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Synthesis of a Novel pH-Sensitive Methacrylate Amphiphilic Polymer and Its Primary Application in Aqueous Two-phase Systems

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Abstract In this study, a novel pH-sensitive and reversible water-soluble polymer(P_{ABC}) forming aqueous two-phase systems(ATPS) was synthesized by using 2-(dimethylamino) ethyl methacrylate, *t*-butyl methacrylate, and methyl methacrylate as monomers and 2,2′-azo-bis-isobutyronitrile as initiator. The P_{ABC} could be recovered by adjusting isoelectric point (PI) to 8.4, and recovery at PI could reach 95%. ATPS was formed by 5% (*w*/*w*) P_{ABC} and 10% (*w*/*w*) PEG20000. The partition coefficient *K* of lysozyme was 6.8, and the partition coefficient *K* of bovine serum albumin could reach 12.5 in the ATPS.

Keywords Aqueous two-phase systems · PH-sensitive · Amphiphilic polymer · Methacrylate · Polymer recycling

Introduction

Aqueous two-phase systems (ATPS) have been well known for several decades, providing a friendly and mild aqueous environment for the unstable proteins or chemicals [1–3]. However, phase-forming polymers need to be recycled and reuse due to the cost of polymer and environmental problem. Thermoseparating ethylene oxide–propylene oxide copolymers (EO–PO) appeared in some reports [4, 5]. Johansson et al. [6] synthesized a hydrophobically modified random copolymer of EO and PO with aliphatic $C_{14}H_{29}$ groups coupled to each end of the polymer (HM–EOPO). Specific ATPS could be formed by one-polymer HM–EOPO and H_2O. The top phase of ATPS contains 100% (*w*/*w*) water, and the bottom phase contains 5–7% (*w*/*w*) HM–EOPO. The polymer could be recycled by temperature-inducing phase separation. Afterward, Persson et al. [7] presented another thermoseparating ATPS with $EO_{50}PO_{50}$ and HM–EOPO. In protein partition systems, 73% $EO_{50}PO_{50}$ and 97.5% HM–EOPO could be recycled, respectively. These thermo-separating ATPS can be applied in some thermostable biomolecules because most biomolecules will inactivate during

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the temperature-inducing phase separation when the polymers were recycled. Mos et al. [8] reported the recycling of polymer by using a poly(ethylene glycol)-volatile salt ATPS. ATPS containing PEG3350 and sodium sulfate was reported by Toboada et al. [9]. The salt Na_2SO_4 could be crystallized and recycled by evaporation of water. An anionic polyelectrolyte (APE) was synthesized by polymerization of *N*,*N*-(dially-*N*-carboethoxymethyl)ammonium chloride by Hasan et al. [10]. ATPS were formed by APE and PEG35000, and the APE could be recycled by the change of pH.

For the pH-sensitive ATPS, a polyampholyte containing methacrylic acid (MAA), methyl methacrylate (MMA), and 2-(dimethylamino)ethyl methacrylate (DMAEMA) was synthesized by using group transfer polymerization (GTP) [11]. The ATPS were formed by the polyampholyte and poly(vinyl alcohol) (PVA).

In this study, we report a novel pH-sensitive, amphiphilic, reversible water-soluble polymer (P_{ABC})-forming ATPS with PEG20000. The polymer (P_{ABC}) was synthesized by using DMAEMA, *t*-butyl methacrylate (TBMA), and MMA as monomers, and 2,2′-azo-bis-isobutyronitrile (AIBN) as initiator. The P_{ABC} could be recycled at a mild pH range.

Materials and Methods

Materials

MMA, AIBN, and tetrahydrofuran were from Lingfeng Chemical Co. (Shanghai, China). Methacrylic chloride was from Best Fine Chemical Co. (Haimen, China). PEG20000 was purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Tetrahydro-furan was dried overnight in the present of calcium hydroxide and then distilled under atmospheric pressure at a b.p. 66–67 °C.

DMAEMA was synthesized by esterification reaction of methacrylic chloride and dimethyl aminoethanol (DMAE) [12–14]. TBMA was synthesized by esterification reaction of methacrylic chloride and *t*-butyl alcohol [15–18]. The obtained DMAEMA and TBMA were purified by distilling from CaH_2. Average yield of DMAEMA and TBMA were 77.1% and 83.0%, respectively.

