aqueous phase. Normally, the droplets disappear rapidly due to the formation of water-methanol bonds. However, we found recently that poly(*N*-isopropylacrylamide) (PNIPAm), well-known as a “smart” polymer, can subtly utilize the interface produced in this transient emulsification process to self-assemble into hollow micro-vesicle based on its “cononsolvency” properties whereby either water or methanol alone are good solvents for PNIPAm, but mixtures of the two are not.^[22–24] This special phase behavior of PNIPAm not only becomes the evidence for the transient emulsification, but also shows that macromolecules can self-assemble at the transient two-phase interface even if they do not have an amphiphilic block structure.

Cononsolvency phenomenon of PNIPAm was first reported by Winnik *et al.* in 1990s.^[22] Generally, the mixed solvents might promote the dissolution of polymers, which could be estimated by Hildebrand solubility equation.^[25] But

* Corresponding authors, E-mail: chenrui@suda.edu.cn (R.C.)

E-mail: chenh@suda.edu.cn (H.C.)

Received November 30, 2020; Accepted December 22, 2020; Published online March 23, 2021

for PNIPAm, methanol was a typical additive to decrease its cloud point within solvent-specific composition ranges.^[22–24] Different theories were put forward to explain the microcosmic essence of cononsolvency. For instance, Wu *et al.* first observed the coil-to-globule-to-coil re-entrance transition of a single PNIPAm chain in water with the increase fraction of methanol by light scattering. They believed that the change of solvent quality is the main reason for the cononsolvency of PNIPAm.^[26] It can be understood as the chelation of methanol with water molecules reducing the hydration of polymers, which makes the cosolvent a poorer solvent than the pure ones. Several models were further proposed to explain this phenomenon in another way, including the preferential adsorption of methanol on PNIPAm chains,^[27] as well as alternative explanations based on geometry.^[28,29] Although different views have been raised, there is still controversy.^[30] Especially, a completely satisfactory mechanism is lacked to explain the existence of the upper critical solution temperature (UCST) in some cononsolvent pairs^[31,32] and the disappearance of cononsolvency at high pressure.^[33,34] Moreover, cononsolvency was commonly used as a strategy for polymer precipitation, except for some cases of microgels synthesis,^[35,36] few practical applications make further use of this property.

In this work, we employed an experimental operation contrary to the mixed steps used in previous cononsolvency study by dropping PNIPAm methanol solution into water instead of adding methanol into PNIPAm aqueous solution. It was accidentally found that PNIPAm could form a metastable liquid layer composed of micro-vesicles at the methanol/water mixing interface. These micro-vesicles could be preserved by chemical crosslinking and a hollow structure was demonstrated by electron microscopy and fluorescence microscopy. A new mechanism called “interfacial cononsolvency” was proposed to explain the formation of hollow micro-vesicles. This mechanism could guide a novel self-assembly strategy for a range of non-amphiphilic polymers. And it was demonstrated that the addition of functional monomers with a small molar ratio has no effect on the self-assembly of copolymers to form hollow micro-vesicles. As an application case, a copolymer consisting of glucose monomer and NIPAm was synthesized in our work to fabricate “sweet” hollow micro-vesicles, which could well mimic the protocells with proteoglycan membrane. We showed that these protocells had good affinity with specific proteins and could selectively regulate the behavior of different bacteria.

EXPERIMENTAL

Materials

N-isopropylacrylamide (NIPAm, 98%) was purchased from J&K (Shanghai, China) and recrystallized from acetone/hexane before use. Azodiisobutyronitrile (AIBN, 98%) was purchased from Macklin (Shanghai, China) and recrystallized from ethanol before use. Dibromoalkanes ($\geq 97\%$), with carbon number ranging from 4 to 9, were purchased from Adamas (Shanghai, China). 1-Bromoalkanes ($\geq 97\%$) and linear alkanes ($\geq 97\%$), with carbon number ranging from 4 to 9, were all purchased from Aladdin (Shanghai, China). LIVE/DEAD BacLight was purchased from Invitrogen (Shanghai, China). 1-Vinylimidazole (VIM, $\geq$

99%), fluorescein *o*-methacrylate (Fm, $\geq 95\%$), and FITC-concanavalin A (FITC-con A) were purchased from Sigma-Aldrich (Shanghai, China) and used directly. Other chemical reagents were purchased from Shanghai Chemical Reagent Company and purified by standard methods before use. Deionized water was prepared using a microporous water purification system with a minimum resistivity of 18.2 M Ω ·cm. Nitrogen gas was of high purity grade.

