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Polymerization of a new thermo-responsive copolymer with *N*-vinylcaprolactam and its application in recyclable aqueous two-phase systems with another thermo-responsive polymer

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Abstract

Background: The recovery characteristics of phase-forming polymers are essential for aqueous two-phase systems (ATPS) to recycle in bioseparation engineering.

Results: A new thermo-responsive copolymer (P_{VBAm}) is suggested based on *N*-vinylcaprolactam, acrylamide, and butyl methacrylate. Together with another thermo-responsive polymer, poly (*N*-isopropyl acrylamide) (P_{N}), it has been applied to form a recyclable ATPS. P_{VBAm} and P_{N} were designed to obtain structures and molecular weights allowing a lower critical solution temperature (LCST). By polymerization optimization, both P_{N} and P_{VBAm} were obtained with recoveries 98.5% and 95% above their LCST (i.e., P_{N} 32.5 °C and P_{VBAm} 40.5 °C), respectively, which allows each ATPS phase to be effectively recycled. The recycled ATPS based on P_{VBAm} and P_{N} was applied to the partitioning of vitamin B₁₂. Under optimized conditions (5% PVBA_m/3.5 %PN ATPS, in the presence of 0.8 M KCl, pH 4.0), the partition coefficient of vitamin B₁₂ reached a value of 5.81.

Conclusion: The new ATPS based on the thermo-responsive copolymer $P_{\text{VBAm}}/P_{\text{N}}$ possessed appropriate recycling characteristics regarding LCST, as well as recovery and phase separation characteristics.

Keywords: Aqueous two-phase systems, Thermo-responsive polymer, *N*-isopropyl acrylamide, *N*-vinylcaprolactam

Background

Aqueous two-phase systems (ATPS) are promising alternatives to extract and purify biomolecules thanks to high biocompatibility and readiness to scaling up (Molino et al. 2013). ATPS have been applied for bioseparation since several decades (Asenjo and Andrews 2012), but not yet applied to industrial processes. Conventional ATPS were traditionally prepared from polyethylene glycol and salts or dextran and applied for the extraction of different biomolecules (Ramesh and Murty 2015). These

original phase-forming components were difficult to recover, due to high cost and the large amounts of salt required, which limited their use for process-scale separations. In recent years, recyclable ATPS have been suggested as alternatives to traditional ATPS, particularly based on thermo-responsive (TR) polymers.

During the past decades, polymers based on ethylene oxide-propylene oxide (EOPO) have been extensively studied in TR ATPS (Koon et al. 2015). Thus, in 1995, Berggren et al. (1995) reported the first preparation of ATPS based on an ethylene oxide-propylene oxide (EOPO) TR copolymer. In a subsequent study, Persson et al. (1999) used EO₅₀PO₅₀ to form ATPS with a hydrophobically modified random copolymer of EO and PO (HM-EOPO). The TR polymers used in these ATPSs

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could be recycled via a temperature-induced phase separation process. The recoveries of EO₅₀PO₅₀ and HM-EPO were 73% and 97.5%, respectively. In a study by Ng et al. (2012) in 2012, EOPO₃₉₀₀ (LCST 50 °C) was used to form ATPS with potassium phosphate, achieving a recovery of 83.1%.

Other kinds of TR polymers can be recycled by temperature-induced precipitation, such as polymers based on *N*-isopropylacrylamide (NIPA) and *N*-vinylcaprolactam, (NVCL). NIPA has been the most widely used monomer for the synthesis of TR polymers (Sulu et al. 2017); because of their good responsive characteristics and favorable LCST (32 °C), NIPA polymers have been studied and used in both laboratory and industrial scale. They have also been reported to form ATPS as affinity polymers (Tan et al. 2017). The use of polymers based on NVCL was reported by Shostakovski (1959) already in 1957. However, despite its long history, the first P_{NVCL} -based application was not published until in 1996 (Makhaeva et al. 1996). In 2008, the behavior of P_{NVCL} in ATPSs was reported for the first time by Foroutan et al. (2008). The LCST of P_{NIPA} and P_{NVCL} (32–34 °C) is near to the physiological temperature range. However, there are still few reports that polymers based on NIPA and NVCL have been applied in bioseparations.

In our laboratory, we have recently developed new ATPS forming polymers based on *N*-isopropyl acrylamide (NIPA) or *N*-vinylcaprolactam (NVCL) as monomers. A thermo-pH-responsive ATPS has been reported, which constitutes a TR polymer poly(*N*-isopropyl acrylamide-*co*-*n*-butyl acrylate) (P_{NB}) and a pH-responsive terpolymer (Miao et al. 2010). Hou reported ATPS formed by thermo-responsive copolymers P_{NE} (*N*-isopropyl acrylamide/ethyl methacrylate) and P_{VAm} (NVCL-acrylamide) (Hou and Cao 2014).

To obtain ATPS suitable for industrial scale separation, new recyclable ATPS comprising two TR polymers, P_{VBAm} (NVCL-butyl methacrylate-acrylamide) and P_{N} (NIPA) with higher LCST, were tried in this study and applied to the partition of vitamin B₁₂ (VB₁₂). The purification of VB₁₂ is traditionally based on organic solvent extraction and, therefore, in order to answer a demand for environmental friendly purification processes is a suitable model for ATPS. Furthermore, the newly designed $P_{\text{VBAm}}/P_{\text{N}}$ ATPS was compared with other reported TR ATPS regarding the polymerization process, the polymer LCST, the recovery, the phase formation and the recycling characteristics.

Methods

Materials

N-isopropyl acrylamide (NIPA) and VB₁₂ (98%) were purchased from Jing Chun Chemical Co. (Shanghai, China).

NVCL was purchased from Ao Te Chemical Co. (Zibo, China), and acrylamide (Am), butyl methacrylate (BMA), 2-2'-azoisobutyronitrile (AIBN), benzene, hexane, petroleum ether, acetone, tetrahydrofuran (THF), and ethanol were obtained from Ling Feng Chemical Co. (Shanghai, China). All chemicals were of analytical grade and used without further purification.

Polymerization of P_{VBAm} and P_{N}

P_{VBAm} was polymerized as illustrated in Fig. 1a. Different ratios of NVCL, BMA and Am were used as monomers dissolved in 150 mL benzene, with different concentrations of AIBN (0.5–5.5%, w/w) as initiator. The solutions were stirred using a magnetic stirrer under a constant flow of nitrogen for 15 min before polymerization. The polymerization reaction was carried out at different temperatures (50–70 °C) for 24 h in a water bath shaker at a speed of 120 rpm. In order to remove residual benzene and impurities, such as unreacted AIBN, the products were dissolved in 100 mL THF and precipitated by the addition of hexane. Finally, the precipitate was washed with ethanol and dried under vacuum to constant weight.

