A Novel Approach for Albumin Determination in Aqueous Media by Using Temperature- and pH-Sensitive *N*-Isopropylacrylamide-*co*-*N*-[3-(dimethylamino)-propyl]methacrylamide Random Copolymers

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ABSTRACT: Random copolymers of *N*-isopropylacrylamide (NIPA) and *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPM) were synthesized by solution polymerization using azobisizobutyronitrile as the initiator in 1,4-dioxane at 60°C. NIPA-*co*-DMAPM copolymer exhibited both temperature and pH sensitivity. Thermally reversible phase transitions were observed both in the acidic and the alkaline pH regions for copolymers produced with different DMAPM/NIPA feed ratios. The pH dependency of the lower critical solution temperature (LCST) was stronger for copolymers produced with higher DMAPM feed concentrations. NIPA-*co*-DMAPM random copolymer was also sensitive to the albumin concentration. In the presence of albumin, thermally irreversible phase transitions were observed in slightly acidic and neutral media. However, reversible transitions were obtained in aqueous media containing albumin at basic pH. The phase-transition temperature of NIPA-*co*-DMAPM copolymer significantly decreased with increasing albumin concentration at both acidic and alkaline pH values. This behavior was explained by albumin binding onto the copolymer chains by means of H-bond formation between the dimethylamino groups of the copolymer and the carboxyl groups of albumin. For a certain range of albumin concentration, the phase-transition temperature exhibited a linear decrease with increasing albumin concentration. By utilizing this behavior, a simple albumin assay was developed. The results indicated that NIPA-*co*-DMAPM copolymer could be utilized as a new reagent for the determination of albumin concentration in the aqueous medium. The proposed method was valid for the albumin concentration range of 0–4000 μg/mL. The protein concentrations commonly utilized in biotechnological studies fall in the range of the proposed method. © 2002 Wiley Periodicals, Inc. J Appl Polym Sci 84: 2060–2071, 2002; DOI 10.1002/app.10503

Key words: thermosensitive polymer; pH sensitivity; *N*-isopropylacrylamide; dimethylaminopropylmethacrylamide; 2-(dimethylamino)ethyl methacrylate; albumin; protein

INTRODUCTION

In recent years, thermosensitive polymers have been promoted as useful tools in biotechnological applications such as enzyme immobilization, thermal affinity separation, controlled drug release, immunodiagnostics, gene therapy, and so forth. Most of these polymers have been produced in the form of homopolymers or copolymers of *N*-isopropylacrylamide (NIPA). A thermosensitive graft copolymer of NIPA and acrylic acid (NIPA-*g*-AA) exhibiting a pH sensitivity was obtained by Chen and Hoffman.[1] Synthesis and thermoassociative properties of NIPA-*g*-AA copolymers were extensively investigated by Durand and Hourdet.[2] The temperature- and pH-sensi-

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tive copolymers of NIPA with 4-pentenoic acid were synthesized for possible use in protein conjugation.[3]

Thermosensitive copolymers were also investigated as potential carriers in enzyme immobilization studies because of their reversible phase separation behavior.[4–14] Succinimide-functionalized thermosensitive polymers obtained by the solution copolymerization of NIPA and *N*-acryloxysuccinimide were evaluated as carrier materials in the immobilization of different enzymes, given their direct coupling ability with the amino groups of enzyme molecules.[4,5] Another thermosensitive graft copolymer carrying carboxyl groups was prepared by coupling poly(acrylic acid-*co*-acrylamide) with poly(NIPA) having an amino group at its end.[6] Lipase from wheat germ was covalently bound onto the produced graft copolymer by using a water-soluble carbodiimide activation.[6] Polyallylamine-*g*-poly(NIPA), characterized by both thermal and pH sensitivity, was prepared by coupling polyallylamine with poly(NIPA) carrying an end-standing carboxyl group.[7] The microspheres obtained from the produced copolymer were tried as solid support for the covalent immobilization of trypsin.[7] We have also produced various thermosensitive gels by the copolymerization of NIPA with functional acrylic monomers (e.g., 2-hydroxyethylmethacrylate, *N*-vinylpyrrolidone, and *N*-vinylphenylboronic acid) and investigated the use of these copolymers as support materials in enzyme and nucleotide immobilization.[15–21]

Hinrichs et al.[22] investigated the complexation behavior between NIPA-*co*-2-(dimethylamino)ethyl methacrylate random copolymer and DNA. In the same study the use of this copolymer as a possible transfection agent was also discussed. The reversible phase separation behavior of poly(NIPA) was utilized for the thermal affinity separation of antibodies for immunodiagnostic purposes.[23,24] A copolymer gel matrix composed of NIPA and a cationic monomer, 2-(dimethylamino)propyl methacrylamide, was synthesized for temperature-modulated insulin release.[25] Poly[NIPA-*co*-2-(dimethyl-)acrylamide]-*block*-poly(D,L-lactide) thermally responsive micelles were utilized as carriers for the controlled release of an antitumor agent, adriamycin.[26]

Significant attention has also been paid to boronic acid–functionalized thermosensitive polymers exhibiting a response to glucose concentration.[27–30] A water-soluble, thermosensitive copolymer of 2-(diethyl)acrylamide containing 15 mol % of 3-acrylamidophenylboronic acid (AcPBA) was prepared by the radical copolymerization of corresponding monomers in ethanol.[27] The linear copolymers of NIPA and AcPBA and the terpolymers of NIPA–AcPBA–dimethylaminopropylacrylamide (DMAPA) also showed a lower critical solution temperature (LCST) change against glucose concentration.[28] In the study by Hisamitsu et al.[29] for the sensing of glucose, a good correlation was observed with the soluble terpolymer of AcPBA, DMAPA, and dimethylacrylamide between the diol complexation rate and the fraction of phenylborate as well as DMAPA in the terpolymers. Thermosensitive terpolymer gels in the form of NIPA–AcpBA–2-(dimethylamino)propyl methacrylamide (DMAPM) were also used in the endothelial cell differentiation as a cell substratum.[30]

In our previous studies, we proposed different polymeric structures with nonspecific interaction or specific recognition abilities for proteins.[31–35] By considering our previous results, we attempted to design a thermosensitive copolymer capable of interacting with protein molecules. Here, the synthesis and characterization of a relatively new copolymer having both temperature and pH sensitivity are described. The copolymer obtained by the solution copolymerization of NIPA and the cationic monomer DMAPM exhibited responsive behavior to albumin concentration in aqueous media. In the present study, the response behavior of NIPA-*co*-DMAPM to the presence of albumin was investigated and the use of this copolymer as a reagent in the quantitative determination of albumin was discussed.