Characterization Methods

The monomers and polymers were characterized by ^1H -nuclear magnetic resonance (^1H -NMR, Varian Mercury-400 spectrometer, Varian, USA) and Fourier transform infrared (FTIR) spectroscopy (Nicolet 6700 FTIR spectrometer, Thermo Fisher Scientific, USA). The hydrodynamic diameter (D_h) of the micro-vesicles was measured by dynamic light scattering (DLS, Zetasizer Nano-ZS90, Malvern) at a concentration of 10^{-3} g/mL, and a scattering angle of 90° . The zeta potential of the microgels was determined with a zeta potentiometer (Zetasizer Nano-ZS90, Malvern), taking the average of at least three measurements. The micro-vesicles were also characterized by transmission electron microscopy (TEM, G-200, Hitachi). Briefly, one drop of suspension (1 mg/mL) was placed on a copper mesh for 30 min to allow for adsorption. The copper mesh was dried at room temperature before observing by TEM. Atomic force microscopy (AFM, Bruker Multimode 8, with the ScanAnalyst mode) and scanning electron microscopy (SEM, S-4700, Hitachi) were also used to characterize the micro-vesicles using standard procedures. Cryo-TEM measurements were conducted at the test center of Shanghai Jiao Tong University.

Preparation of PNIPAm of Variable Molecular Weights

PNIPAm was prepared by free radical polymerization. Typically, NIPAm and AIBN were dissolved in methanol in a 400:1 molar ratio, and nitrogen purged for 30 min; polymerization was carried out under nitrogen at 70°C for 24 h. To vary molecular weight, polymers were prepared using different solvents; namely, *tert*-butanol, 1,4-dioxane, tetrahydrofuran, *N,N'*-dimethylamine, and dimethyl sulfoxide.^[37] The crude reaction solutions were dialyzed against water for periods in excess of one week using cellulose ester membrane (molecular weight cut-off 3500 Da), and freeze-dried. The ^1H -NMR spectra of the different polymers are shown in Fig. S1 (in the electronic supplementary information, ESI) and FTIR spectra in Fig. S2 (in ESI). Molecular weight and polydispersity index data are listed in Table 1.

Preparation of P(NIPAm-co-VIM) with VIM as Crosslinking Sites

Monomers in different molar ratios and AIBN were dissolved in methanol in a 400:1 molar ratio with nitrogen purging for 30 min, and then reacted under nitrogen at 70°C for 24 h. The crude product solution was dialyzed against water for periods in excess of one week using a cellulose ester membrane (molecular weight cut-off 3500 Da), and freeze-dried. ^1H -NMR spectra are shown in Fig. S1 (in ESI) and FTIR spectra in Fig. S2 (in ESI). Molecular weight and polydispersity index data are listed in Table 1.

Synthesis of (Methacrylamido)glucopyranose (MAG)

MAG, a double bond monomer containing glucose units, was synthesized as reported.^[38] Briefly, 5 g of D-(+)-glucosamine

Table 1 Molecular weights and polydispersity index (PDI) of various samples.

Sample	Polymer (reaction solvent)	M_n (GPC)	PDI
1	PNIPAm (<i>tert</i> -butanol)	2.16×10^5	2.75
2	PNIPAm (methanol)	4.53×10^4	2.96
3	PNIPAm (DMF)	2.71×10^4	2.12
4	PNIPAm (DMSO)	7.60×10^4	3.57
5	P(NIPAm-co-VIM) (methanol) ^a	2.51×10^4	2.12
6	P(NIPAm-co-VIM-co-Fm) (methanol) ^b	2.35×10^4	2.63
7	P(NIPAm-co-VIM-co-MAG) (methanol) ^c	4.86×10^4	4.71

^a P(NIPAm-co-VIM) with feed containing 7% VIM, showed a typical molecular weight of $\sim 2.5 \times 10^4$. The VIM content in the copolymer, calculated from ¹H-NMR data, was about 9.9%. For VIM feed ratios of 4%, 13%, 18% and 23%, the VIM content in the polymer was 4.7%, 12.3%, 23.6% and 25.2%, respectively. The VIM content of the copolymer determines the crosslink density, and in this regard, preliminary experiments showed that feed ratios above 7% ensured sufficiently strong micro-vesicle shells. Excessive VIM feed content was unfavorable, and could not exceed 18%. ^b The contents of VIM and Fm in the copolymer were about 13.2% and 5.9%, respectively. ^c The contents of VIM and MAG in the copolymer were about 11.4% and 8.8%, respectively. All the polymers listed above could form the middle layer when their methanol solution was added into water.

hydrochloride and 3.2 g of potassium carbonate were dissolved in 120 mL of anhydrous methanol in a 250 mL round-bottom flask and placed in an ice/ethanol bath. After 30 min when the temperature reached -10°C , 1.8 mL of methacryloyl chloride was added dropwise. The reaction was allowed to continue for 4 h. After removing potassium carbonate by filtration, the product solution was concentrated to 15 mL. The product was purified by column chromatography (methanol:dichloromethane=1:4, V:V) and a white solid (2.8 g) was obtained by evaporation. The ¹H-NMR spectrum of MAG is shown in Fig. S3 (in ESI).

Preparation of P(NIPAm-co-VIM-co-Fm) and P(NIPAm-co-VIM-co-MAG)

The preparation of P(NIPAm-co-VIM-co-Fm) and P(NIPAm-co-VIM-co-MAG) was similar to that for P(NIPAm-co-VIM) described above. The content of the functional groups was controlled by varying the monomer ratios. For P(NIPAm-co-VIM-co-Fm), VIM comprised 7 mol% of the total monomer and Fm 1%. For P(NIPAm-co-VIM-co-MAG), VIM comprised 7 mol% of the total monomer and MAG 5%. ¹H-NMR spectra are shown in Fig. S4 (P(NIPAm-co-VIM-co-Fm), in ESI) and Fig. S5 (P(NIPAm-co-VIM-co-MAG), in ESI). Molecular weights and polydispersity indices are listed in Table 1.

