Colloid Polym Sci 276:470–475 (1998)

ORIGINAL CONTRIBUTION

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Synthesis of temperature-sensitive micron-sized monodispersed composite polymer particles and its application as a carrier for biomolecules

Received: 6 August 1997
Accepted: 16 January 1998

Part CLXXIII of the series
"Studies on Suspension and Emulsion"

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Abstract Temperature-sensitive micron-sized monodispersed composite polymer particles were prepared by seeded copolymerization of dimethylaminoethyl methacrylate and ethylene glycol dimethacrylate with 1.77 μm-sized monodispersed polystyrene seed particles. The change in surface property at temperature above and below 35 °C was examined by differential scanning calorimetry, trypsin activity and the adsorption/desorption behaviors of low molecular weight cationic emulsifier as well as biomolecules.

Key words Composite particle – temperature-sensitive – biomolecules – specific activity – adsorption

Introduction

Stimuli responsive polymers are considered to have many potential applications, specially in medicine, biotechnology, and environmental problems [1–5]. Recently poly-*N*-isopropylacrylamide (poly-NIPAM) is getting more attention because it has an attractive temperature-sensitive property [6–9]. Such temperature-sensitive poly-NIPAM in aqueous medium has its lower critical solution temperature (LCST) at 32 °C. At temperature below the LCST poly-NIPAM gel swells with water and expands and at temperature above the LCST the gel deswells.

In 1974 [10], we reported that poly-dimethylaminoethyl methacrylate (PDM) in an aqueous medium also exhibited a similar temperature-sensitive property as poly-NIPAM and utilized it as a temperature-sensitive flocculant for the treatment of waste water. Recently we have prepared temperature-sensitive submicron-sized composite polymer particles by seeded emulsion copolymerization of dimethylaminoethyl methacrylate (DM) and ethylene glycol dimethacrylate (EGDM) with polystyrene (PS) seed particles [11–15]. The shell consisting of (DM-EGDM) copolymer, swelled with water and expands at temperature below the LCST (35 °C) and above it shrank. It is well known that the temperature-sensitive composite particle has such similar surface property, i.e. at temperature below the LCST, the particle surface is hydrophilic and above, it is hydrophobic. We also observed an excellent adsorption/desorption behaviors of low molecular weight emulsifiers and biomolecules as compared to the poly-NIPAM based core-shell composite particles [21].

In this article, we attempt discussing the production of temperature-sensitive micron-sized monodispersed composite polymer particles by considering their usefulness as a carrier for chromatography. For the purpose, seeded copolymerization was carried out of DM and EGDM with 1.77 μm-sized monodispersed PS seed particles produced by dispersion polymerization. EGDM was used as a cross-linking agent to prevent the dissolution of PDM in water below the LCST.

Experimental

Materials

Styrene (S) was distilled under reduced pressure in a nitrogen atmosphere. DM and EGDM were of reagent grade and used as received. 2,2′-Azobis(isobutyronitrile) (AIBN), 2,2′-azobis(2-amidinopropane) hydrochloride (V-50), polyvinyl pyrrolidone (PVP), tricaprylyl methyl ammonium chloride (aliquat$^{(R)}$ 336) and trimethyl stearyl ammonium chloride (TSAC) were also of reagent grade. Albumin from eggs (AL) and lactalbumin hydrolysate (LA) of Wako Pure Chemicals Co. were preserved in the refrigerator and used as received. Trypsin, type 1 (10,600 units/mg solid) of Sigma Chemical Co. and substrate α-*N*-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) of Nacalai Tesque, were preserved in the refrigerator and used without further purification. Deionized water was distilled with a Pyrex distillator. Other chemicals used were of analytical grade.

Preparation of micron-sized monodispersed PS seed particles

Dispersion polymerization of S was carried out in the presence of PVP and aliquat$^{(R)}$ 336 for 24 h under a nitrogen atmosphere at a stirring rate of 80 rpm in a round bottomed four necked flask under the conditions listed in Table 1 which was changed a little from those reported by Almog and coworkers [16]. The seed particles were washed repeatedly by serum replacement with distilled deionized water. The PS seed particles were observed with a JEOL JEM-2010 transmission electron microscope (TEM).

Seeded copolymerization of DM and EGDM

Seeded copolymerization of DM and EGDM was carried out in the presence of PS seed particles at 60 °C under the conditions listed in Table 2. The produced composite polymer particles were washed repeatedly by serum replacement with distilled deionized water to remove any traces of ionized salt. The solid content of the redispersed particles was adjusted in the range 15–20 g/l. The conductance of the purified composite emulsion was found to be under 5 μS/cm.