EXPERIMENTAL

Materials

The monomer, *N*-isopropylacrylamide (NIPA; Aldrich Chemical Co., Milwaukee, WI) was recrystallized from *n*-hexane–acetone solution. The comonomer *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPM; Aldrich) was used without further purification. Azobisizobutyronitrile (AIBN; BDH Chemicals, Poole, UK) and 1,4-dioxane (Merck A.G., Darmstadt, Germany) were selected as the initiator and the solvent in the solution copolymerization, respectively. Methanol and diethyl ether, both supplied from Merck, were used as solvent and precipitant, respectively, in the purification of copolymers. Bovine serum albumin

(BSA; Sigma Chemical Co., St. Louis, MO) was used as the model protein. Distilled/deionized water was used for the protein assays.

Preparation of NIPA-*co*-DMAPM Copolymer

A series of NIPA-*co*-DMAPM copolymers were obtained by solution copolymerization by varying the DMAPM/NIPA feed ratio between 0/100 and 18.8/81.2 mol/mol. A typical procedure was as follows: NIPA (2.0 g) and DMAPM (0.35 mL) were dissolved in 1,4-dioxane (25 mL) placed in a sealed cylindrical polymerization reactor (total volume: 100 mL) and AIBN was added to this homogeneous solution. After complete dissolution of AIBN (0.035 g), the medium was purged with nitrogen for 10 min. The sealed reactor was placed into a shaking bath equipped with a temperature-control system. The reactor was heated to the polymerization temperature in approximately 40 min by shaking at 120 cpm. The copolymerization was conducted at 60°C for 24 h at 120 cpm shaking rate under a nitrogen atmosphere. After cooling to room temperature, dioxane was removed in a rotary evaporator under vacuum at 40°C until the total solution volume was reduced to 2–3 mL. The resulting product was then dissolved in methanol (20 mL) and the copolymer was precipitated by adding diethyl ether (20 mL). The dissolution/precipitation procedure was repeated three times for the removal of impurities in the precipitated copolymer. The copolymer purity was checked by recording FTIR spectra of the monomers and the copolymers produced with different DMAPM/NIPA mol/mol ratios. FTIR study indicated that the purified copolymer included no monomeric compound.

Characterization of NIPA-*co*-DMAPM Copolymer

The isolation yields of copolymers were determined by a gravimetric procedure. The copolymer isolated by the above procedure was dried in vacuum at 40°C for 48 h and weighed. The copolymer yield was calculated by taking the ratio of the isolated copolymer weight to the initial monomer weight charged into the reactor. The number-average molecular weights of copolymers were determined by viscosimetric measurement. For this purpose, the copolymers were dissolved in tetrahydrofuran (THF; Aldrich) by stirring the solution magnetically for 24 h at room temperature. The viscosities of copolymer solutions prepared in the concentration range of 0.25–1.0 g/dL were measured at 27°C. The viscosity-average molecular weights of copolymers were calculated according to the following equation[36]:

$$[\eta] = 5.8 \times 10^{-5} M_{\eta}^{0.78} \quad (1)$$

In most of the copolymerizations, DMAPM feed concentration was lower than 10% mol (except the last experiment performed with the DMAPM feed concentration of 11.7% mol). By considering this property, the above relation derived for poly(NIPA) homopolymer was also used for the molecular weight determination of poly(NIPA-*co*-DMAPM) copolymers produced in our study. The LCST measurements were performed in a UV–Vis spectrophotometer (Model 230; Hitachi, Tokyo, Japan) equipped with a heating system and a thermometer. For this purpose, a 1% (w/w) aqueous solution of the NIPA-*co*-DMAPM copolymer was put into a quartz cuvette with a volume of 2.5 mL. The temperature of the solution was increased at a rate of 1°C/min, starting from room temperature, and the absorbance of the solution was periodically recorded at a wavelength of 550 nm. LCSTs of the copolymers were calculated from the absorbance–temperature curves by using the method described in the literature.[3,37] These measurements were performed at pH values of 5, 7, and 11. pH values of 5 and 7 were obtained by adding 0.1*N* HCl solution to the freshly prepared copolymer solutions; 0.05*N* NaOH solution was used for adjusting the pH to 11.

Albumin–Copolymer Interaction

The phase-transition temperatures of aqueous solutions containing BSA and NIPA-*co*-DMAPM copolymer [i.e., at a concentration of 1% (w/v)] were determined in a UV–Vis spectrophotometer by measuring absorbances of the solutions at a wavelength of 540 nm as a function of temperature.[3,37] BSA concentration was varied between 0 and 4000 μg/mL and these measurements were performed at pH values of 5, 7, and 11, using NIPA-*co*-DMAPM random copolymers produced with different DMAPM/NIPA feed ratios.