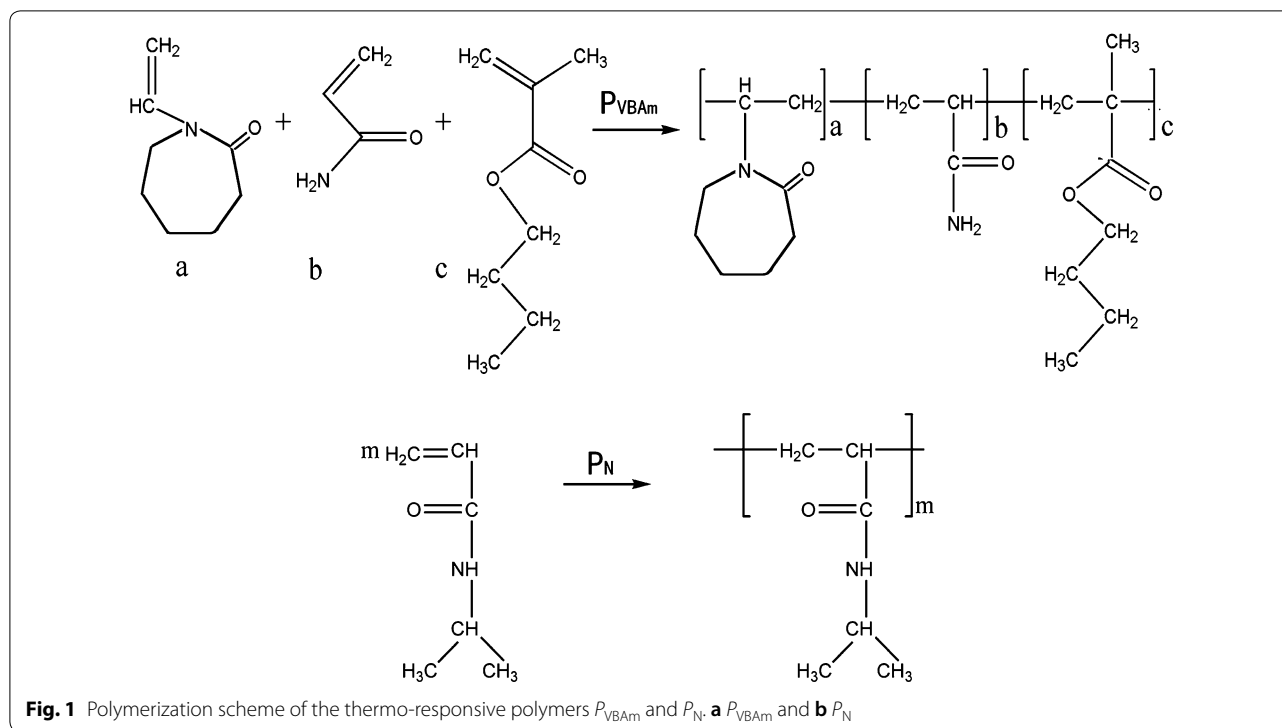
P_{N} was synthesized as illustrated in Fig. 1b. First, 5.0 g *N*-isopropyl acrylamide was dissolved in 150 mL benzene in the presence of different concentrations of AIBN (0.5–5.5%, w/w). After 15-min nitrogen de-oxygenation, the polymerization reaction applied was identical to that of P_{VBAm} . The products obtained were dissolved in 100 mL acetone, precipitated by the addition of hexane, and dried under vacuum at 55 °C to constant weight.

Characterization of P_{VBAm} and P_{N}

The P_{VBAm} and P_{N} reaction products were analyzed using a Nicolet 6700 FTIR spectrometer (Thermo Electron Corporation, UK). The molecular weights of the copolymers were determined using an Ubbelohde viscometer and calculated according to the Kuhn–Mark–Houwink equation (Scholte et al. 1984). The LCST of the copolymers was determined by measuring the transmittance of 5% P_{VBAm} and 5% P_{N} at different temperatures, using a spectrophotometer (Shimadzu, Japan).

Recycling of P_{VBAm} and P_{N}

The recoveries of P_{VBAm} and P_{N} were determined by measuring the amount obtained after thermo-precipitation above LCST. Thus, the precipitate was filtered and dried to constant weight. To this end, solutions of P_{VBAm} and P_{N} in the pH range 4.0–10.0 were prepared and thermo-precipitated. The effect of different concentrations of salt (KCl, NaCl, Na₂SO₄, K₂SO₄, Na₂HPO₄, and NaH₂PO₄) on polymer recovery was further investigated on a 10% solution of P_{VBAm} and P_{N} .



Construction of phase diagram

The Cloud Point Method (Pfenning et al. 1998) was used to construct a P_N/P_{VBA_m} ATPS phase diagram. Thus, a binodal curve was drawn on the basis of the change in turbidity when two phases were formed. To this end, a 10% solution of P_N was added dropwise to a 10% solution of P_{VBA_m} and mixed in test tubes using a vortex mixer. The concentrations of P_N and P_{VBA_m} at each turbidity point were calculated according to the additional mass and volume in the system. Finally, the binodal curve was drawn based on the concentrations of P_N and P_{VBA_m} . The tie line of the M point was obtained as follows: P_N and P_{VBA_m} were mixed for several minutes and settled at room temperature until a clear phase was obtained, followed by determination of the phase volume ratio. The concentrations of P_N and P_{VBA_m} in the top and bottom phases were measured by UV spectrophotometry (Shimadzu, Japan). The concentration of P_N was measured at 190 nm with reference to a standard curve. The concentration of P_{VBA_m} was calculated by subtracting the absorbance of P_N from the total absorbance of P_N and P_{VBA_m} at 210 nm. Finally, a tie line was drawn based on the concentrations of P_N and P_{VBA_m} in the top and bottom phases, respectively.

Partition of vitamin B₁₂

The P_{VBA_m}/P_N ATPS was applied to the partitioning of vitamin B₁₂. The initial concentration of VB₁₂ in the

ATPS was 0.2 mg/mL. The effects of pH and inorganic salts on the partition of VB₁₂ were investigated. After the ATPS reached equilibrium at room temperature, aliquots were sampled from the top and bottom phases using a micro-syringe. The concentration of VB₁₂ in both phases was determined by measuring the absorbance of the samples using a UV-vis spectrophotometer at 361 nm (Jin and Guo 2007). The partition coefficient of VB₁₂ was calculated as the ratio of the concentrations in the top and bottom phases, respectively. The recovery yield (Y_R) was calculated according to the following equation:

$$1/Y_R = 1 + 1/K * V_b/V_t$$

where K is the partition coefficient, V_b is the volume of the bottom phase and V_t is the volume of top phase.