Table 1 Preparation of PS seed particles by dispersion polymerization[a)]

Ingredients (g)	
Styrene	40.0
PVP	1.6
Aliquat$^{(R)}$	0.4576
AIBN	0.4
Ethanol	200.0
Water	10.0

a) 70 °C, 24 h, N_2, 80 rpm.
Abbreviations: PVP, polyvinyl pyrrolidone; aliquat$^{(R)}$, tricaprylyl methyl ammonium chloride; AIBN, 2,2′-azobis(isobutyronitrile).

Table 2 Preparation of PS/P(DM-EGDM) composite particles by seeded copolymerization[a)]

Ingredients (g)	
PS seed[b)]	19.12
DM	1.695
EGDM	0.0524
V-50	0.4
Water	98.0

a) 60 °C, 8 h, N_2, 80 rpm.
b) Solid content, 104.6 g/l.
Abbreviations: PS, polystyrene; DM, dimethylaminoethyl methacrylate; EDGM; ethylene glycol dimethacrylate; V-50, 2,2′-azobis(2-amidinopropane) hydrochloride.

Preparation of PDM

Solution polymerization of DM was carried out in toluene using AIBN: DM, 30 wt %; AIBN, 0.2 wt % per DM, in a nitrogen atmosphere at 70 °C for 48 h. The product was then precipitated in petroleum ether and dried at room temperature for 24 h. This was redried for 24 h under vacuum at 50 °C.

Differential scanning calorimetric (DSC) measurement

Calorimetric measurements of concentrated PS seed, PS/P(DM-EGDM) composite emulsions (90 g/l) and PDM aqueous solution were carried out at pH 9 with a 4100 MC-DSC of CSC Co. as a function of temperature. The volume of the sample measured was about 0.8 ml. In each case the obtained spectrum was subtracted from the spectrum of KOH aqueous solution having pH 9.

Determination of enzymatic activity

A mixture was prepared from 10 ml of each purified seed or composite emulsion and trypsin aqueous solution, and the pH was immediately adjusted at 7 using 0.02 N KOH. The mixture was kept at certain temperatures for 45 min and then enzymatic activity was determined by the pH stat method [17] using BAEE as a substrate according to the following procedure.

A known amount of the free or adsorbed trypsin was added to 100 ml of 10^{-3} mol/l BAEE aqueous solution. Then the pH was adjusted at 7 and maintained using a TOA Electronics HSM-10A pH-stat with 0.02 N KOH under magnetic stirring at the respective temperatures. The activity was calculated from the amount of KOH consumed to neutralize the acid liberated from the hydrolysis of BAEE. The unit of specific activity was expressed as μmole/min/mg-trypsin adsorbed.

Adsorption of cationic emulsifier

A mixture was prepared from 10 ml of each purified seed or composite emulsion (solid content 13.3 g/l) and cationic TSAC emulsifier aqueous solution. The concentration of the emulsifier in the mixture was maintained below the critical micelle concentration. The pH value of the mixture was adjusted at 9 by using a buffer solution. The adsorption/desorption behaviors onto the composite particles were examined repeatedly by alternatively measuring the conductances at 40 °C and 25 °C. Before each measurement, the mixture was kept at the respective temperatures for 1 h. The conductance was measured by a digital conductivity meter (TOA Electronics Ltd. CM-40S).

The amount of emulsifier adsorbed was calculated by subtracting the emulsifier concentration in the medium from the initial concentration calculated under the assumption that the adsorbed emulsifier molecules do not contribute to the conductance. The emulsifier concentration in the medium was obtained from the measurement of conductance using calibration curve, representing the relationship between the concentration and conductance, for the emulsifier aqueous solution at 40 °C and 25 °C.

Adsorption/desorption of biomolecules

A mixture was prepared from 10 ml of each purified composite emulsion (solid content 13.3 g/l) and biomolecule aqueous solution. The pH value of the mixture was immediately adjusted at the isoelectric point with a buffer solution. The mixture was allowed to stand at 40 °C for 3 h, and then centrifuged at 4000 g, 40 °C for 10 min. In order to remove completely wafting particles, the supernatant was centrifuged twice at 7000 g, 40 °C. Biomolecules concentration in the medium was measured by ultraviolet spectrophotometry at 280 nm. The same measurement was carried out at 25 °C. The amount of biomolecules adsorbed was calculated by subtracting the biomolecule concentration in the medium from the initial concentration.