Preparation of ABC Triblock Polymer P_{ABC}

Fifty milliliter of freshly distilled tetrahydrofolate (THF) were syringed into a 250-ml conical flask containing a small amount (about 0.25 g) of AIBN. Then, 8.4 ml of DMAEMA (50 mmol), 4.0 ml of TBMA(25 mmol), and 1.3 ml of MMA(12.5 mmol) were slowly added into it in turn with stirring. The mixture was then stirred for 24 h under N_2 at 60 °C water bath. Then, the polymer was precipitated in mineral ether with b.p. 30–60 °C. The precipitate was dried for 3 days in a vacuum oven at room temperature. The white polymer powder was obtained after grinding. The polymer samples were characterized according to their molecular weight (MW) and composition using Fourier transform infrared (FTIR), ^{1}H NMR, and gel permeation chromatography (GPC).

Phase Compositions and Phase Diagram

The ATPS was formed by 5% P_{ABC} (*w*/*w*) and 10% PEG20000 (*w*/*w*). The solid polymers were dissolved in acidified water to prepare an acidic solution [pH below the isoelectric

point (PI), typically pH 6.0] at a concentration of 5% *w*/*w*. A 10% *w*/*w* PEG20000 solution was also prepared by distilled water. The ATPS was formed by 3 ml P_{ABC} solution and 2 ml PEG20000 solution in the tube. After vortex mixing, each tube was centrifuged for 30 min, left to equilibrate for 24 h in a water bath at 25 °C, and examined for phase separation. The P_{ABC} mainly existed in bottom phase. The phase diagram was prepared by using the cloud-point method [19]. The polymer P_{ABC} concentration was determined by using a UV/vis spectrometer at 234 nm at 20 °C. The PEG20000 concentration was determined by using a UV/vis spectrometer at 535 nm according to the method of Cao et al. [20].

Recycling of Polymer

The PI of polymer could be measured according to Patrickios et al. [21]. The pH of top phase and bottom phase was adjusted to the PI of P_{ABC}, respectively. Then the precipitation will appear. The P_{ABC} concentration was calculated by measuring the absorbance of the supernatant. These data were used as reference to calculate the whole recovery of polymer.

Partition of Protein and Amino Acids

The biomolecules partition in ATPS is described by the partition coefficient *K*. In this experiment, the partition of two proteins (lysozyme and bovine serum albumin, BSA) and three amino acids (Tyr, Phe, and Trp) was investigated in ATPS. The initial concentration of lysozyme and BSA in ATPS was 0.4 and 0.6 mg/ml, respectively. The initial concentration of Tyr, Phe, and Trp in ATPS was 1.0, 0.1, and 0.5 mg/ml. The effect of salt species on phase partition was investigated by using NaCl, Na_3PO_4, $NaClO_4$, and Na_2SO_4. Four aliquots of ATPS with different salt types were studies at the range of salt concentrations from 10 to 200 mM. When ATPS were formed after equilibration, the sample was taken from the top phase and the bottom phase by a syringe. The concentration of substances partitioned was determined.

Analysis Methods

The activity of trypsin was determined by *N*-benzoyl-L-arginine ethyl ester method [22]. A 2-mg/ml trypsin solution was prepared by dissolving the appropriate amount of trypsin powder in distilled water. Then, 0.5 ml trypsin solution was added into the ATPS formed by 3 ml (5%, *w*/*v*) P_{ABC} and 2 ml (10%, *w*/*v*) PEG20000. Then, the mixture was stirred and stood to form two phases. The activity of trypsin in top and bottom phase was measured every 1.5 h.

The concentration of Tyr and Phe was determined by measuring the absorbance at 275 and 257 nm, respectively. The concentration of BSA and lysozyme was determined by using Coomassie brilliant blue analysis according to Watter et al. [23].

Results and Discussion

Polymer Synthesis

The components of the triblock polymer P_{ABC} were DMAEMA, TBMA, and MMA. The hydrophobicity of the monomer increases in the following sequence: DMAEMA < TBMA < MMA. DMAEMA and TBMA are hydrophobic. DMAEMA is less hydrophobic than TBMA

due to more nitrogen atoms on the side chain of DMAEMA. MMA is the most hydrophobic component and completely water-insoluble. The structure of three monomers was as follows as Fig. 1.

In our experiments, the optimal reaction conditions were investigated by using orthogonal design tests. The pH-sensitive and reversible water-soluble polymer (P_{ABC}) was synthesized under the optimal condition according to the orthogonal experimental design.

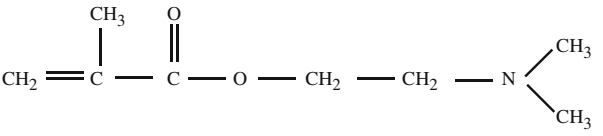
After lots of preliminary experiments, four mainly influencing factors including the polymerization temperature (*A*), the primary ratio of three monomer (*B*), the polymerization time (*C*), and the ratio of initiator (*D*) were investigated at three levels using the orthogonal form of $L_9(3^4)$ in the orthogonal design tests. Table 1 depicts the result of the orthogonal design tests. The value of monomer ratio (*B*) was determined by DMAEMA/TBMA/MMA. The value of the result (*Y*) was defined by the yield and the dissolve percentage of polymer.