Each experimental condition was subject to three parallel repetitions and all measurements were repeated at least three times until reproducible data were obtained. The data are expressed as the mean \pm standard deviation (SD) and analyzed by Excel.

Results and discussion

Characterization of P_{VBA_m} and P_N

In this study, the TR copolymer P_{VBA_m} is designed and synthesized using three functional monomers. Among them, NVCL forms the main backbone of P_{VBA_m} and is responsible for the thermo-responsive function; BMA

contains a hydrophobic group that is used to adjust the solubility and the LCST. Finally, Am contributes to the hydrophilic properties of the copolymer. The TR copolymer P_N is polymerized using only one monomer, i.e., NIPA.

Viscosity average molecular weight

During polymerization, the viscosity average molecular weight (M_v) of the polymer is strongly influenced by the reaction temperature and the amount of the initiator AIBN. Thus, the M_v of P_{VBAm} and P_N decreases at higher reaction temperatures (Fig. 2a). And higher AIBN concentrations leads to higher M_v in the low concentration range and then its M_v decreased with an increase in AIBN concentration (Fig. 2b). The polymer molecular weight strongly influences the phase formation: thus phases are not formed by polymers with too low M_v . In contrast, ATPS formed by polymers with high M_v result in a very viscous solution that is detrimental to effective mass transfer. With respect to phase formation

and viscosity, 1.5% and 4.0% (w/w) AIBN are preferably used for polymerization of P_N and P_{VBAm} , respectively, and with a reaction temperature of 65 °C. For optimum performance a M_v of P_N and P_{VBAm} , respectively, are 5.41×10^4 and 8.91×10^4 Da and with an NVCL/BMA/Am monomer ratio: 5:0.5:0.2 with polymerization yields of 96.8% and 92.8% for P_N and P_{VBAm} , respectively.

Structure of P_{VBAm}

The synthesized P_{VBAm} was characterized by Fourier transform infrared (FTIR) spectroscopy and the results are shown in Fig. 3. The spectrum features a minor peaks at 3330 cm^{-1} , corresponding to free amino groups, and a band at 3200 cm^{-1} is identified as the characteristic band of primary amines ($-\text{NH}_2$). These findings indicate that Am is present in P_{VBAm} . The peaks at 1196 cm^{-1} and 1010 cm^{-1} , respectively, are attributed to the C–O–C stretching of aliphatic ester groups, which indicate the presence of BMA in the chain of P_{VBAm} . The peak at 720 cm^{-1} is assigned to the $-\text{CH}_2$ rocking of long chain

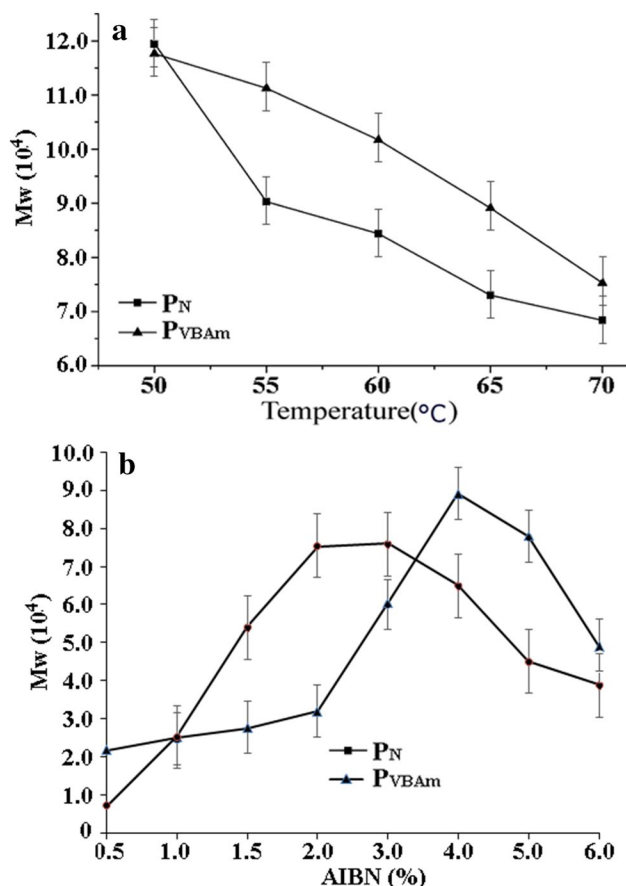
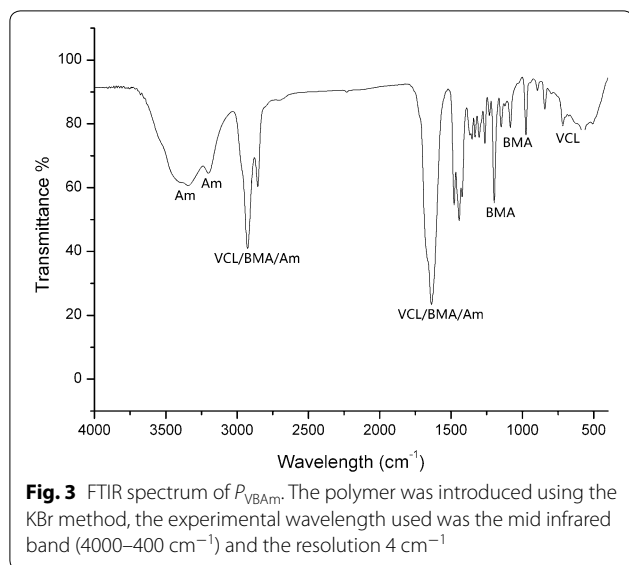