Phase Separation and Dye Diffusion Experiments

PNIPAm sample ($M_w=2.2 \times 10^5$) were dissolved in 2 mL of methanol (2 mg/mL) and slowly added dropwise to 2 mL of ultrapure water. Allura red (dissolved in methanol, 5 mg/mL, 50 μL) was added to the upper methanol phase to observe the dye diffusion in this special mixture. The diffusion state was observed within 12 h.

Preparation of Stable Hollow Micro-vesicles

Hollow micro-vesicles were prepared based on the cononsolvency of PNIPAm in methanol/water mixtures. Typically, P(NIPAm-co-VIM) was dissolved in 20 mL of methanol (optimized concentration: 5 mg/mL) and the solution was added dropwise to water. After the middle white layer is formed, 5 μL of chemical crosslinker was added and then the upper and

lower layers were mixed by quickly shaking. After dialysis in water, the hollow PNIPAm micro-vesicles were obtained.

The final volume ratio methanol/water was controlled in the range 0.2–2.5 to ensure the formation of a turbid suspension based on cononsolvency. The length of chemical crosslinkers (controlled by the number of carbon atoms) would affect the stability of the resulting micro-vesicles in the pure water phase and therefore, 1,7-dibromoheptane, 1,8-dibromo-octane, 1,9-dibromononane would successfully retain the hollow structure of vesicle structure. 1-Bromoalkanes and linear alkanes were used as controls which demonstrated that the hydrophobic effect of the additives under non-crosslinking state could not stabilize the formation of micro-vesicles. In addition to methanol, a variety of water-soluble organic solvents could also be used to prepare hollow vesicles. A common feature was that the PNIPAm had cononsolvency in these solvents. Detailed data from these experiments are shown in Table 2. Hollow micro-vesicles comprising P(NIPAm-co-VIM-co-Fm) and P(NIPAm-co-VIM-co-MAG) were prepared similarly with a final volume ratio of methanol to water of 1:1.

Table 2 Conditions for preparation of hollow micro-vesicles.

Parameter	Condition
Carbon number of crosslinker (N) ^a	$7 \leq N \leq 9$
Concentration of polymer (C)	$0 < C \leq 20 \text{ mg/mL}$
Feed molar ratio of VIM (R_1)	$7\% \leq R_1 \leq 18\%$
Volume ratio of methanol to water (R_2)	$0.2 < R_2 < 2.5$
Water/Organic solvent pairs ^b	Water/methanol, ethanol, tetrahydrofuran
Temperature (T) ^c	$T_{wc} < T < T_{mc}$

^a Crosslinker must be a dibromoalkane. 1-Bromoalkanes and linear alkanes did not stabilize the shell of the micro-vesicles. ^b In addition to methanol/water pairs, micro-vesicles can be prepared in various water/organic solvent pairs. ^c T_{wc} : melting temperature of water; T_{mc} : boiling temperature of methanol.

Adsorption of FITC-con A on “Sweet” Micro-vesicles

20 μL of sweet micro-vesicle suspension (1 mg/mL) was added to 60 μL of HEPES buffer solution (10 mmol/L, pH=7.5, containing 1 mmol/L Ca^{2+} , 0.15 mmol/L Na^+ , and 0.01 mmol/L Mn^{2+}). 20 μL of FITC-con A (1 mg/mL) was then added, and the mixed solution incubated in the dark for 1 h. The free FITC-con A was separated by centrifugation (106 g, 2 min) and re-suspended in HEPES buffer solution. A drop of the suspension was taken for characterization by fluorescence microscopy (Olympus IX71).

Bacterial Culture and Pretreatment.

Small quantities of frozen *E. coli* MG1655 strain and *S. aureus* were immersed in sterile Luria-Bertani broth medium (LB), and then cultured in a shaking-bed incubator with a shaking rate of 190 oscillations per min for 12 h. The cultured bacteria were centrifuged at 3824 g for 2 min. After discarding the supernatant, the bacteria were re-suspended in sterile water. The centrifugation and washing process was repeated three times. Finally, the bacterial suspension was diluted with sterile water to a concentration of $1 \times 10^7 \text{ cells mL}^{-1}$ as measured by microplate reader at a wavelength of 600 nm.

Specific Recognition of Sweet Micro-vesicles by Bacteria

The bacterial suspension was first stained with LIVE/DEAD

BaCLight. Then 20 μL of sweet micro-vesicle suspensions (1 mg/mL) were added to 1 mL of bacterial suspension (concentration 1×10^7 cells·mL⁻¹) and the mixtures incubated in a thermostatted shaker for 0.5 h. Aggregation behavior was observed with an Olympus IX71 fluorescence microscope. The adhesion of bacteria on sweet micro-vesicles was observed by laser confocal microscopy and scanning electron microscopy (SEM).