It could be deduced from Table 1 that the importance of factors could be ranked according to their influence on yield as follows: $B > D > A > C$. From the orthogonal experimental results, the initial optimal polymerization conditions are

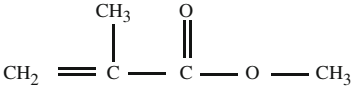
A, 60 °C; *B*, 4:2:1; *C*, 24 h; *D*, 2%.

The ratio of three monomers (*B*) is the most significant factor (highest value of S_i.) A water-soluble polymer was obtained when the ratio is 4:2:1, while water-insoluble polymers were obtained when the ratio is 2:2:1 or 4:4:1. The difference of polymer behavior could be attributed to the hydrophobicity of the polymer structure. The polymer solubility decreased with the increasing of the ratio of TBMA and MMA because the hydrophobicity of the monomers increases as DMAEMA < TBMA < MMA.

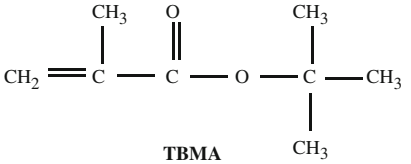
Table 1 also shows that temperature (*A*) is another important factor. The polymer yield increased with the enhancement of temperature. But the polymers obtained were partly dissolved in water when the polymerization temperature reached 70 °C. This partial dissolution was attributed to two reasons: Firstly, with the temperature rising, the activity of the free radical increased; the reaction rate and reaction conversion ratio were increased



DMAEMA



MMA



TBMA

Fig. 1 Structure of three monomers (DMAEMA, MMA, TBMA)

Table 1 Orthogonal design optimization of P_{ABC} synthesis.

B-Monomer ratio(DMAEMA/TBMA/MMA)					
Exp. no.	Polymerization temperature (*A*)	Monomer ratio (*B*)	Polymerization time (*C*)	Initiator ratio (*D*)	Result (Y_i)
1	50 °C	4:2:1	12 h	0.50%	17.8
2	50 °C	2:2:1	18 h	1%	25.8
3	50 °C	4:4:1	24 h	2%	28
4	60 °C	4:2:1	18 h	2%	67.7
5	60 °C	2:2:1	24 h	0.50%	26.1
6	60 °C	4:4:1	12 h	1%	30.3
7	70 °C	4:2:1	24 h	1%	65.6
8	70 °C	2:2:1	12 h	2%	27.5
9	70 °C	4:4:1	18 h	0.50%	25.8
Sum of level 1 (I_j)	71.6	151.1	75.6	69.7	
Sum of level 2 (II_j)	124.1	79.4	119.3	121.7	
Sum of level 3 (III_j)	118.9	84.1	119.7	123.2	
S_j	557.86	1,072.46	428.31	618.74	

greatly; and more hydrophobic monomers were polymerized in polymer structure. Secondly, with the temperature rising, the side reactions results in more side chains, and cross-linked structures were enhanced enormously. In our experiments, the optimal temperature was 60 °C.

Characterization of Polymer P_{ABC} by FT-IR, ^{1}H NMR, and GPC

Figure 2 shows the FT-IR spectrum of the polymer by Nicolet Magna-IR spectrometer. The analysis as follows: IR (cm^{-1}), 2,900 (C–H ester), 1,750 (C=O), 1,450 (C–H, polymer chain), 1,380 (C–H, *t*-butyl), 1,250 (C–O, ester), 1,150 (C–N), 750 (C–H, polymer chain).

Figure 3 shows the ^{1}H NMR spectrum of the polymer by using CD?Cl_3 as solvent. The analysis as follows: 1H NMR (δ ppm), 4.2 (6H, dimethyl-amino), 3.6 (3H, methyl near to