RESULTS AND DISCUSSION

The chemical structure of NIPA-*co*-DMAPM random copolymer is shown in Figure 1. A series of NIPA-*co*-DMAPM copolymers were synthesized

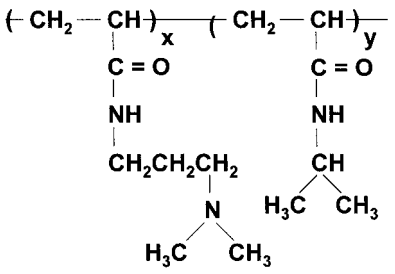

Figure 1 Chemical structure of NIPA-*co*-DMAPM random copolymer.

by changing the DMAPM/NIPA feed ratio between 0/100 and 11.7/88.3 mol/mol. The effects of DMAPM feed concentration on the copolymer yield and the viscosity-average molecular weight are given in Figure 2. As seen here, satisfactorily high copolymer yields were achieved in all cases. The copolymer yield slightly increased with increasing DMAPM feed concentration. Low DMAPM feed concentrations provided copolymers with an average molecular weight of approximately 3×10^5. However, the copolymers with lower molecular weights were obtained with the DMAPM feed concentrations higher than 5% mol. The order of magnitude of the molecular weights found in this study was close to that measured for both poly(NIPA) and poly(NIPA-*co*-dimethylaminoethylmethacrylate) random copolymers produced under similar conditions.[36,38]

As mentioned in the literature, there have been significant controversies in the molecular weight determination of poly(NIPA) by GPC.[36,39,40,41] Some authors reported that the GPC method could not be used to obtain molecular weight information for this polymer because of filtration problems.[36,39] Recently, molecular weight characterization of poly(NIPA) was extensively investigated by Ganachaud et al.[36] In their study, the molecular weights determined by GPC were compared with those obtained by matrix assisted laser desorption/ionization mass spectrometry-time of flight (MALDI-TOF) and theoretically predicted values.[36] This comparison indicated that MALDI-TOF and GPC provided similar molecular weight distributions, at least for the low molecular weight range, whereas discrepancies arose for higher molecular weight. Note that we also tried to measure the molecular weights of NIPA–DMAPM copolymers by GPC by using Shimpack 802 and 804 columns with THF as the eluent. However, we could not obtain meaningful results. As a consequence, we prefer to measure the molecular weight by viscosimetric method in which the Mark–Houwink–Sakaruda parameters were taken as the values determined for poly(NIPA) in the study performed by Ganachaud et al.[36] In most of our copolymerizations, the DMAPM feed concentration was kept lower than 10% mol. By considering the low DMAPM contents of the copolymer samples, this ap-

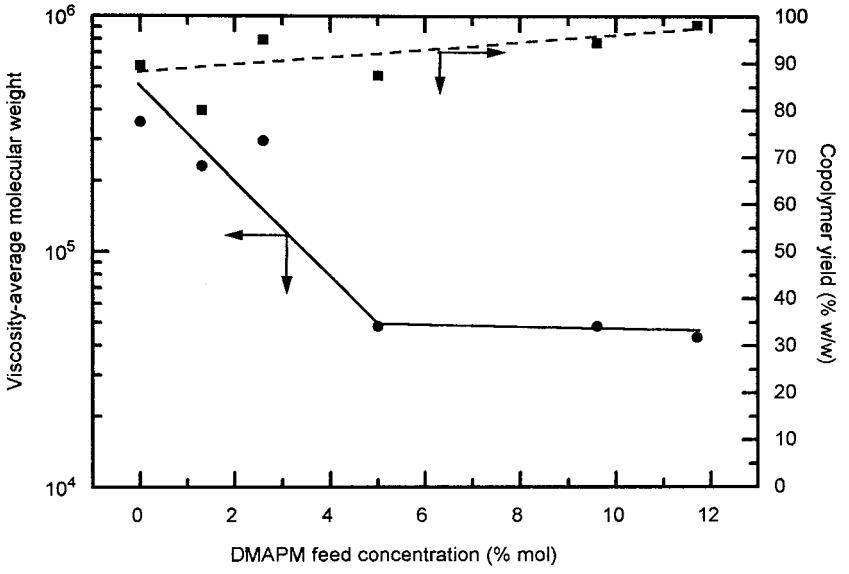

Figure 2 Effects of DMAPM feed concentration on the copolymer yield and the viscosity-average molecular weight.

proach was selected for molecular weight determination.

The temperature response of the copolymers was studied at pH values of 5, 7, and 11. Absorbances of aqueous solutions containing 1% (w/w) copolymer were measured at 550 nm. Figure 3 exemplifies typical stimuli-responsive behavior, which is obtained for the copolymer produced with the DMAPM/NIPA feed ratio of 9.6/91.4 mol/mol. As seen here, the absorbance increased with increasing temperature, given that the transparent copolymer solution became turbid. Based on these curves, LCSTs were determined as the temperature at which 10% of the total absorbance increase was observed.[3,37] It should be noted that a sharper phase transition could be achieved at pH 11 relative to those observed at pH 5 and 7. After completion of phase transition, final absorbance values of 1% (w/w) copolymer-containing aqueous media at pH values of 5, 7, and 11 were measured as 0.196, 0.656, and 3.01, respectively. Note that the lower final absorbance was obtained with a lower slope (i.e., with lower dA/dT) in the aqueous medium at lower pH. The slope of the phase-transition curve and the magnitude of final absorbance measured at the end of phase transition should be related to the degree of phase separation taking place between the copolymer and water at LCST. These results indicated that the copolymer solubility was higher in the aqueous medium with lower pH, probably as a result of the protonation of basic dimethylamino groups in the copolymer structure. Note that all transitions were thermally reversible so that the turbid copolymer solutions at the temperatures higher than LCST again turned into the transparent form when the temperature was decreased below the LCST.