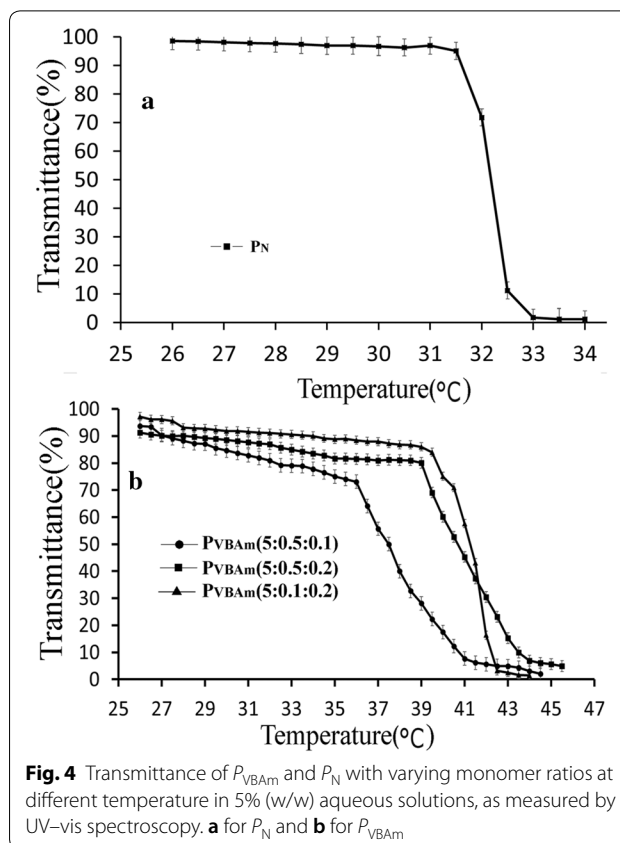
Fig. 2 Effect of **a** temperature and **b** concentration of AIBN on M_v of P_{VBAm} and P_N . The polymerization reaction was carried out for 24 h at a shaking speed of 120 rpm. P_{VBAm} was polymerized with an NVCL/BMA/Am monomer ratio: 5:0.5:0.2. M_v was measured at 25 °C. In **a** 4% (w/w) AIBN was used as the initiator; in **b** the reaction was carried out at 65 °C



$-(\text{CH}_2)_4-$, which indicates the presence of NVCL in the prepared P_{VBAm} . The peaks at 2925 cm^{-1} and 2854 cm^{-1} , respectively, are attributed to the C–H symmetric and asymmetric stretching of methylene groups, the peak at 1648 cm^{-1} is assigned to the C=O stretching vibration and the peak at 1440 cm^{-1} is assigned to the C–H bending vibration. The above three kinds of absorption maxima are present in all Am, BMA and VCL polymers. Based on the FTIR analysis, it can be concluded that all three monomers are successfully polymerized.

Lower critical solution temperature

The lower critical solution temperature (LCST) of the two TR polymers was studied to explore their thermo-induced precipitation temperature. Solutions of P_{VBAm} and P_N are transparent at temperatures below their LCST and become turbid above their LCST. As shown in Fig. 4a, the temperature increase from 26 to 34 °C for P_N indicates an LCST of P_N at 32.5 °C. To obtain a P_{VBAm} preparation with higher LCST, polymerization with different monomer ratios was studied (Table 1; Fig. 4b). Thus, the LCST of P_{VBAm} with an NVCL/BMA/Am monomer ratio of 5:0.5:0.1 is 37.8 °C. Increasing the content of Am led to increased energy requirements to destroy the hydrogen bonds between the amide groups and the water molecules. Consequently, the LCST of P_{VBAm} with an NVCL/BMA/Am ratio of 5:0.5:0.2 increased from 37.8 to 40.5 °C. In contrast, decreasing the content of BMA in P_{VBAm} led to a minor increase of LCST from 40.5 to 41.0 °C. However, the low ratio of BMA resulted in lower molecular weight and low recovery of P_{VBAm} (Table 1). P_{VBAm} with an NVCL/BMA/Am ratio of 5:0.5:0.1 cannot form a two-phase system with P_N regardless of the



concentration. So, based on the results of LCST, recovery, and ATPS formation, an NVCL/BMA/Am monomer ratio of 5:0.5:0.2 is selected with an LCST of 40.5 °C, which is appropriate for its application in a high temperature environment. According to the above results, it is possible to conclude that when the temperature is below the LCST of both P_{VBAm} and P_N , they can form ATPS, and when the temperature exceeds their LCST, P_{VBAm} and P_N will be precipitated from either top or bottom phase.

Recovery of P_{VBAm} and P_N

Favorable recovery characteristics of the phase-forming polymers are essential for proper recycling of aqueous two-phase systems (ATPS). In this study, the recovery of the TR polymers was explored by changing the solution temperature. When the solution was heated above their LCST, P_{VBAm} and P_N became insoluble forming clots. When the temperature was reset below their LCST, the clots were re-dissolved. The conclusion is that P_{VBAm} and P_N are thermo-responsive and can be recycled by temperature change. It is also concluded that adjustment of pH and the addition of inorganic salts can improve the recovery of P_{VBAm} and P_N .

Table 1 Recovery rate and LCST of polymers prepared with different monomer ratios

NVCL/BMA/Am (w/w)	Mv(10 ⁴)	Recovery rate (%)	LCST (°C)
5:0.1:0.2	1.89 ± 0.3	73.2 ± 1.3	41.0 ± 0.5
5:0.5:0.2	8.91 ± 0.5	94.7 ± 1.2	40.5 ± 0.5
5:0.5:0.1	8.93 ± 0.6	95.8 ± 0.9	37.8 ± 0.5

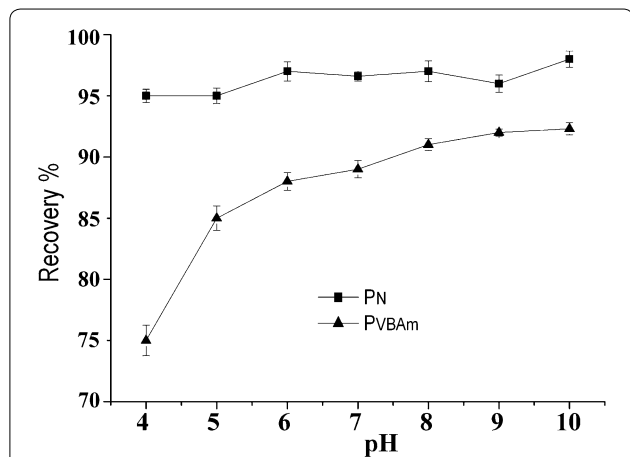


Fig. 5 Effect of pH on the recovery of the two thermo-responsive polymers without salt. The recoveries were carried out at 38 °C for P_N and 46 °C for P_{VBAm}

As shown in Fig. 5, with an initial pH value of the polymer solution around 5.0–6.0, the recovery of P_N was not obviously influenced by pH change reaching > 95% in the pH range 4.0–10.0, and 98.0% at pH 10. However, in contrast, the recovery of P_{VBAm} shows an obvious increase at higher pH values, reaching a maximum recovery of 92.3% at pH 10.0. For the TR copolymer, an increase in temperature is the main factor for inducing P_{VBAm} aggregation by breaking hydrogen bonds between P_{VBAm} and water molecules. Besides temperature, the solution pH also influences the strength of the hydrogen bonds. In general, the greater the polarity of the molecule, the greater the effect is of the hydrogen bond on its solubility. Although P_{VBAm} does not contain ionic groups, it contains a terminal amide bond, so its solubility is more influenced by hydrogen bonding than P_N which has got an internal amide bond. The result is a larger effect of pH on the recovery of P_{VBAm} . Under acidic conditions, there are enough hydrogen ions in the solution, so P_{VBAm} cannot easily precipitate, because of the strong hydrogen bonding between the water molecules and P_{VBAm} . With the increase of pH, the hydrogen bonds are weakened and the inter-molecular hydrogen bonds of P_{VBAm} become strong, making P_{VBAm} precipitate more easily.