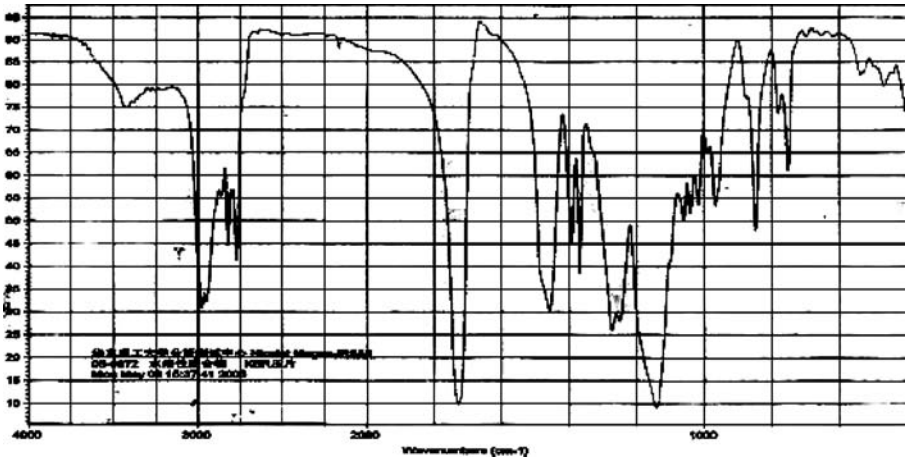


Fig. 2 The FT-IR spectrum of polymer P_{ABC}

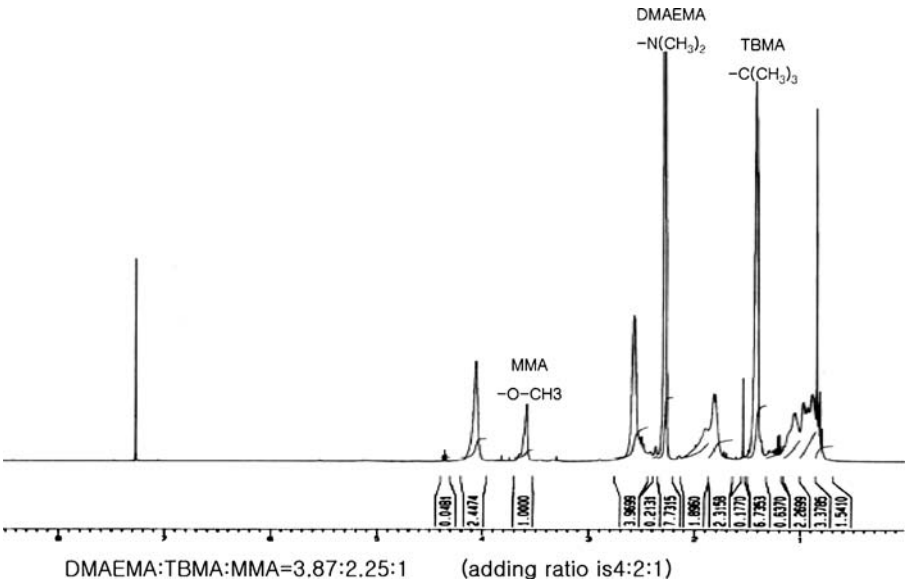


Fig. 3 The ^{1}H NMR spectrum of polymer P_{ABC}

carbonyl), 2.4 (2nH, polymer chain), 1.45 (9H, *t*-butyl), 0.85 (3H, methyl). The proportion of monomers in the polymer was determined by calculating the peak's area. The composition of polymer was proved as DMAEMA/TBMA/MMA=3.87:2.25:1 that was similar as adding ratio (4:2:1).

The IR and nuclear magnetic resonance (NMR) spectrums indicate the presence of the *t*-butyl, amino, carbonyl, and ester groups in the polymeric backbone. First, the dimethylamino group of DMAEMA can be indicated by ^{1}H NMR at 4.2 ppm; the ester group of AMAEMA, TBMA and MMA can be indicated by IR at 1,250 and 2,900 cm^{-1}. Second, the C–H stretching vibration of *t*-butyl group can be indicated by IR at 1,380 cm^{-1}, and the hydrogen atoms of *t*-butyl group can be indicated by NMR at 1.45 ppm. In the same way, it can also demonstrate the polymer chain by the IR (at 750,1450 cm^{-1}) and the NMR (at 2.4 ppm). Third, the proportion of monomers in the polymer was proved as similar as the adding ratio according to Fig. 3. Finally, the polymer was confirmed to be a triblock copolymer by the spectrums, and the general formula of triblock polymers is shown in Fig. 4.

The triblock polymer MW was characterized using HP-1100 GPC. The eluent was THF at the flow rate of 1 ml min^{-1}. The result is shown in Table 2. The MW distributions of polymer are unimodal (data not shown). The polydispersity of polymer P_{ABC} was 1.1; it means that the molecular of the polymer have a narrow distribution. Because of the steric hindrance between TBMA and DMAEMA, the rate of prolongation of polymer chain was reduced, and the monomers polymerized with a narrow polydispersity.