Mannose Competition Experiment

Water solutions of mannose with varying concentrations were first prepared. Sweet micro-vesicle suspension (20 μL , 1 mg/mL) and mannose solution (2 μL , 1 mg/mL) were added into 1 mL of bacterial suspension (1×10^7 cells·mL⁻¹) and the mixture was incubated in a thermostatted shaker for 2 h.

RESULTS AND DISCUSSION

Interfacial Cononsolvency Phenomenon

Our findings are based on an interesting phenomenon that when PNIPAm-methanol solution is added slowly to water, the methanol and water do not fully mix (as shown in Movie S1 in ESI, when methanol droplets fall into water, they quickly turn to white emulsion and float on the surface of water without being affected by the large amount of water around them even if these droplets sink a certain distance by the gravity acceleration). A thin, white emulsion layer first forms above the aqueous phase; a transparent methanol phase then forms on the white layer (Fig. 1a). ¹H-NMR spectra showed that the upper methanol phase contains only tiny amounts of water. Correspondingly, the lower water phase contains almost no methanol. The middle phase is a viscous layer consisting of micro-vesicles (Fig. 1b) with hydrodynamic diameter in the range of 0.9–2.0 μm (Fig. 1c). A dye diffusion experiment showed that the viscous middle layer effectively inhibited flow within the different layers for at least 12 h (Fig. 1d). This result

indicates that the middle layer is a third phase independent of methanol and water and prevents the fusion of methanol and water. In addition, the turbidity and viscosity of the middle layer were only related to the mass concentration of PNIPAm with no dependence on PNIPAm molecular weight in the range of 2.7×10^4 – 2.2×10^5 (Table 1). And the middle layer could still be formed when PNIPAm aqueous solution was added into the methanol and therefore, it was not a physical phenomenon caused by solvent density. For a liquid mixed system, it is quite weird that the two miscible solvents can be stably separated and the key is the dense micro-vesicles in the middle layer. In order to explain this intriguing phenomenon, the structure of the observed PNIPAm micro-vesicles should be confirmed firstly.

Investigation and Characterization

Since the structure of the micro-vesicles formed by PNIPAm in the cononsolvent was metastable (maintained only at methanol/water volume ratios in the range from 0.2:1 to 2.5:1 after mixing the lower water phase and upper methanol phase by shaking, as shown in Movie S2 in ESI), a small chemical modification of the PNIPAm chain was needed to preserve the metastable interfacial phase transition for characterization. The modification has two requirements. First, a small number of crosslinking sites should be introduced to stabilize the micro-vesicle structure in pure water, which is accomplished by adding a monomer with suitable reactivity. Second, the added monomer should not affect the cononsolvency behavior of PNIPAm. Based on these requirements, we used 1-vinyl imidazole (VIM) to provide the cross-linking sites, and prepared a P(NIPAm-co-VIM) copolymer (Table 1, sample 5) by random radical copolymerization (Scheme 1a). The random copolymer had good water solubility and appropriate proportion of cross-linking sites. Besides, its phase transition temperature was around 40 °C, and it still showed cononsolvency property in

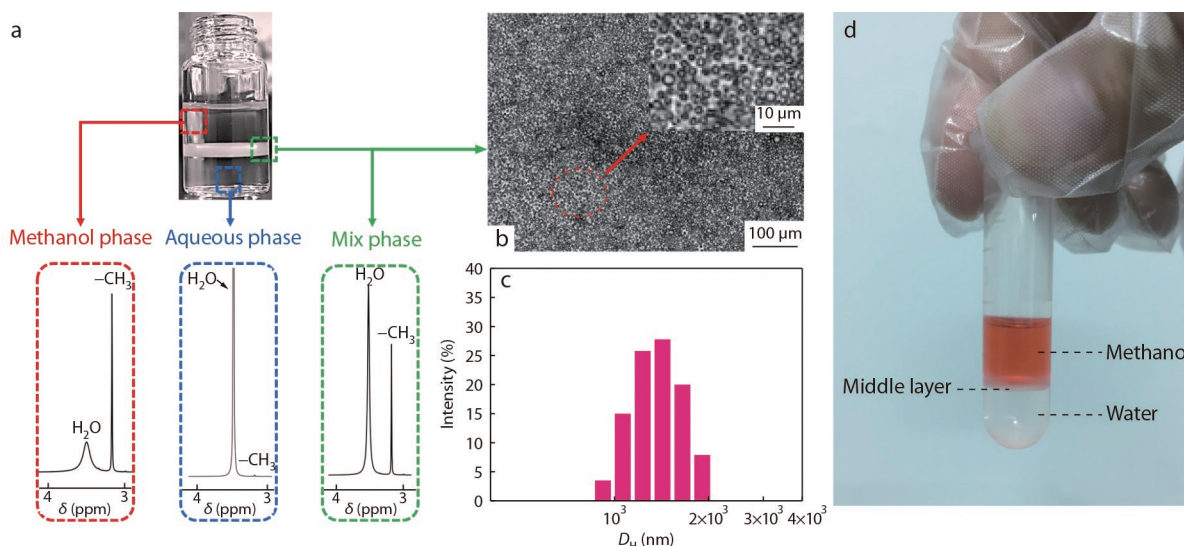
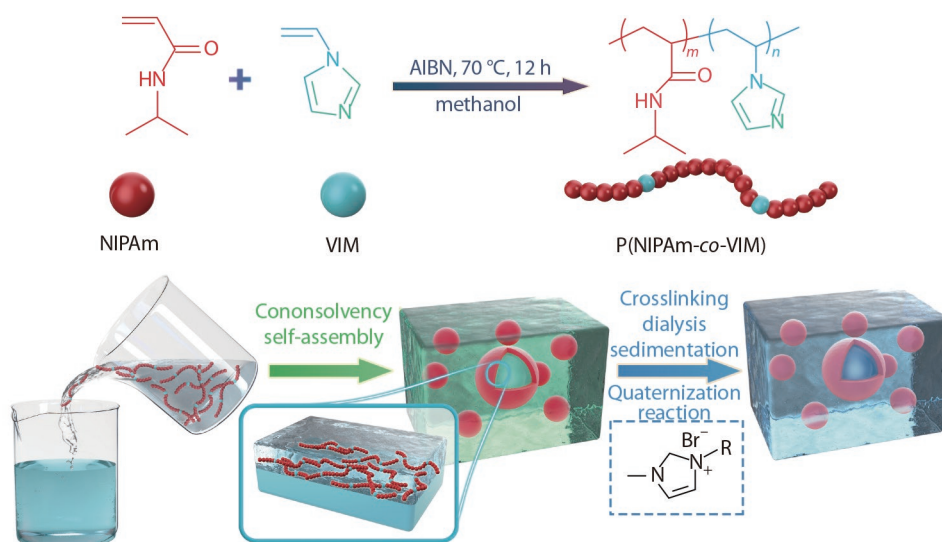