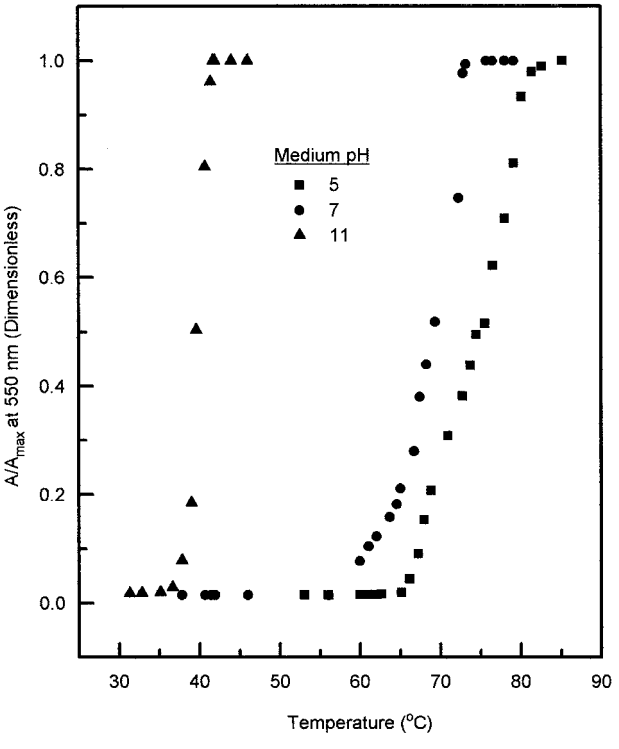

Figure 3 Temperature responsivity of copolymer produced with DMAPM/NIPA feed ratio of 9.6/91.4 mol/mol.

Table I Lower Critical Solution Temperature (LCST) Values Measured at Different pH for the Copolymers Produced with Different DMAPM/NIPA Feed Ratios

DMAPM/NIPA (mol/mol)	LCST (°C)		
	pH 5	pH 7	pH 11
0/100	31.3	31.3	31.3
1.3/98.7	35.2	34.3	32.4
2.6/97.4	39.3	37.2	33.1
5.0/95.0	57.6	57.9	37.0
9.6/91.4	67.4	61.1	37.6
11.7/88.3	—[a]	—[a]	64.8

[a] No LCST was observed up to 90°C with 1% (w/w) copolymer solution at the studied pH.

The effects of DMAPM feed concentration on the LCST of NIPA-*co*-DMAPM copolymer at three different pH values are given in Table I. As seen here, LCST increased with increasing DMAPM feed concentration at all pH values. However, LCST increase was clearer in the aqueous media at pH 5 and pH 7. The final compositions of the copolymers were probably close to the compositions of initial monomer mixtures, given that the determined copolymer yields were satisfactorily high. This case involves an increase in the dimethylamino content of copolymer with the increasing DMAPM feed concentration. The protonation of a higher number of DMAPM units in the copolymer structure probably led to a significant increase in the copolymer solubility at acidic pH (i.e., pH 5). Hence, LCST increased by the increasing DMAPM content at this pH. On the other hand, the polarity of resulting copolymer probably increased with the increasing DMAPM content because of the highly polar character of dimethylamino groups. LCST increases observed at both pH 7 and pH 11 can be explained by the

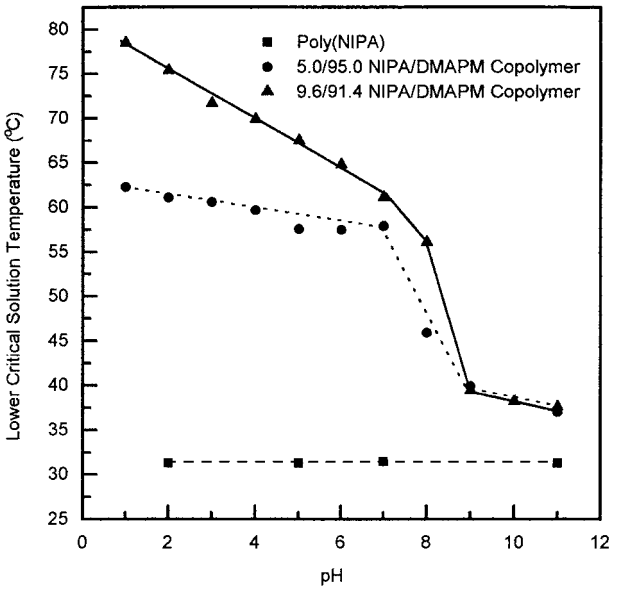

Figure 4 Variation of LCST of NIPA-*co*-DMAPM random copolymer with pH.

higher water solubility of copolymers carrying higher numbers of polar groups (i.e., DMAPM units). With the NIPA-*co*-DMAPM copolymer obtained by using DMAPM/NIPA feed ratio of 11.7/98.3 mol/mol, no phase transition could be observed up to 90°C in the aqueous media at pH 5 and pH 7. This copolymer exhibited a LCST only at 64.8°C at pH 11. It is normally expected that the DMAPM content of this copolymer is the highest among the copolymers exhibiting a LCST behavior in the aqueous media. For this reason, a relatively weak thermosensitivity was observed.

The variation of LCST of NIPA-*co*-DMAPM random copolymer with pH is given in Figure 4. This behavior was exemplified for the copolymers produced with two different DMAPM/NIPA feed ratios (i.e., 5.0/95.0 and 9.6/91.4 mol/mol). Here, poly(NIPA) homopolymer was included for comparison. As seen in Figure 4, the LCST drastically decreased with increasing pH for both NIPA-*co*-DMAPM copolymers, whereas no significant change was observed in the LCST of poly(NIPA) homopolymer. As discussed above, the solubility of NIPA-*co*-DMAPM copolymer was higher because of the protonation of basic dimethylamino groups at low pH. Therefore higher LCST values observed at acidic pH can be explained by the protonation effect on the copolymer. In the pH region between 7 and 9, a sharp decrease was observed in the LCST. By starting from the neutral pH value, the disappearance of protonation effect on the basic dimethylamino groups was probably responsible for this decrease. In the alkaline pH region between 9 and 11, no significant difference was observed between the LCST values of both copolymers. However, NIPA-*co*-DMAPM copolymers had slightly higher LCSTs relative to those of poly(NIPA) in the same region. The polar character of DMAPM units in the copolymer structure might be the reason for the LCST difference observed between the NIPA-*co*-DMAPM copolymer and the homopolymer of NIPA in this region.