The Maximal Absorb Wavelength (λ_m) and the Standard Curve

The maximal absorb wavelength (λm) of polymer P_{ABC} was determined by an automatic UV/vis spectrometer. Figure 5a shows that the maximal absorbance of P_{ABC} was at 234 nm. The polymer has strong ultraviolet absorbance. It could be explained that a strong K absorption band was generated at 200–250 nm by the carbonyls in the polymer chains.

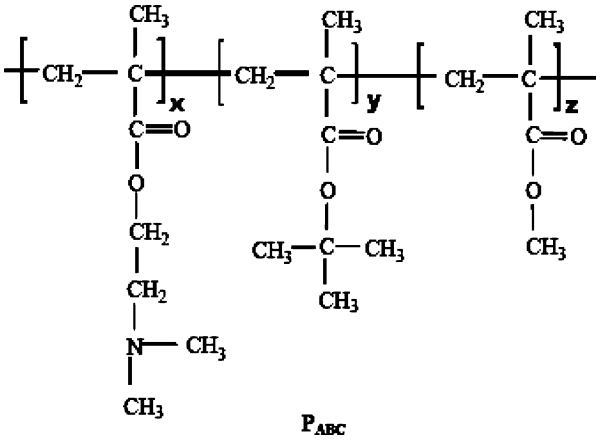


Fig. 4 The general formula of polymer P_{ABC}

Figure 5b showed the standard curve of polymer at 234 nm. There is a linear relationship between concentration and absorbance at 234 nm in a certain concentration range (0.01–0.1%). The relationship could be indicated by a linear equation as follows:

$$A = 0.0334 + 5.25067 * \mathrm{C} \quad \left(R^2 = 0.996\right)$$

where A is the absorbance and C is the P_{ABC} concentration (%, *w*/*v*) The concentration of the polymer in top and bottom phases could be calculated by using this equation. In this report, the concentration of polymer P_{ABC} was determined by this standard curve.

Phase Diagram

The phase diagram for the P_{ABC}/PEG20000/water system is shown in Fig. 6. This phase diagram was prepared by using 5% polymer solution (*w*/*w*) at 20 °C and 10% PEG20000 solution (*w*/*w*). The phase separation is driven by repulsion between the polymer P_{ABC} and PEG20000. The PEG20000 mainly is in the top phase, and the P_{ABC} polymer mainly is the bottom phase. The volume ratio of the top and bottom phase could be estimated by the length ratio of line MB and MT on the tie line TMB. From the phase diagram, the optimal primary volume ratio of the P_{ABC} solution (5%, *w*/*w*) and PEG20000 solution (10%, *w*/*w*) was selected to be 3:1. The volume ratio of the top and bottom phases will be 2:1 after phase equilibrium.

Recycling of Polymer

Recovery at the mild PI (=8.4) was studied using pure water–P_{ABC} mixtures and P_{ABC}/PEG20000 systems, respectively.

The recovery of polymer P_{ABC} in the water–P_{ABC} mixtures was measured three times, and the result was 94.9%, 95.0%, and 95.5%, respectively. The polymer could be recycled for 40 times according to the recoveries.

Table 2 GPC MW of P_{ABC}.

Polymer	M_W	M_n	M_W/M_n
P_{ABC}	5,400	5,900	1.10

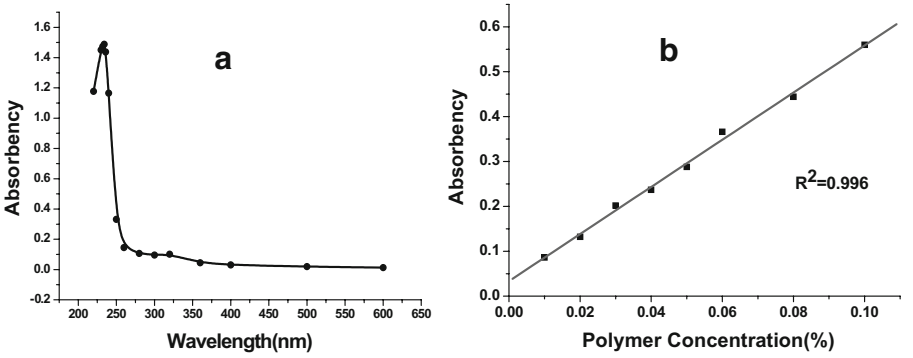


Fig. 5 The absorbable curve (**a**) and standard curve (**b**) of polymer P_{ABC}

Figure 7 shows the recovery of P_{ABC} in the P_{ABC}/PEG20000 systems. The primary P_{ABC}/PEG20000 systems were mixed, and after phase separation, the P_{ABC} and PEG20000 were isolated in separate test tubes. The P_{ABC} concentration in the primary system and in the supernatant of the top and bottom phases after adjusting the phase pH (P_{ABC} precipitation) were determined by the standard curve method. The recycled polymer was reused in the next recycle experiment after isolation, desiccation. The recycling of the polymer was repeated five times by using the same polymer sample. The results in Fig. 7 showed that the recovery of P_{ABC} was stable at 90% and could be reused many times. In the fifth recycling experiment, the recovery of P_{ABC} was down to 87%. This could be due to the impurity (mainly PEG20000) of the sample increased after several recycle times. This problem will be investigated in later.