Fig. 1 (a) Typical stratification after adding PNIPAm-methanol solution ($M_w = 2.2 \times 10^5$, 5 mg/mL) to water, and ¹H-NMR spectra of the three phases (DMSO solvent, δ 3.33 H₂O, δ 3.16 CH₃). The thickness of the middle layer is dependent on the polymer concentration. (b) Viscous layer structure consisting of micro-vesicles observed by the optical microscope. (c) Size distribution of vesicles in middle layer from dynamic light scattering after diluting with a 1:1 (V/V) methanol/water solution. (d) The water-soluble dye allura red could not pass through the middle layer within 12 h.

water/methanol mixture. Then, the polymer was dissolved in methanol (5 mg/mL) and mixed with water (Scheme 1b). The final methanol/water volume ratio was controlled in the range of 0.2–2.5 to ensure the formation of a turbid emulsion. Subsequently, a small quantity of 1,8-dibromooctane was added as chemical crosslinker. This small molecule, which is insoluble in the water/methanol mixture, penetrated rapidly into the collapsed PNIPAm chains and crosslinked the polymer via the quaternization reaction between the bromooctane and the imidazole groups of VIM residues. This strategy has been

proved feasible in many cases.^[39–41] Finally, pure P(NIPAm-co-VIM) suspension was obtained by repeated dialysis and sedimentation (Fig. 2a). In the control experiments using dibromo crosslinkers with less than seven carbon atoms, it was found that the emulsion was not stable and dissolved completely during dialysis (Table 2). This was mostly attributed to the molecular chain distance larger than the length of crosslinker as reported before.^[42] These results indicate that the dibromo functionality is required to immobilize the polymer chains in place and that the selection of crosslinker is



Scheme 1 Preparation of P(NIPAm-co-VIM) micro-vesicles.