Albumin–Copolymer Interaction

In the absence of BSA, the copolymers prepared with different DMAPM/NIPA feed ratios exhibited thermally reversible phase transitions at pH values between 5 and 11. In the presence of BSA, the phase transition induced by the temperature change was also reversible at pH 11. However, thermally irreversible transitions were observed in the presence of BSA, at pH 5 and pH 7. In this pH region, the transparent copolymer solution containing BSA became turbid at the phase-transition temperature (PTT) and remained in this form at temperatures higher than PTT. When pH was kept constant at 5 or 7, the copolymer solution did not return to the transparent form by decreasing the temperature below the PTT determined during the temperature increase. The term *LCST* is usually used for the polymers exhibiting thermally reversible phase transition in aqueous media. Instead of this term, however, we prefer to include a more general term, *phase-transition temperature* (PTT), for describing the temperature at which the copolymer irreversibly passed into the insoluble form in the aqueous medium containing BSA.

The effect of albumin concentration on the thermoresponsive behavior of 95.0/5.0 (mol/mol) NIPA-*co*-DMAPM copolymer is given in Figure 5. This figure shows the variation of PTT with the albumin concentration at pH values of 5, 7, and 11. As seen here, PTT decreased with increasing albumin concentration. This decrease is probably explained by albumin binding onto the copolymer chains by means of the interaction between dimethylamino groups of copolymer and carboxyl groups of albumin. A possible scheme for this interaction is given in Figure 6. As seen here, BSA binding onto the copolymer chains was probably controlled by the formation of hydrogen bonds between the C-terminal carboxyl groups of

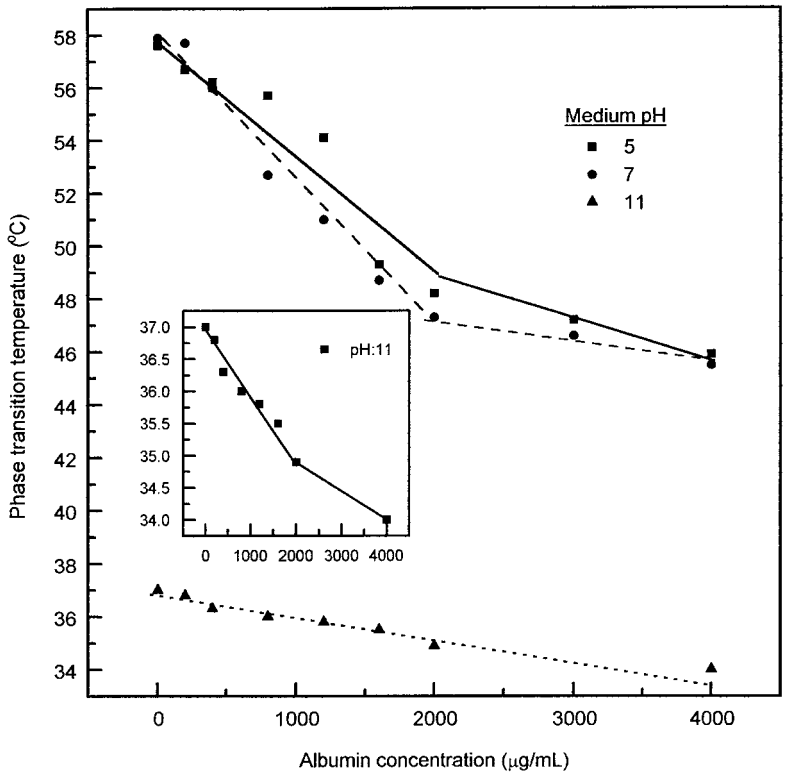

Figure 5 Variation of phase-transition temperature of NIPA-*co*-DMAPM copolymer with the albumin concentration (DMAPM/NIPA feed ratio: 5.0/95.0).

albumin and DMAPM units in the copolymer. Thermally irreversible phase transitions observed in the presence of BSA at both pH 5 and pH 7 possibly indicated that the formed hydrogen bonds were stronger in this pH region. On the other hand, the electrostatic interactions may also play a role for the binding of BSA to the copolymer molecules. BSA is known as a hydrophobic protein. The binding of albumin molecules

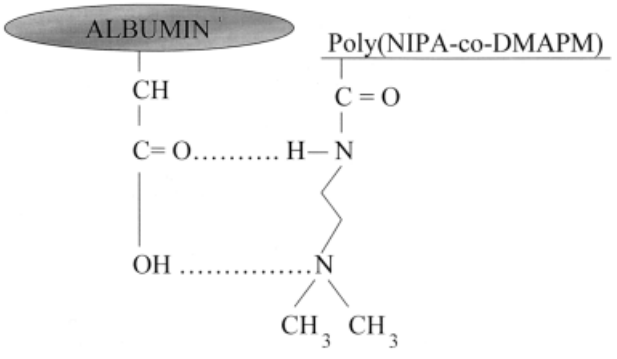

Figure 6 Schematic representation of chemical interaction between NIPA-*co*-DMAPM random copolymer and albumin.

onto the NIPA-*co*-DMAPM chains probably resulted in a decrease in the solubility of copolymer. Therefore a decrease was observed in the PTT, given that the albumin molecules bound to the copolymer chains probably increased with increasing albumin concentration.