The presence of inorganic salts also influences the polymer recovery. As shown in Fig. 6, the recoveries of both P_{VBAm} and P_N are improved by the addition of

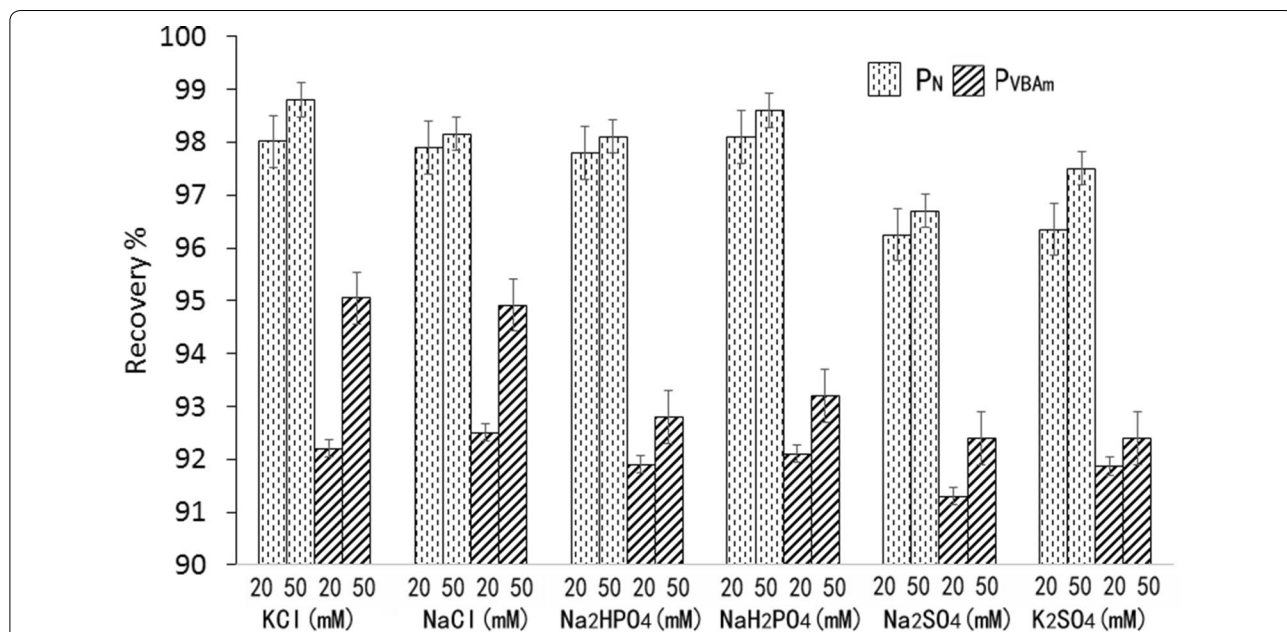
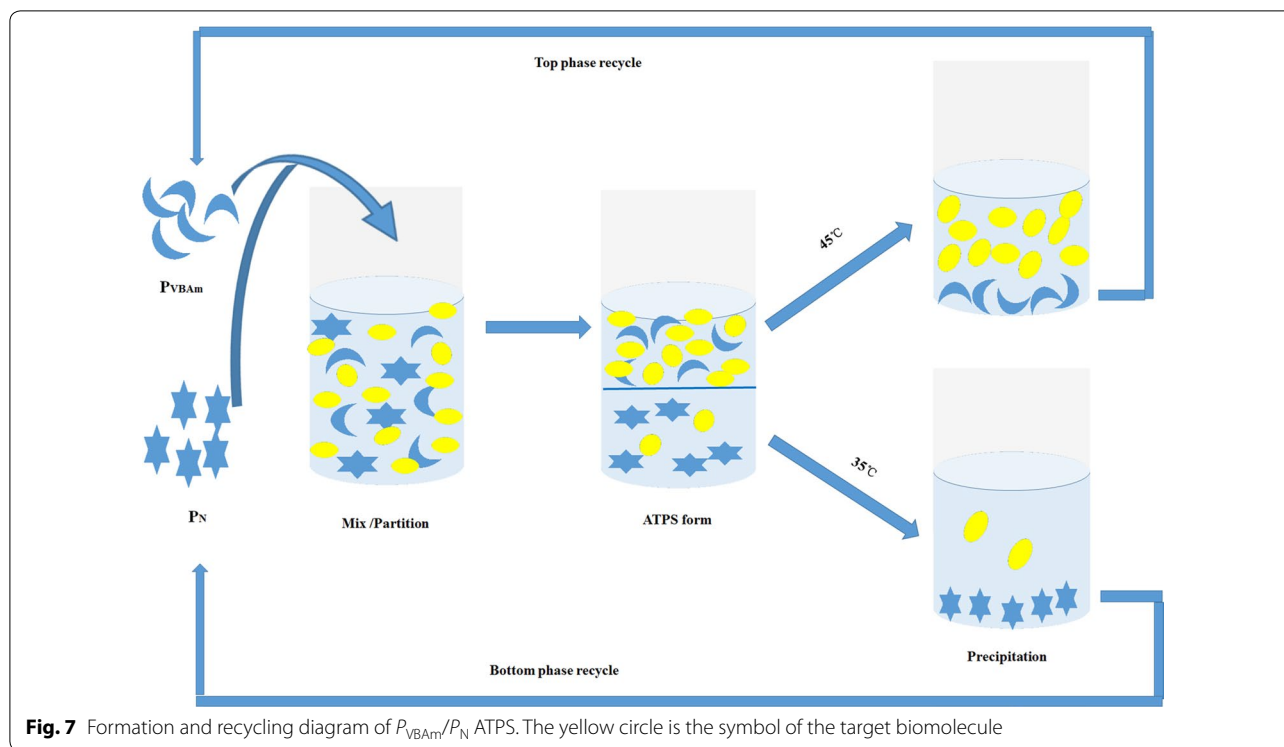