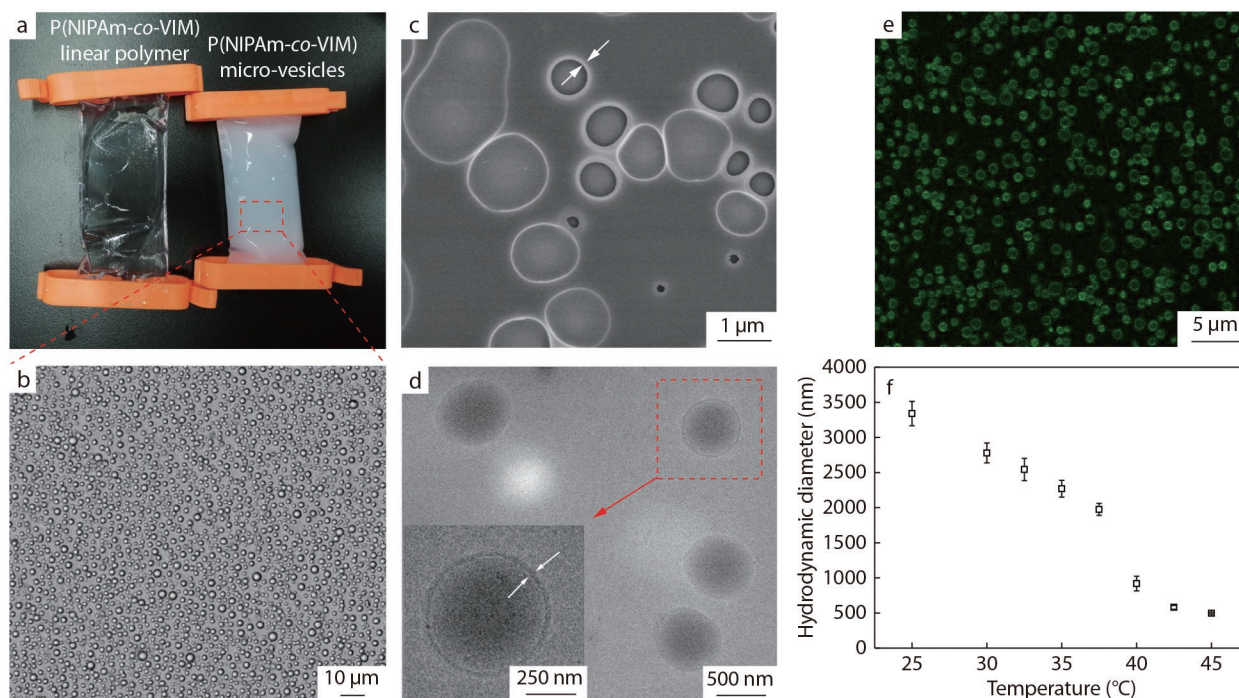


Fig. 2 (a) Photographs comparing linear P(NIPAm-co-VIM) solution and micro-vesicle suspension after dialysis. (b) Optical microscope image of micro-vesicles. (c) TEM image of micro-vesicles. (d) Cryo-TEM image of micro-vesicles. (e) Confocal microscopy images of fluorescently labeled micro-vesicles. (f) Temperature dependence of the hydrodynamic diameter of hollow micro-vesicles in water (10^{-3} mg/mL). Data are mean $\pm$ SE ($n=3$).

dependent on its length. In addition, excess PNIPAm can lead to the crosslinkage between micro-vesicles and thus forming aggregates. Therefore, as described above, the concentration of PNIPAm in methanol was kept below 20 mg/mL (Table 2). And the optimized concentration was ~5 mg/mL. Other parameters for the formation of micro-vesicles, such as feed molar ratio of VIM (R_1), volume ratio of methanol to water (R_2), water/organic solvent pairs and temperature were all presented in Table 2. For R_1 , too much VIM will lead to the loss of the cononsolvent effect of the PNIPAm chains. And less R_1 could not allow the crosslinking of different PNIPAm chains. For R_2 , either too much water or too much methanol in the water-methanol mixture will dissolve the PNIPAm chains that no micro-vesicle could be formed. In addition to methanol, micro-vesicles could also be formed in other solvent mixtures such as water/ethanol, water/tetrahydrofuran, as cononsolvency could also occur in these solvent mixtures. Finally, the temperature had little effect on the formation of micro-vesicles as the phase transition temperature of PNIPAm was broadened in the cononsolvent pairs.^[22]

Observation of the P(NIPAm-co-VIM) suspension in the optical microscope showed that the crosslinked micro-vesicles remained stable permanently in aqueous solution at room temperature, with diameters ranging from 0.5 μm to 3.0 μm (Fig. 2b). TEM (Fig. 2c) and cryo-TEM (Fig. 2d) images confirmed that the micro-vesicles had a very thin shell with a thickness of about 19 nm, indicating that they were hollow (white arrows in figures). AFM also showed the morphology of the micro-vesicles in the dry state in detail (Fig. S6, in ESI). Collapse of the micro-vesicles was a typical feature of the hollow structure.^[43,44] Besides, thermo-sensitivity of the micro-vesicles showed interesting features, *i.e.*, a temperature-dependent decrease in hydrodynamic diameter with an irreversible “burst” above the LCST (40 °C) (Fig. 2f). This phenomenon was consistent with our observation that vesicles have very thin shells. Moreover, as verification, P(NIPAm-co-VIM) micro-vesicles labeled with FITC (Table 1, sample 6), observed in the confocal microscope, showed a green fluorescent shell with a black inner cavity (Fig. 2d). Our findings were partly relevant with Winnik’s recent work.^[45] In their work, the nonequilibrium liquid-liquid phase separation of poly(*N*-isopropylacrylamide) in water/methanol mixtures after resting for some time would cause the polymers to form vesicles. However, according to our observations, micro-vesicles with hollow structures were formed at the initial moment of mixing water and methanol, and here we collected sufficient evidence for these micro-vesicles through the cross-linking strategy.

Explanation and Mechanism

It is of interest to understand why PNIPAm self-assembles as a shell at the transient interface between methanol and water rather than completely collapsing into globules when the solvents are mixed as described in previous inferences. We propose a plausible mechanism based on the thermodynamics process within polymers and solvent molecules at the shell interface.

$$\Delta G_{\text{total}} = \Delta H_{\text{total}} - T\Delta S_{\text{total}} \quad (1)$$