Results and discussion

Figure 1 shows TEM photographs of PS seed and PS/P(DM-EGDM) composite particles. Both seed and the composite particles were monodispersed and their diameters were, respectively, 1.77 and 1.80 μm. Their coefficients of variation were, respectively, 1.6% and 1.9%. No P(DM-EGDM) copolymer particle was byproduced. These indicate that the seeded emulsion copolymerization of DM and EGDM mainly proceeded in PS seed particles.

Figure 2 shows magnified DSC curves of washed PS seed and PS/P(DM-EGDM) composite emulsions at pH 9. In the case of PS seed emulsion no endothermic peak appeared, whereas in the case of the composite emulsion a small but clear endothermic peak appeared at about 34 °C which corresponds to the LCST of PDM aqueous solution. The endothermic peak of the composite emulsion is related to the deswelling of the composite particles. The assignment of the smaller peak around 26 °C is unclear. The swelling/deswelling ratio for submicron-sized PS/P(DM-EGDM) composite particles was previously measured by dynamic light scattering (DLS) [11] but here for micron-sized composite particles we could not succeed to get reliable result as the hydrodynamic diameter exceeded the upper limit of DLS.

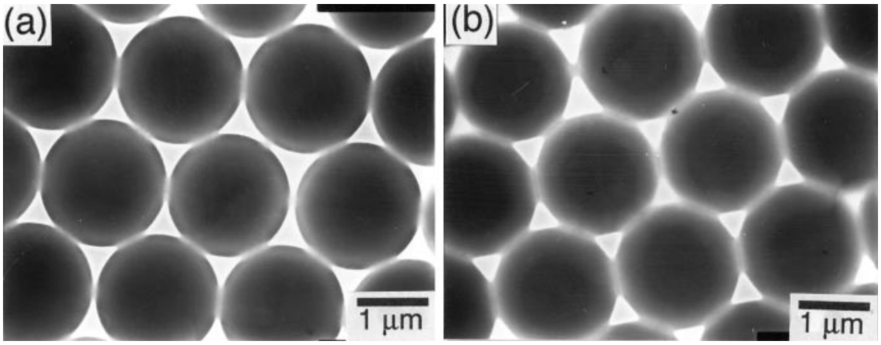


Fig. 1 Transmission electron micrographs of unwashed PS seed (a) and PS/P(DM-EGDM) composite (b) particles

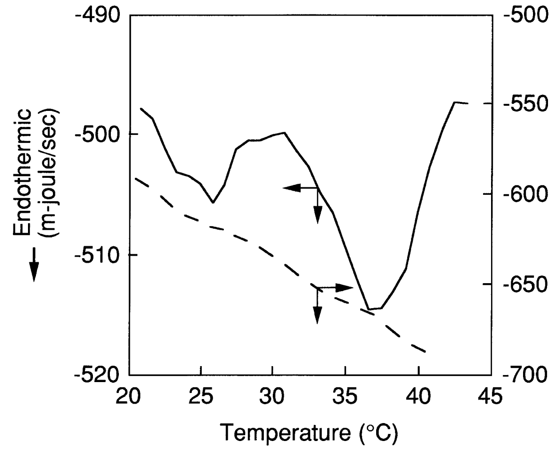


Fig. 2 DSC curves of purified PS seed (——) and PS/P(DM-EGDM) composite polymer (-----) emulsions at pH 9, and at a scanning rate of 5 °C/min. Solid content $\sim$90 g/l

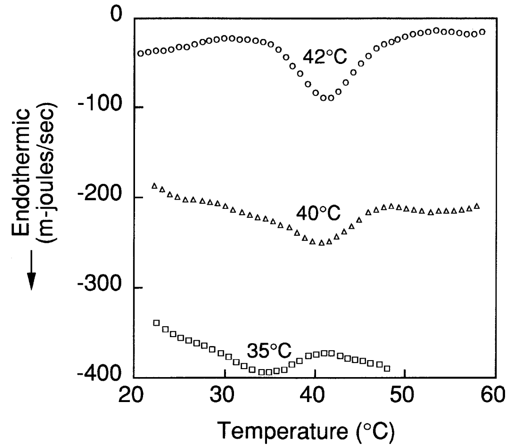


Fig. 3 DSC curves of PDM aqueous solutions at pH 9, and at a scanning rate of 5 °C/min. PDM concentration (wt%): (○) 10; (△) 13; (□) 20