Fig. 6 Effect of different inorganic salts and concentration (20 and 50 mM) on the recovery of the two thermo-responsive polymers at pH 10. The recoveries were carried out at 38 °C for P_N and 46 °C for P_{VBAm}

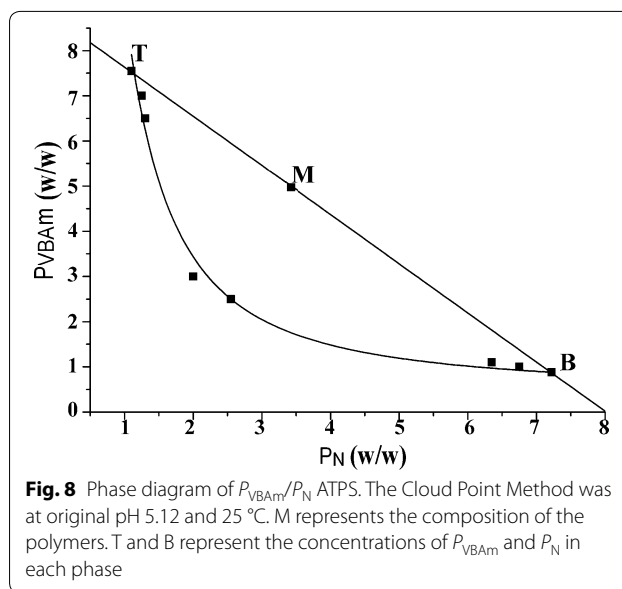


salts, except for Na_2SO_4 and K_2SO_4 , and they are slightly higher in 50 mM than in 20 mM. The recoveries of P_{VBAm} and P_N in the presence of 50 mM KCl increased to 98.5% and 94.7%, respectively. However, the effect of inorganic salts on recovery is not obvious for P_N , probably owing to the absence of ionizable groups.

In conclusion, through optimization, both P_{VBAm} and P_N can be recovered effectively by precipitation, and the ATPS formed by P_{VBAm} and P_N can be easily recycled. The scheme of P_{VBAm}/P_N ATPS formation and recycling is shown in Fig. 7.

Phase diagram of P_{VBAm}/P_N ATPS

Phase diagrams of ATPS indicate their biphasic composition, the concentration of phase components in the top and bottom phases, and the ratio of phase volumes, and they are very useful for guiding phase formation and molecular partitioning. The phase diagram of the P_{VBAm}/P_N ATPS is shown in Fig. 8. The area above the binodal curve represents the two-phase region. Within a pH range of 4.0–6.0 (initial pH of the ATPS is 5.12), a clear phase layer is observed. As shown in the diagram, very low concentrations of both P_{VBAm} and P_N are required to form ATPS, which means a lower application cost. For example, in the diagram M represents the composition of the polymers in the ATPS (5.0% P_{VBAm} and 3.5% P_N), which was used for partitioning in the following experiment. Furthermore, T and B on the binodal



curve represent the concentrations of P_{VBAm} and P_N in each phase, 7.5% P_{VBAm} and 1.1% P_N in the top phase and 0.9% P_{VBAm} and 7.2% P_N in the bottom phases, indicating that P_N or P_{VBAm} are mainly enriched in one phase, which is very beneficial for the recycling of the phase-forming polymer. The ratio of the phases is calculated according to the length ratio of the lines MB and MT on the tie line TMB, giving a ratio of the M system to be 2:1.

Partition of vitamin B₁₂ in P_{VBAm}/P_N ATPS

For the development of a low cost and environmental friendly separation method, in order to avoid organic solvent extraction, the recyclable P_{VBAm}/P_N ATPS has been applied to the partitioning of vitamin B₁₂ including the influence of pH and inorganic salts.

Taking the stability of VB₁₂ and the phase formation range of ATPS into consideration, pH is kept in the range 4.0–6.0. In this range, vitamin B₁₂ tends to partition to the top phase enriched in P_{VBAm} . The partition coefficient decreases slightly from 2.48 to 1.74 with an increase of pH from 4.0 to 6.0 (Fig. 9a). The decreasing K trend can be attributed to charge effects. VB₁₂ contains a phosphate anion and six terminal amide bonds, and P_{VBAm} also contains a terminal amide bond. The above groups show slightly more negative charge at increasing pH values. Therefore, VB₁₂ moved to the bottom phase, owing to electrostatic repulsion, resulting in a slight decrease of the partition coefficient.

Inorganic salts are frequently used in ATPSs to improve the partition coefficient (K). Different cations and anions (NaCl, Na₂SO₄, KCl, K₂SO₄, and NH₄Cl) were explored in the range 10–100 mM (Table 2). The effect of NaCl and KCl on K displayed an upward trend, whereas reduced K values were obtained for other salts with increasing concentrations. The highest K (2.62) was observed in 100 mM KCl. Therefore, the effect of higher KCl concentrations (0.1–1.0 M) on partition behavior was further investigated, as shown in Fig. 9b. The K value increased gradually with an increase of KCl concentration, reaching 5.81 in 0.8 M KCl, whereas two phases could not be formed at higher concentrations (0.9 and 1.0 M). Inorganic ions can generate an electrostatic potential difference over the interface and the Donnan effect requires electrostatic neutrality between the two phases (Iqbal et al. 2016; Hou et al. 2014). Thus, an uneven concentration of inorganic salts in the two phases influenced the partition of VB₁₂. High salt concentration also causes salting-out which may also influence the partition. In addition, other forces, such as hydrogen bonds, may also influence the partition behavior of VB₁₂ in such a complex system (Wang et al. 2008). The recovery yield of VB₁₂ was 92.1% when K was 5.81 and V_b/V_t was 0.5.