Effect of Salts Species on the Partition of Biomolecules

Salts are frequently used in ATPS to improve partitioning of target molecules between the phases [2, 23]. Experiments of partition of the protein and amino acid in the presence of inorganic salts were investigated. The results are shown in Fig. 8. Concentration of NaCl,

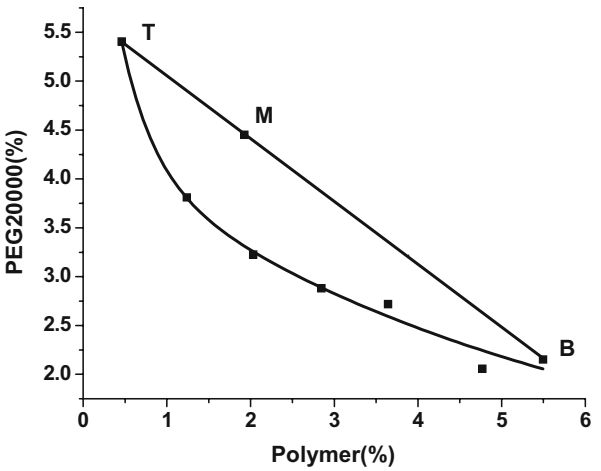


Fig. 6 The phase diagram of P_{ABC}/PEG20000

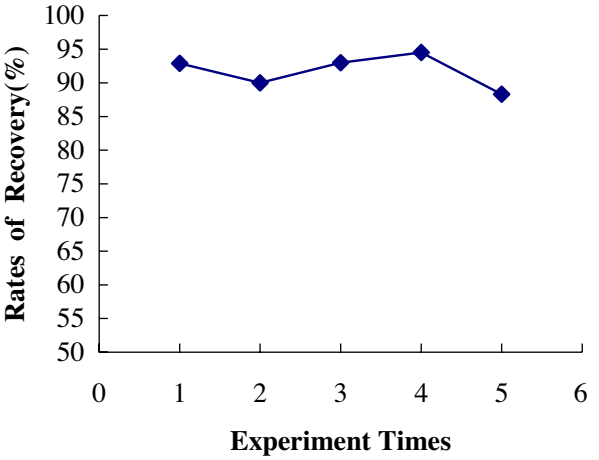


Fig. 7 The recovery of P_{ABC} in ATPS

Na_3PO_4, $NaClO_4$, and Na_2SO_4 was fixed at 100 mM. The ionic strength (*I*) of every salt at 100 mM was listed in Table 3.

ATPS could not be formed at higher Na_3PO_4 concentration due to its strong electric charge effect; the polymer will precipitate at high $NaClO_4$ concentration due to its strong salting out effect.

Different species of ions have different partition behaviors in the two phases. Electroneutrality must be maintained in each phase. The electrochemical driving force in partitioning has been explained by the formation of an electrostatic potential difference over the interface [24–27]. This potential difference is created by the different species of the ions in the two phases. The electrostatic potential difference will affect the partitioning of proteins or other charged molecules in the phase system.