As shown in Eq. (1), when a small number of droplets was added, the temperature of the whole system can be regarded as constant. Therefore, the Gibbs free energy of the whole metastable system, ΔG_{total} , are determined by the en-

thalpy ΔH_{total} and the entropy ΔS_{total} . Due to the existence of interfacial energy between water and methanol at their initial contact, the PNIPAm chains tend to accumulate at the interface. It is useful to consider the different hydrogen bonds that may form in this system, *i.e.*, methanol-water, O—H...O with average bond energy 21 kJ/mol, and water/methanol-PNIPAm, O—H...N with average bond energy 8 kJ/mol.^[46] The energy of the water-methanol bond is greater than that of the PNIPAm-water/methanol bond, which means the ΔH_{total} is negative. Therefore, by an enthalpy-driven process the water may quickly “capture” methanol from the polymer chains, causing transient de-solvation of the polymers (Fig. 3b). For a single polymer chain, the solvent molecules are rapidly replenished as described in the hydrogen bond competition models of Tanaka *et al.*^[47,48] and Mukherji *et al.*^[49,50] For an assemblage of many polymer chains, however, the combination of inter-chain hydrogen bonding and hydrophobic interactions causes rapid self-assembly to form a thin shell (Figs. 3c and 3d). In other words, the hydrophobic interactions briefly limit the further binding of the amide bond to solvent molecules. The most direct evidence, as shown in Fig. 1, is that for a very short time, the collapsed PNIPAm chains produced by cononsolvency is soluble neither in methanol nor in water (Fig. 3a). The PNIPAm shell consisting of collapsed chains is a natural barrier which restricts the further mixing of water and methanol. It means that less solvent molecules bind to the polymer chains and those free solvent molecules falling away from the polymer chains increase the system entropy in a short period of time and therefore, ΔG_{total} is negative which indicates that the formation of the shell is spontaneous. The addition of crosslinkers enhances the hydrophobic interactions between chains and promotes the retention of the thin polymer shell. The whole process that the PNIPAm chains self-assemble at the water/methanol interface can be donated as a new concept, “interfacial cononsolvency”. This concept clearly presents the phase transition behavior of PNIPAm chains during cononsolvency appears and explains why hollow structures are formed.

It should be noted that, in this process, non-amphiphilic polymers can self-assemble at the transient interface of two miscible solvents. Different from the precipitation process that PNIPAm chains might aggregate when its poor solvents are added, this is originated from its cononsolvent effect as both water and methanol can be good solvents. For other polymers such as poly(*N,N'*-dimethyl acrylamide), poly(*N,N'*-diethyl acrylamide), polyacrylamide and polyvinylcaprolactam, they could not form hollow structures and no phase transition occurs when their aqueous solutions were added into methanol. These results further demonstrate the interfacial cononsolvent functions during this process.

Applications

Due to the feasible modification of the PNIPAm chain, functional units can be easily copolymerized into the chains. As an application, 2-(methacrylamido)glucopyranose (MAG), a glucose with carbon-carbon double bond as a comonomer was modified into the PNIPAm chains by random copolymerization (Table 1, sample 7). And then hollow micro-vesicles containing glucose groups (sweet micro-vesicles) were prepared by the reported interfacial cononsolvency method. It was shown that

the addition of sugar units did not affect the formation of sweet micro-vesicles (Fig. 4a). Sugar code is an important “language” in intercellular communication, and sweet micro-vesicles might well simulate the recognition and signal transduction functions of protocells interacting with living cells through the glycoprotein and proteoglycan receptors on the cell membranes.^[51] As verification, we first used FITC-concanavalin A (FITC-con A), a lectin with high affinity for glucose,^[52] to estimate the density of carbohydrate recognition sites on the surface of protocells. The green fluorescence on “sweet” protocells showed the presence of glucose units (Fig. 4b).

Then, we selected *E. Coli* MG1655 as the signal communication object of sweet micro-vesicles, with *fimH* on its fimbriae as a special protein with high affinity for mannose ($K_d=2.3 \mu\text{mol/L}$) and glucose ($K_d=9.24 \text{ mmol/L}$).^[53,54] We found that *E. Coli* MG1655 could be recognized and bounded to the surface of sweet micro-vesicles (Figs. 4c, 4e and 4f, Fig. S7 in ESI). This aggregation was disrupted with the addition of mannose since mannose and *fimH* have higher binding constants. However, *S. aureus* lack this special glucose binding lectin and do not aggregate (Fig. 4d). This aggregation behavior is considered as an important mode of communication between bacteria, and as