Recovery comparison of P_{VBAm}/P_N ATPS with other TR ATPS

NIPA and VCL are widely employed as thermo-sensitive polymers for smart fibers, surfaces and hydrogels (Kim and Matsunaga 2017). However, there are very few reports regarding their application in ATPS (Tan et al. 2017). In our laboratory, six different TR polymers have been synthesized and applied in ATPS (as shown in Table 3). Among those polymers, *N*-Isopropyl acrylamide (NIPA) or *N*-vinylcaprolactam (VCL) was used as the

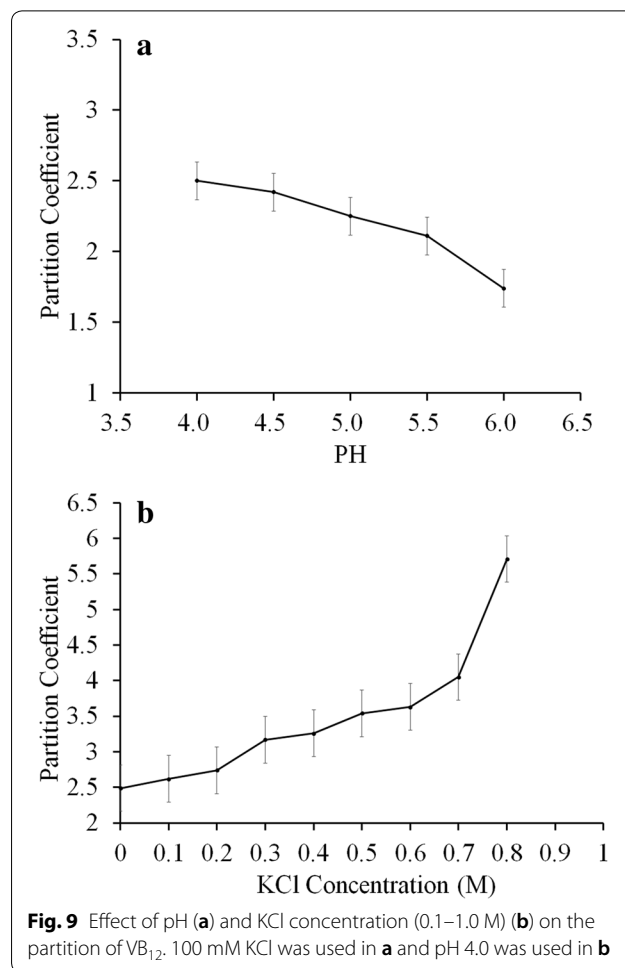


Fig. 9 Effect of pH (a) and KCl concentration (0.1–1.0 M) (b) on the partition of VB₁₂. 100 mM KCl was used in a and pH 4.0 was used in b

thermo-responsive monomer. Factors such as monomer component, LCST, recovery and molecular weight are important for TR polymers to form ATPS. To this end, P_{VBAm} and P_N were compared with other published TR polymers regarding the above factors.

Influence of polymerization medium

Either organic solvents or water can be used as medium for TR polymer polymerization. Although water is environmental friendly, we have found that the polymerization yields and recoveries in water are not as high as in organic solvents. In this study, P_{VBAm} and P_N are polymerized in benzene, giving higher yields than in water (Table 3). Moreover, when polymerized in benzene, P_{VBAm} and P_N shrunk to a clot when heated above their LCST and could easily be removed, whereas when polymerized in water, they needed to be centrifuged before recovery. In another TR ATPS (Table 3), P_{NBAA} polymerized in benzene also showed high yields after forming clots that can be easily collected, whereas P_{NE}

Table 2 Effect of inorganic salt at varying concentrations on the partition coefficient of VB₁₂

Con. (mM)	KCl	K ₂ SO ₄	NaCl	Na ₂ SO ₄	NH ₄ Cl
10	1.60	2.15	1.84	2.43	2.44
20	1.67	1.80	1.85	2.41	1.68
30	1.70	1.94	1.85	2.40	1.48
40	1.70	1.57	1.87	2.38	1.37
50	1.78	1.49	1.89	2.03	1.62
60	1.88	1.56	1.83	2.01	1.55
70	1.84	1.48	2.03	1.97	1.49
80	2.30	1.51	2.12	1.97	1.45
90	2.55	1.50	2.18	1.93	1.36
100	2.62	1.51	2.23	1.89	1.44

The standard deviations for different salts were similar, average ± 0.3

polymerized in water only gave 85.8% yield and needed to be centrifuged to be collected.

Influence of Mw on TR polymers

The polymer molecular weight (Mw) relates to both solution viscosity and solubility. We have found that P_{NE} with Mw 1.89×10^6 will need several hours to dissolve from a dry solid and its solution is very viscous, thus it is not suitable for ATPS formation. The Mw of other polymers tested, as shown in Table 3, showed a span of four orders of magnitude, but did not show obvious differences in viscosity and solubility. It has been reported that polymers with low Mw show higher LCST and form ATPS at higher concentrations (Iqbal et al. 2016).

Influence of LCST on the recovery of TR polymers

The most important characteristic of TR polymers is their low critical solution temperature (LCST). When the temperature is above LCST, the polymers become insoluble and precipitate. Although a low LCST can reduce the risk of denaturation of biomolecules during temperature-induced precipitation, LCST values below 30 °C, such as for P_{NBAA} and P_{NE} (Table 3), may cause the polymer to precipitate at high room temperatures. For example, a hot summer day would affect the formation of ATPS. Therefore, because P_{NBAA}/P_{NDB} and P_{NE}/P_{VAm} ATPS contain a polymer with low LCST, they are unsuitable to be applied to partition at high ambient temperatures, as shown in Tables 3 and 4. The LCST of P_{VBAm} in this study was 40 °C. Such LCST is suitable for the formation of ATPS at higher temperatures, preserving the activity of biomolecules during temperature-induced polymer precipitation. The LCST value of the widely applied TR copolymer EO₅₀PO₅₀ is about 50 °C and temperature-induced phase separation may occur at about 65 °C (see Table 3), which is higher than safe temperatures for many biomolecules. Therefore, TR polymers with LCST in the range of 30–40 °C are more suitable to form ATPS for biomolecule separation.

Recovery state of TR polymers

All TR polymers reported in Table 3 achieved more than 95% recovery with optimized salts in the solution. However, the degree of recovery differs significantly between polymers composed of NIPA or VCL and polymers based on the EOPO series. When the solution temperature