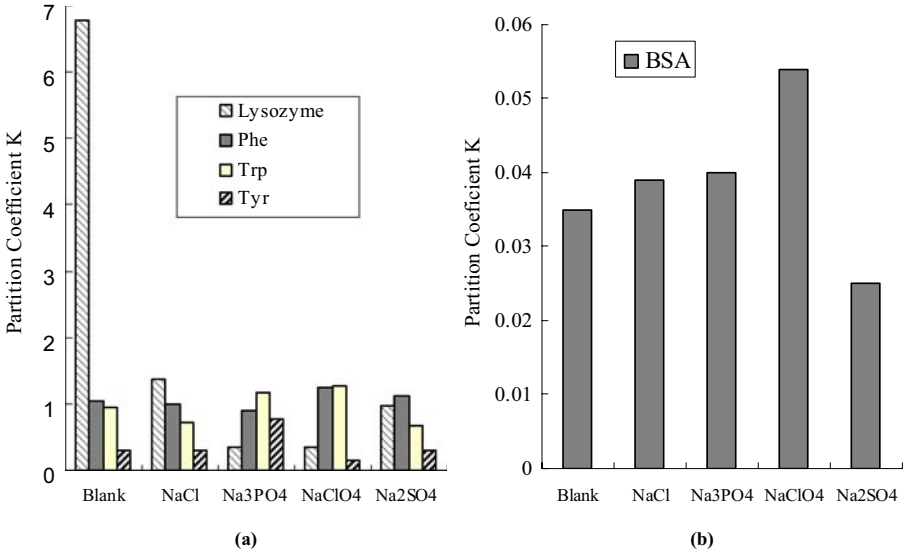


Fig. 8 **(a)** Partition of Lysozyme, Phe, Trp and Tyr in P_{ABC}/PEG20000 systems in the presence of differentsalts. **(b)** Partition of BSA in P_{ABC}/PEG20000 systems in the presence of different salts

Table 3 The ionic strength of salts at 100 mM.

	NaCl	Na_3PO_4	$NaClO_4$	Na_2SO_4
I (mol/kg)	0.1	0.6	0.1	0.3

Figure 8 indicated the partition of lysozyme and BSA in ATPS. The partition coefficient of lysozyme could reach 6.8 without a salt in ATPS, but the partition coefficient of BSA was always less than 0.5. This difference could be attributed to different charge effect that lysozyme (PI=10.7) was positively charged at pH 7.0 [28], and BSA (PI=4.8) was a negatively charged protein at pH 7.0 [28].

BSA has a low K value and is always partitioned in the bottom phase in the presence of salts. This could be attributed to the interaction between the interfacial electric potential (Donan effect) and the charges on BSA. On the other hand, the repulsion force between the BSA molecules and the PEG20000 molecules is also a reason. Therefore, the BSA molecules prefer to exist in the bottom phase.

From Fig. 8(a), the amino acids Phe and Trp were not enriched obviously in either phase, and their partition coefficients were near 1. This could be attributed to the fact that the amino acid Phe and Trp were small molecules. Their partition coefficients were not changed much. According to the Brownstedt theory, ($\ln K = \exp^{(-M_\lambda/kT)}$, the partition coefficients K was correlative with their molecules (M). Amino acids are small molecules. The contribution of M to $\ln K$ is not as high as macromolecules. Tyr is slightly biased to the bottom phase. Perhaps, there is a similar hydrophobicity between Tyr and the methacrylate polymer.

Effect of Salts Concentration on Partition of Biomolecules

Partition experiments of biomolecules at different salt concentration were preformed, and the result is shown in Fig. 9. Figure 9(a) indicated the partitioning curve of BSA at different Na_2SO_4 concentration. The partitioning coefficient of BSA was increased slowly with the increase of Na_2SO_4 concentration and then rapidly reached 12.5 in the 150–200 mM range of Na_2SO_4 concentration. Similar salt effect was observed at the BSA partition in PEG/

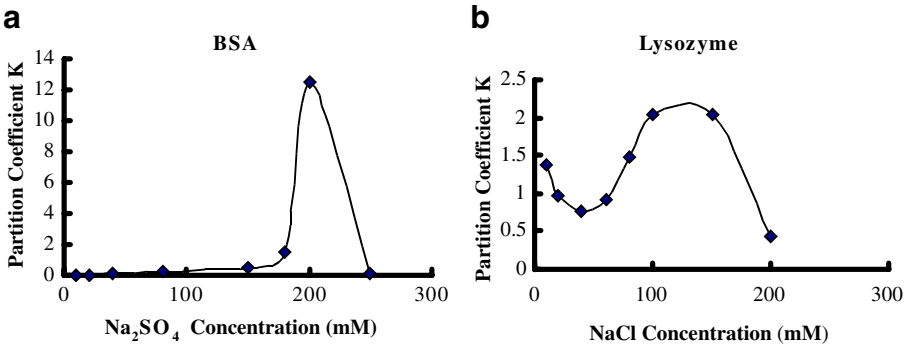


Fig. 9 **(a)** Effect of salt concentration on partition of BSA in P_{ABC}/PEG20000 systems. **(b)** Effect of salt concentration on partition of Lysozyme in P_{ABC}/PEG20000 systems

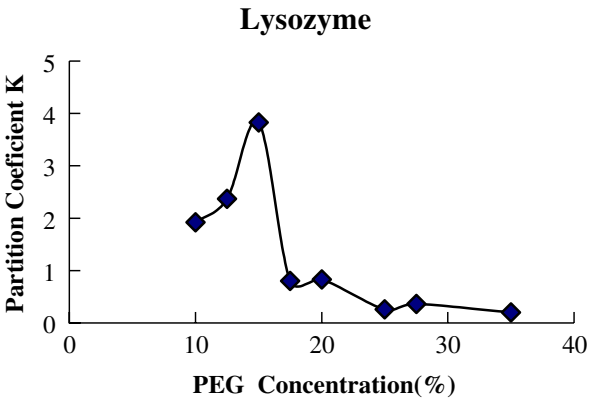


Fig. 10 Effect of PEG concentration on partition of protein in P_{ABC}/PEG20000 systems