However, the linearity of PTT decrease by the albumin concentration is important in the quantitative determination of albumin. Subsequently, a series of curve fittings were performed by using a least-squares algorithm for finding linear relations between PTT and albumin concentration. The following conventional expression was used in the curve fitting:

$$\mathrm{PTT} = a_0 + a_1 C_{\mathrm{alb}} \qquad (2)$$

In eq. (2), PTT (°C) and C_{alb} (μg/mL) are the phase-transition temperature of 1% (w/w) copolymer solution and the albumin concentration in the aqueous medium at a certain pH, respectively. In the linear regressions, the parameters a_0 and a_1 and the correlation coefficient r of eq. (2) were estimated. In each regression, PTT values obtained in a certain range of albumin concentra-

Table II Parameters of Linear Relations Between Phase-Transition Temperature and Albumin Concentration for the Copolymer Produced with the DMAPM/NIPA Feed Ratio of 5.0/95.0 mol/mol

pH	C_{alb}[a] (μg/mL)	a_0[b] (°C)	a_1[c] (°C μL^{-1} μg^{-1})	r[d]
5	0–1200	57.43621 ± 0.21111	−0.00265 ± 0.00031	0.97971
5	0–2000	58.21485 ± 0.71899	−0.00479 ± 0.00064	0.95819
7	0–1200	58.34138 ± 0.40846	−0.00631 ± 0.00047	0.98650
7	0–2000	58.08055 ± 0.37158	−0.00568 ± 0.00033	0.99166
11	0–2000	36.89608 ± 0.09100	−0.00096 ± 0.00008	0.98279
11	0–4000	36.73473 ± 0.12132	−0.00074 ± 0.00006	0.97519

[a] C_{alb}, selected albumin concentration range in which the linear regression was performed.
[b] a_0, first parameter in the linear relation between phase-transition temperature (°C) and albumin concentration (μg/mL) .
[c] a_1, second parameter of the linear relation between phase-transition temperature (°C) and albumin concentration (μg/mL) .
[d] r, coefficient of variation determined by the linear regression.

tion and at a certain pH were utilized. The estimated model parameters are presented in Table II for the copolymer produced with DMAPM/NIPA ratio of 5.0/95.0 mol/mol.

As seen in Figure 5, PTT linearly decreased with increasing albumin concentration in the range of 0–2000 μg/mL at both pH 5 and pH 7. Here, the linear regressions were performed for two different ranges of albumin concentration (i.e., 0–1200 and 0–2000 μg/mL) (Table II, first four regressions). Among these, the best correlation coefficient (i.e., 0.99166) was obtained in the regression performed for the albumin concentration range of 0–2000 μg/mL, at pH 7. Under these conditions, PTT decreased from 58 to 47°C when the albumin concentration was increased from 0 to 2000 μg/mL (Fig. 5). Hence, the magnitude of PTT decrease was 11°C. The magnitude of change in the PTT is also important because more accurate determination of albumin concentration is possible only when the PTT change occurs in a wider range.

At pH 11, PTT decreased linearly in the albumin concentration range of 0–4000 μg/mL (Fig. 5). The PTT change at pH 11 is shown in a small plot introduced into Figure 5 because the variation range of PTT was narrower relative to those observed at pH 5 and pH 7. For pH 11, two separate linear regressions were performed by selecting the albumin concentration ranges of 0–2000 and 0–4000 μg/mL (i.e., the last two regressions in Table II). A satisfactory correlation coefficient was obtained for the albumin concentration range of 0–4000 μg/mL. The results in Table II indicate that the copolymer produced with DMAPM/NIPA ratio of 5.0/95.0 could be utilized as an appropriate reagent for the determination of albumin concentration in aqueous media.

The variation of PTT with the albumin concentration is shown for the copolymer produced with a higher DMAPM/NIPA feed ratio (i.e., 9.6/91.4 mol/mol), shown in Figure 7. As seen here, PTT exhibited a linear decrease with the albumin concentration, especially in the range of 0–1200 μg/mL at pH 5 and pH 7. Thus the linear regressions were also made for this copolymer, the results of which are presented in Table III. In the presence of this copolymer, the albumin concentration range, in which the linear relation works, is narrower relative to that of the previous copolymer (i.e., 0–2000 μg/mL). By using the data collected for pH 5 and pH 7, satisfactorily high correlation coefficients could be obtained only in the linear regressions performed for the albumin concentration range of 0–1200 μg/mL. The regressions based on a wider albumin concentration range (i.e., 0–2000 μg/mL) provided relatively lower correlation coefficients (i.e., close to 0.96). The PTT of 9.6/91.4 DMAPM/NIPA copolymer also exhibited a linear decrease at pH 11 (Fig. 7). This behavior was also shown by a small plot included in this figure. At pH 11, the linear regressions performed by selecting the albumin concentration ranges of 0–2000 and 0–4000 μg/mL also provided satisfactorily high correlation coefficients (i.e., last two regressions in Table III).

The effect of albumin concentration on the thermoresponsive behavior of copolymer produced with the DMAPM/NIPA feed ratio of 11.7/88.3 mol/mol is given in Figure 8. As seen here, this copolymer did not give PTT with the low albumin concentrations at either pH 5 or pH 7. Additionally, a linear relation between PTT and albumin concentration could not be obtained at