Table 3 Characteristics of the thermo-responsive polymers

Polymers	Monomers	Polymerization medium	Yield (%)	LCST (°C)	Recover (%)	Mv (10 ⁴)	References
P_N	NIPA	Benzene	96.8	32.5	98.5	5.4	This study
P_{VBAm}	VCL/BMA/Am (5:0.5:0.2)	Benzene	92.8	40.5	94.7	8.9	This study
P_{NBAA}	NIPA:BMA:Aa (3.0:0.12:0.05)	Benzene	90.8	25.5	98.2	9.7	Hou and Cao (2014)
P_{NDB}	NIPA:DMAEMA:BMA(3.0:0.6:0.4)	THF	88.6	31.3	96.1	5.6	Hou and Cao (2014)
P_N	NIPA	Water	93.2	33.5	98.3	12	Xu et al. (2016)
P_{VBAm}	VCL/BMA/Am (5:0.5:0.3)	Water	89.8	45.0	96.6	2.8	Xu et al. (2016)
P_{NE}	NIPA:EMA (3.0:0.05)	Water	85.8	28.7	97.5	19	Hou et al. (2014)
P_{VAm}	VCL/Am (3.0:0.4)	Methanol	92.6	35.6	96.3	2.8	Hou et al. (2014)
EO ₅₀ PO ₅₀	EO:PO(0.5:0.5)	/	/	50.0	95.0	0.39	Persson et al. (2000)
HM-EOPO	EO:PO(0.66:0.14/C14H29)	/	/	14.0	97.5	0.8	Persson et al. (1999)
L44	EOPOEO (EO = 40%)	/	/	65.0	98.5	0.22	Wang et al. (2015)
MAH-CD-NIPA	NIPA:MAH-CD (3.5:5)	Water	46.7	42.0	/	/	Tan et al. (2017)
Poly-VI-VCL	VCL:VI (4.8:0.2)	Iso-propanol	/	37	/	/	Pietruszka et al. (2000)

"/" means that no corresponding information can be found in the published paper

Table 4 Characteristics of the thermo-responsive ATPS

ATPS type	System composition top/bottom	Phase recovery state	Partition coefficient	References
Thermo-thermo	5% P_{VBAm} /4% P_N	Solid	5.81/ VB_{12}	This study
Thermo-thermo	2% P_{VBAm} /2% P_N	Solid	6.87/polylysine	Xu et al. (2016)
Thermo-thermo	5% P_{NBAA} /5% P_{NDB}	Solid	2.61/demeclocycline	Hou and Cao (2014)
Thermo-thermo	5% P_{NE} /5% P_{VAm}	Solid	0.33/cefprozil	Hou et al. (2014)
Thermo-Starch	6%EOPO/13%Reppal PES ₁₀₀	Solid	5.86/lysozyme	Persson et al. (2000)
Thermo-thermo	5%EOPO/5%HM-EOPO	Solid	0.24/Apo	Persson et al. (1999)
Thermo-salt	20%UCON(EO ₅₀ PO ₅₀)/4.0%K ₂ HPO ₄	Solution	0.238/laccase	Moreiraa et al. (2013)
Thermo-salt	24%L44(EOPEO)/20.0%K ₂ HPO ₄	Solution	/LBP	Wang et al. (2015)
Affinity Thermo-Dextran	10%MAH-CD-NIPA/10% dextran T40	Solid	1.7/mandelic acid	Tan et al. (2017)
Affinity Thermo-Dextran	9%poly-VI-VCL/5% starch S/AA	Solid	/R-Amylase Inhibitor	Pietruszka et al. (2000)

increases to above LCST, polymers composed of NIPA or VCL become insoluble and precipitate. When ATPS are formed based on such polymers, for example the P_{VBAm}/P_N in this study, and after partition and phase separation has occurred, each phase containing NIPA or VCL is heated to above its LCST, so that the phase forming polymer precipitates and is separated from the biomolecules, after which the polymer can be dissolved and recycled to form ATPS again. On the other hand, when ATPS are formed using polymers based on the EOPO series, after partition and phase separation, the phase containing the EOPO copolymer (5–20%) when heated above its LCST does not precipitate, but remain in solution, with the top phase containing almost all water-soluble biomolecules, whereas the bottom phase contains about 40–60% EOPO polymer. This means that polymers based on NIPA or VCL, such as P_N , P_{VBAm} , P_{NBAA} , P_{NDB} , P_{NE} , show temperature-induced precipitation, and polymers based on EOPO, such as EO₅₀PO₅₀, HM-EOPO, L44, L31, do not show temperature-induced phase separation (Table 4).

ATPS formed by different polymer types

The ATPS (P_{VBAm}/P_N , P_{NBAA}/P_{NDB} , and P_{NE}/P_{VAm}) in Table 4 are composed of two TR polymers and, after phase separation, each polymer is enriched in one phase and can be recovered by temperature-induced precipitation. However, for other ATPS based on one TR polymer and a none-TR component, such as dextran, starch, and salt, only one phase can be subject to temperature-induced recovery and the other phase is still difficult to recover. It has also been reported that ATPS with two TR polymers (EOPO and HM-EOPO) both can be recovered by temperature-induced precipitation. However, the LCST of HM-EOPO was only 14 °C (Table 4).

In addition, different types of ATPS require different polymer concentration to form ATPS. As shown in

Table 4, when both phase-forming polymers are TR polymers, such as P_{VBAm}/P_N ATPS, a lower concentration of the TR polymer ($\leq 5\%$) is needed for ATPS formation. On the other hand, a higher polymer concentration is often needed for the ATPS formed with one TR polymer and another component such as dextran or salt.

Conclusions

A new TR polymer P_{VBAm} with suitable LCST is synthesized and can be recycled with high recovery, and a new recyclable ATPS is designed using a combination of P_{VBAm} and the TR polymer P_N . Compared to previous TR polymers tested in our laboratory, the LCST of the two polymers in this study is more favorable (32.5 °C and 40.5 °C) and is higher than the previously tested polymers (28.7 °C and 35.6 °C). Therefore, they have the ability to form ATPS easier than previous polymers at high room temperatures. Furthermore, the properties of the two polymers in this study differ to such an extent that they can be easily separated from the system. A VB_{12} partition coefficient of 5.81 was obtained using the 5.0% $P_{VBAm}/3.5\%$ P_N ATPS. Thus, the P_{VBAm}/P_N ATPS has got appropriate recycling characteristics regarding LCST, recovery and phase separation. These data are valuable for the promotion of ATPS to be applied in industrial bioseparation processes.

Abbreviations

NIPA: *N*-isopropylacrylamide; NVCL: *N*-vinylcaprolactam; P_{VBAm} : Poly (NVCL-butyl methacrylate-acrylamide); P_N : Poly (*N*-isopropylacrylamide); VB_{12} : vitamin B₁₂; ATPS: Aqueous two-phase systems; TR polymer: thermo-responsive polymer; M_v : viscosity average molecular weight; LCST: lower critical solution temperature.

Authors' contributions

JW conceived, designed, and wrote the paper, WD performed parts of the experiments, DH performed a part of polymerization, ZW performed a part of partition experiments, XC was involved in project planning, experimental designing, manuscript revisions, and editing. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data obtained or analyzed during this study are included in this article.

Consent for publication

All of authors have read and approved to submit it to bioresources and bioprocessing.

Ethics approval and consent to participate

Not applicable.

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