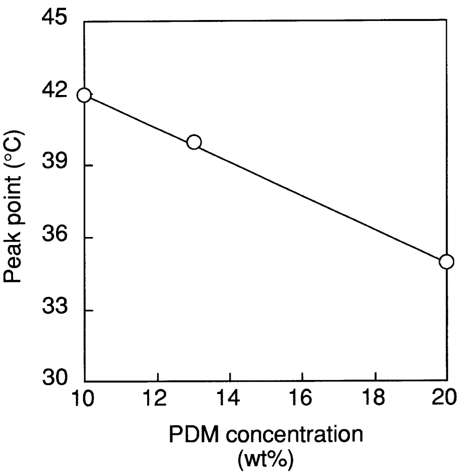


Fig. 4 A relationship between PDM concentration and the endothermic peak in the DSC curves shown in Fig. 3

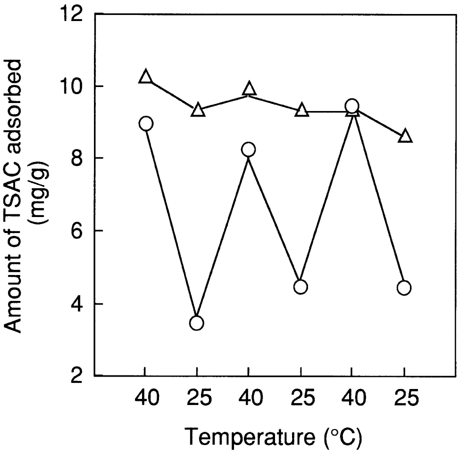


Fig. 5 Amounts of cationic trimethyl stearyl ammonium chloride (TSAC) adsorbed on PS seed (△) and PS/P(DM-EGDM) composite (○) particles at pH 9, measured alternatively at 40 °C and 25 °C. PS seed, 13.3 g/l; TSAC, 2.2 g/l; PS/P(DM-EGDM), 13.3 g/l; TSAC, 1.7 g/l

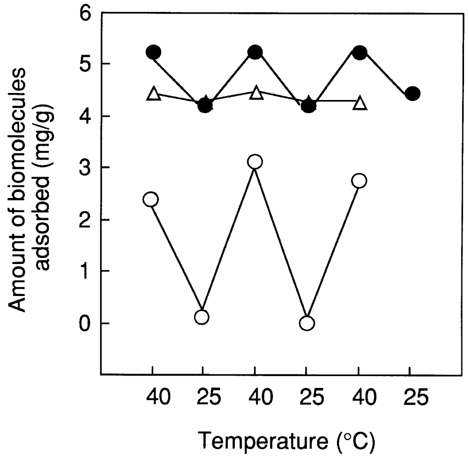
Figure 3 shows DSC curves of PDM aqueous solutions at pH 9. The endothermic peak appeared around 40 °C and deviated to lower temperatures with an increase in PDM concentration. Unexpectedly it also indicates that the amount of endothermic peak decreased with an increase in PDM concentration though the overall endothermic value increased. This decrease is possibly based on highly viscous solution with increasing PDM concentration. Since the measurement was carried out under static condition, the smooth heat transfer through the solution possibly hindered and affected the results. As shown in Fig. 4 a linear relationship was established from Fig. 3 between the PDM concentration and the temperature at which the endothermic peak appeared. This tendency agreed with the result that the temperature at which PDM aqueous solution became turbid decreased with the increase in the PDM concentration [10]. These results indicate that with the increase in the PDM concentration, the hydrophobic interactions among the side groups increased intermolecularly and thereby lowered the transition temperature. In these ways, the results shown in Figs. 2 and 4 indicate that the composite particle surface has a dense P(DM-EGDM) copolymer shell. EGDM as a hydrophobic substrate which is only 3 wt% in the shell is supposed to have negligible effect on transition temperature [11].

Figure 5 shows the amounts of TSAC emulsifier adsorbed on PS seed and PS/P(DM-EGDM) composite particles at pH 9 measured alternatively at 40 °C and 25 °C. In the case of PS seed particles the amounts of adsorptions at 40 °C and 25 °C were high and the difference was appreciably low. This small difference between the amount of adsorptions at 40 °C and 25 °C is possibly based on the difference of state of TSAC emulsifier in the aqueous phase with changing temperature [18], whereas in the case of the composite particles the amounts at 40 °C were always much higher than those at 25 °C. The adsorption and desorption behaviors were almost reversible among the consecutive measurements.