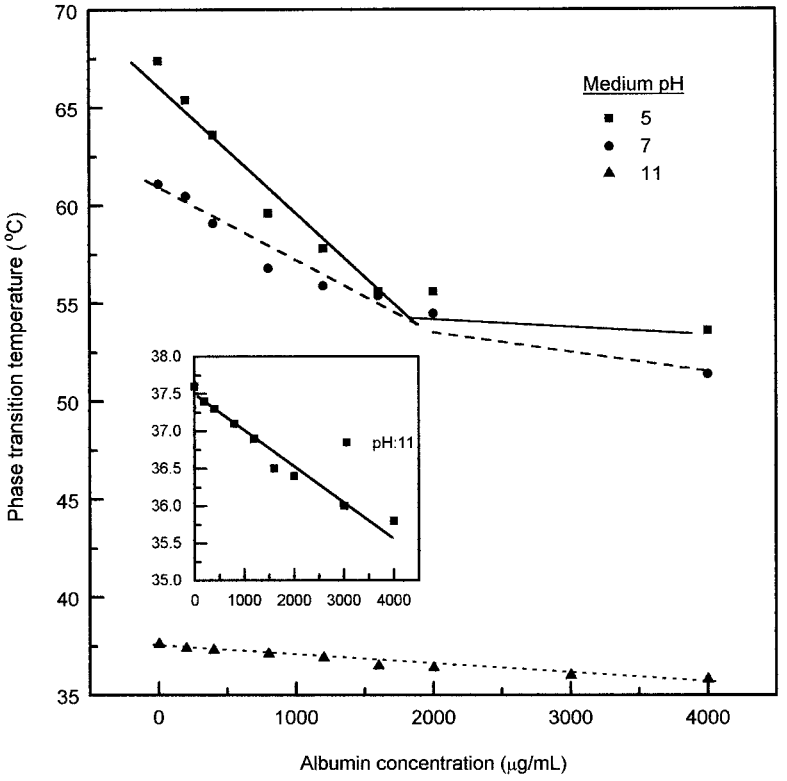

Figure 7 Variation of phase-transition temperature of NIPA-*co*-DMAPM copolymer with albumin concentration (DMAPM/NIPA feed ratio: 9.6/91.4).

pH 11. Therefore linear regressions were not performed for this copolymer.

The results in Tables II and III indicate that NIPA-*co*-DMAPM copolymer could be utilized as an appropriate reagent for the determination of albumin concentration only by measuring the temperature at which the transparent albumin–copolymer solution became turbid. The results of the linear regressions indicated that the maximum concentration range in which the albumin concentration could be estimated accurately was 0–4000 μg/mL. The magnitudes of protein concentrations used in the biotechnological applications usually fall in the range of the proposed method.[42–45] The precise determination of PTT involves the use of a visible spectrophotometer equipped with a heating system and a thermometer capable of measuring the cell temperature with an accuracy of 0.1°C. Alternatively, the detection of PTT can be roughly made by using a simple thermometer and by following the turbidity of the copolymer–albumin solution visible to the naked eye. To support this idea, the phase-transition behavior of 9.6/91.4 NIPA-*co*-DMAPM copolymer in an aqueous medium having an albumin concentration of 4000 μg/mL is given in

Table III Parameters of Linear Relations Between Phase-Transition Temperature and Albumin Concentration for the Copolymer Produced with the DMAPM/NIPA Feed Ratio of 9.6/91.4 mol/mol

pH	C_{alb} (μg/mL)	a_0 (°C)	a_1 (°C μL^{-1} μg^{-1})	r
5	0–1200	67.02758 ± 0.45936	−0.00821 ± 0.00068	0.98985
5	0–2000	66.19181 ± 0.82211	−0.00618 ± 0.00073	0.96675
7	0–1200	61.09397 ± 0.31589	−0.00464 ± 0.00047	0.98511
7	0–2000	60.60546 ± 0.45111	−0.00338 ± 0.00040	0.96644
11	0–2000	37.56212 ± 0.03804	−0.00066 ± 0.00003	0.99220
11	0–4000	37.45709 ± 0.06924	−0.00046 ± 0.00004	0.97982

Figure 9. Here, the absorbance–temperature curves were obtained in the aqueous medium containing 1% (w/w) copolymer. As shown in Figure 9, the phase transitions observed in the presence of albumin were sufficiently sharp at all tried pH values. In the existence of such a sharp phase transition, the PTT of copolymer can be determined even by naked eye, without the use of a visible spectrophotometer. Then it should be possible to estimate the albumin concentration quickly by using a calibration chart including the PTT of copolymer versus albumin concentration.

As mentioned above, thermally irreversible phase transitions were observed at both acidic and neutral pH values with the produced copolymer in the aqueous medium containing albumin. For this reason, a permanent turbidity occurred in the copolymer–albumin solutions heated to temperatures higher than the PTT. The turbid character of the solution did not change when these solutions were cooled to temperatures below the PTT. The absorbance values originating from the permanent turbidity were measured at 550 nm and plotted against the albumin concentration. The variation of permanent absorbance by the albumin concentration is given in Figure 10 for the copolymers produced with the DMAPM/NIPA feed ratios of 5.0/95.0 and 9.6/91.4 mol/mol. As seen in Figure 10(A), the variation of permanent absorbance by the albumin concentration did not exhibit a linear tendency at either pH value. In the copolymer obtained with the DMAPM/NIPA feed ratio of 9.6/91.4, a linear relation between the permanent absorbance and albumin concentration could be observed at pH 5 for the albumin concentration range of 0–2000 μg/mL [Fig. 10(B)]. The performed linear regression gave the linear model constants of $a_0 = 0.02387 \pm 0.04088$ and $a_1 = 1.23451 \pm 0.03638$, with a correlation coefficient of 0.99784. This behavior can be also utilized for the estimation of albumin concentration in the range of 0–2000 μg/mL.

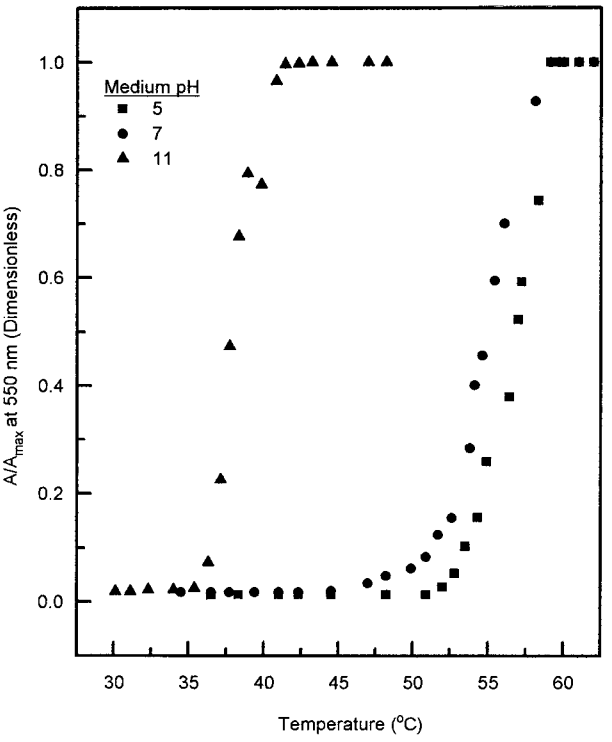

Figure 9 Phase-transition behavior of NIPA-*co*-DMAPM copolymer in aqueous solution including 4000 μg/mL of albumin (DMAPM/NIPA feed ratio: 9.6/91.4 mol/mol).