$(NH_4)_2SO_4$ systems at NaCl addition by Chang et al. [29]. This could be explained by the influence of the electrostatic potential difference over the interface caused by the accumulation of salt Na_2SO_4. However, the *K* value decreased rapidly below 1.0 when the concentration of salt reached 250 mM. This is probably due to the repulsion force between the BSA and PEG20000 molecules and the salting out effect by the high salt concentration.

The partition of lysozyme in ATPS in the presence of NaCl is shown in Fig. 9(b). The partition of lysozyme varies with the different salt concentration. The partition coefficient of lysozyme increased lowly at the range of 50–150 mM and fell down after the concentration exceeds 150 mM. The maximal partition coefficient of lysozyme was observed as 2.0 at the range of 100–150 mM salt concentration. This curve could be attributed to the change of interface electrostatic potential difference caused by the uneven contribution of Na^+ and Cl^- ions in ATPS. The interface electrostatic potential difference between the top phase and bottom phase was increased by adding the salt NaCl. The electrostatic potential difference affected the lysozyme biasing to the top phase at low salt concentration. However, when the concentration of salt was above 150 mM, the repulsion force and the steric hindrance will preponderate the potential difference, and the lysozyme will be biased to the bottom phase.

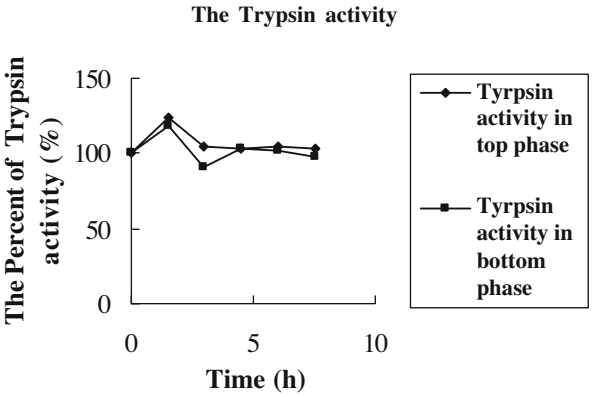


Fig. 11 Enzyme activity in P_{ABC}/PEG20000 ATPS

Effect of PEG20000 Concentration on Partition of Lysozyme

The partition of lysozyme in different PEG20000 concentration is shown in Fig. 10. The partition coefficient of lysozyme reached the maximal value (3.8) when the concentration of PEG20000 was 15%. The *K* value decreased with the increase of PEG20000 concentration after the concentration of more than 15%. The repulsion force between the lysozyme and PEG20000 will increase with the increase of concentration of PEG20000. These make the lysozyme in the top phase transfer to the bottom phase with the increasing of PEG20000 concentration.

Enzyme Activity in ATPS

It was necessary to study the change of enzyme activity to confirm that the polymer is biocompatible for biomolecules. Figure 11 indicates the change of trypsin activity in the top and bottom phases. The relative activity of trypsin still maintained more than 90% of the original activity even after 8 h. At the first 2 h, the activity of trypsin exceeded 100%, especially in the top (PEG) phase; the activity of trypsin was kept more than 100% of the original activity during the experiment. This experiment showed that the ATPS does not have any harmful effect to the biomolecules.

Conclusions

A novel pH-sensitive, amphiphilic, reversible water-soluble random polymer (P_{ABC}) was synthesized by using DMAEMA, TBMA, and MMA as monomers. The average MW of P_{ABC} was about 5×103, and the PI was at pH=8.4. Mixtures of P_{ABC} and PEG20000 in water could form ATPS. The P_{ABC} was a pH-inducing phase separation polymer, and it could be recycled by adjusting the pH of systems to its PI (=8.4). Ninety-five percent of the polymer could be recovered from the P_{ABC} solution. About 90–95% of the polymer was recovered by recycling experiments at the no-protein ATPS. The large partitioning coefficient *K* was obtained when the proteins was partitioned to the P_{ABC}/PEG20000 ATPS. The partitioning coefficient *K* of lysozyme reached 6.8 in ATPS without salt, and the *K* of BSA reached 12.5 in the P_{ABC} /PEG20000 ATPS when the concentration of salt was at 200 mM. This ATPS is both low in cost and without any environmental problem because the polymer P_{ABC} could be recycled and reused.

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