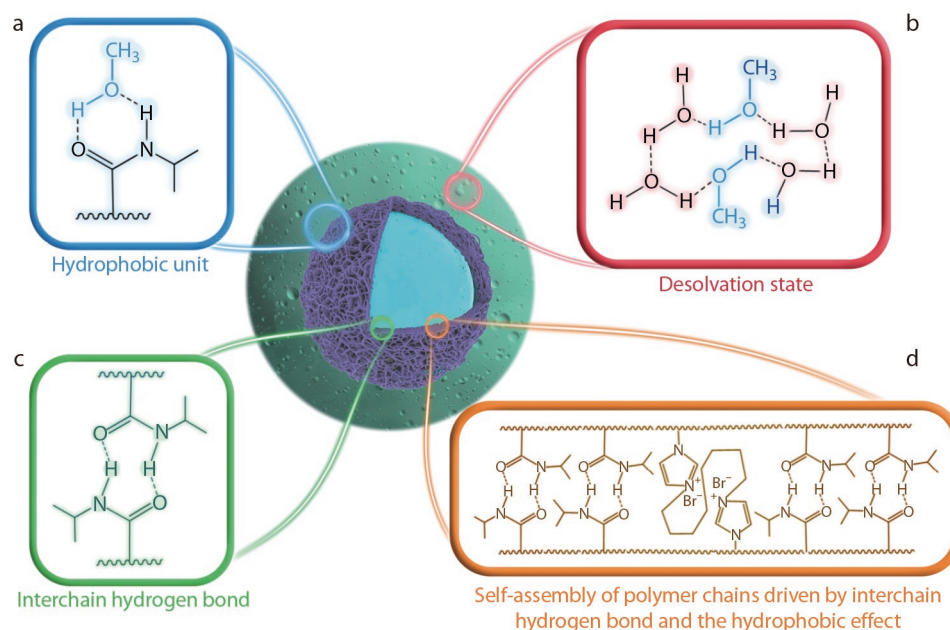


Fig. 3 Self-assembly mechanism of PNIPAm chains when PNIPAm-methanol solution is mixed with water.

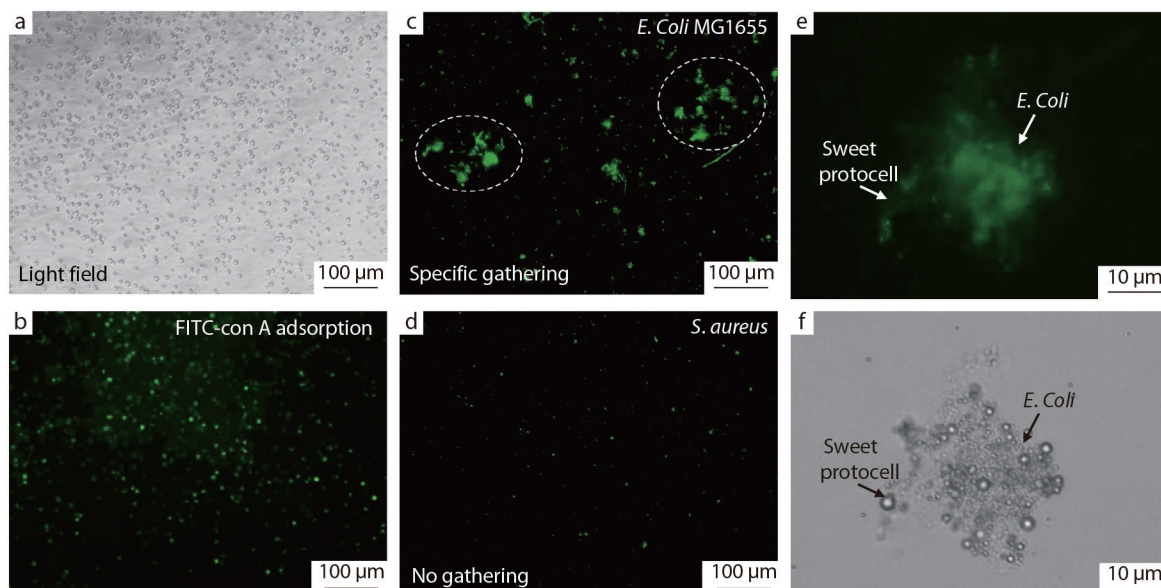


Fig. 4 (a) “Sweet” protocells adsorbed with FITC-con A observed under visible light. (b) “Sweet” protocells adsorbed with FITC-con A observed under fluorescence field; (c) *E. Coli* MG1655 specifically “gathered” under the attraction of “sweet” protocells; (d) *S. aureus* lacking sugar-binding lectins on its fimbriae did not “gather”. (e, f) Details on the interaction between *E. Coli* MG1655 and “sweet” protocells observed under visible light and fluorescence.

a way to regulate their proliferation, adhesion, and virulence.^[55] It is possible that the sweet micro-vehicles developed in this work may become a highly efficient tool for regulating these functions.

CONCLUSIONS

In conclusion, we proposed, for the first time, the concept of interfacial cononsolvency and fabricated the hollow micro-vesicles consisting of NIPAm monomers based on this special mechanism. It is shown that nonamphiphilic macromolecules can self-assemble at a transient interface through H-bonding and hydrophobic interactions with solvent molecules. This transient interface is known to occur when water mixes with other water-soluble organic solvents; it may even be seen at “water/water” interfaces (water mixes with water).^[56] This template-free method can be readily used for the high-efficiency preparation of hollow polymer micro-vesicles, and can be considered as a possible pathway for the creation of natural protocells. Unlike protocells formed by block copolymers, our findings make the addition of functional receptors on the protocell surface convenient and the distribution of these receptors reasonable. The results of these studies may have applications in cell biology research, especially in the construction of artificial cells, or other biological vesicle models with intelligent features. In addition, this finding may enable us to better understand the principle of life self-assembly in the absence of oil-water interface in the early life formation process.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://doi.org/10.1007/s10118-021-2541-z>.

ACKNOWLEDGMENTS

This work was financially supported by the National Natural Science Foundation of China (Nos. 21905192, 21935008 and 21674074), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) and China Postdoctoral Science Foundation (No. 2019M661925).

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