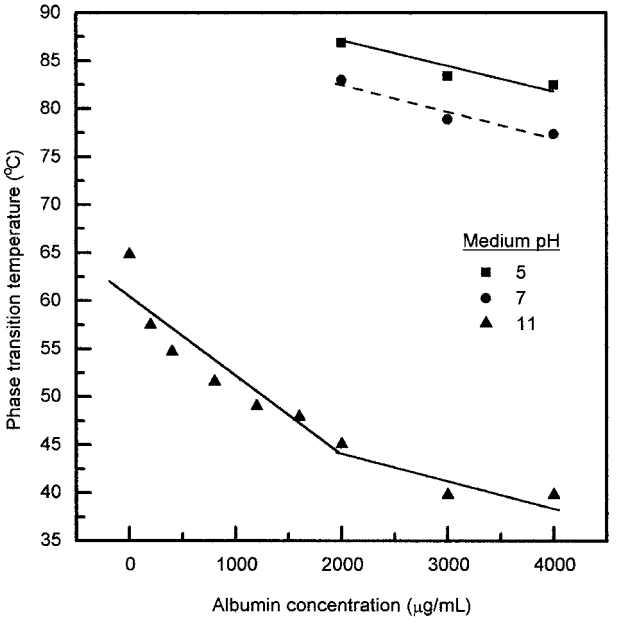

Figure 8 Variation of phase-transition temperature of the NIPA-*co*-DMAPM copolymer with albumin concentration (DMAPM/NIPA feed ratio: 11.7/88.3).

CONCLUSIONS

A random copolymer of NIPA and DMAPM exhibiting both temperature and pH sensitivity was synthesized. The produced copolymer was also sensitive to the albumin concentration in the aqueous media. In a certain range of albumin concentration, the phase-transition temperature

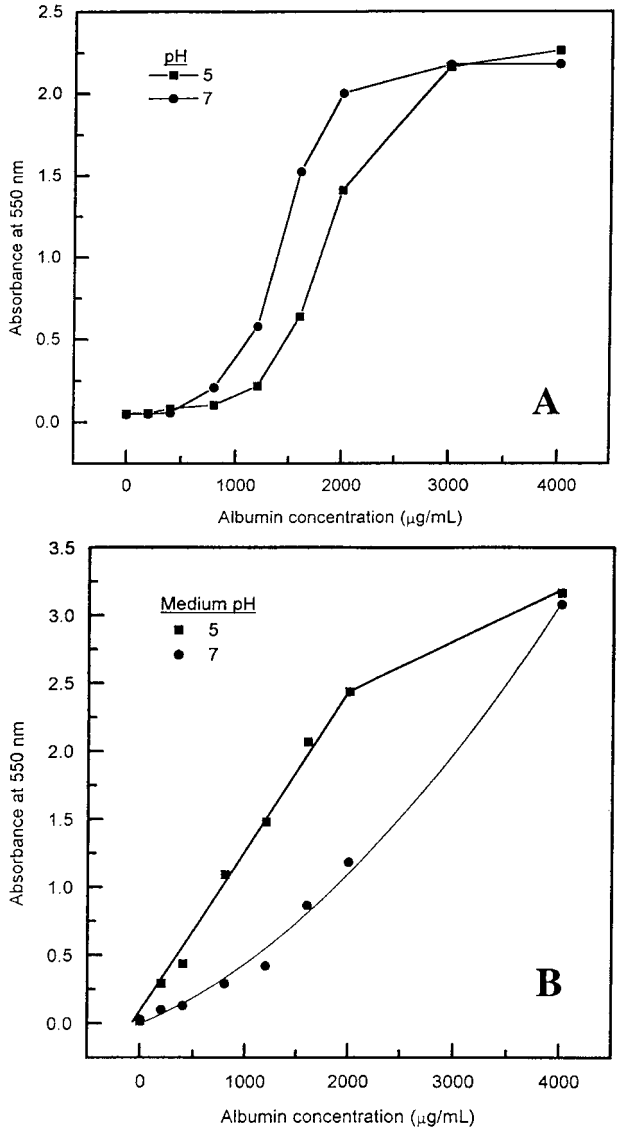

Figure 10 Effect of albumin concentration on the final absorbance of the aqueous medium measured by the completion of phase transition of NIPA-*co*-DMAPM copolymer in the presence of albumin. DMAPM/NIPA feed ratio (mol/mol): (A) 5.0/95.0, (B) 9.6/91.4.

of copolymer showed a linear change by the albumin concentration at different pH values. Although the Lowry method is one of the most customary analytical procedures used for the determination of protein concentration in the aqueous media, a new analytical method involving the use of a temperature- and pH-responsive polymeric reagent was proposed as an alternative to determine the albumin concentration in aqueous media. The method was developed based on the chemical interaction between the dimethylamino groups of the copolymer and the carboxyl groups of the protein molecule. For this purpose, albumin was selected as the model protein. For the generalization of the proposed method, the interactions of produced copolymer with other commonly used proteins such as fibrinogen, lactalbumin, and fibronectin are still under investigation.

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