Figure 6 shows the amounts of AL and LA as macromolecular adsorbents onto the composite and seed particles. The measurements were carried out at the isoelectric point to eliminate the effect of ionic interaction between the particle surface and biomolecules. For composite particles, the amount of adsorbed were always higher at 40 °C than at 25 °C. Especially, in the case of LA most of the adsorbed biomolecules at 40 °C were desorbed from the particle surface at 25 °C. The adsorption and desorption behaviors were almost reversible among the consecutive measurements, whereas in the case of PS particles, the difference between the amount of adsorptions of LA at 40 °C and 25 °C were negligible though the overall amount of adsorptions were always much higher than those on the composite particles. These comparative adsorption/desorption behavior suggest that at temperature below the LCST composite particle surface is hydrophilic and above the LCST it is hydrophobic.

Figure 7 shows the specific activities of trypsin adsorbed on PS seed (△) and PS/P(DM-EGDM) composite (●) particles for 45 min and of free trypsin measured immediately (□) and after 45 min of standing (■) at pH 7 as a function of temperature. In the case of the PS particles specific activity slightly increased linearly with an increase in the temperature. This is simply because the hydrolysis rate of BAEE as a substrate by trypsin increased with the increase in the temperature [19]. In the case of PS/P(DM-EGDM) composite particles the specific activity sharply increased at around the LCST and then almost levelled off at 36 °C, indicating a phase transition at the particle surface from hydrophilic to hydrophobic one. In the case of conjugated trypsin on poly-NIPAM hydrogel microspheres, Kawaguchi et al. [20] reported a decrease in enzymatic activity at around the LCST. This was because above the transition temperature as the hydrogel collapsed some of the conjugated trypsin trapped inside the gel. For PS/P(DM-EGDM) composite particles an increase in enzymatic activity indicates that at temperature above the LCST only the hydrophobic parts of enzyme molecules are loosely bound to the particle surface with the remaining hydrophilic parts in solution and at lower temperature most of the enzymes are free in solution. Since the measurement was carried out at pH 7, the hydrophobicity of the composite particle surface at temperature above the LCST is considered to be comparatively lower than that of PS particles because of the partial ionization of amine group in DM unit. The specific activities (●) of the adsorbed trypsin on PS/P(DM-EGDM) composite particles at the temperatures above LCST were also much higher than those (■) of the free trypsin measured after 45 min and nearly equal to those (□) of the free trypsin measured immediately. These indicate that the micron-sized

Fig. 6 Amounts of lactalbumin hydrolysate adsorbed on PS/P(DM-EGDM) composite (○) and PS seed (△) particles at pH 6.8, and of egg albumin adsorbed on PS/P(DM-EGDM) composite (●) particles at pH 4.9, measured alternatively at 40 °C and 25 °C, respectively. The particles, 13.3 g/l; the proteins, 0.3 g/l



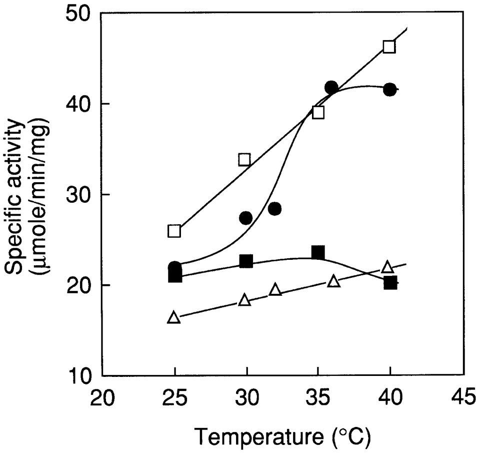


Fig. 7 Specific activities of adsorbed trypsin on PS seed (△) and PS/P(DM-EGDM) composite (●) particles for 45 min and of free trypsin measured immediately (□) and after 45 min of standing (■) at pH 7 at temperatures around the LCST of PDM

PS/P(DM-EGDM) composite particle surface is hydrophilic enough even at temperature above the LCST to prevent conformational change of adsorbed trypsin from hydrophobic interaction.

From these results, it is concluded that the micron-sized monodispersed PS/P(DM-EGDM) composite particles can be expected to be a temperature-sensitive carrier for biomolecules.

Acknowledgements The authors would like to thank Prof. Dr. K. Akasaka of the Faculty of Science, Kobe University, for his kind help in DSC measurement.

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