Water-Solution Properties of a Hydrophobically Modified Poly(*N*-isopropylacrylamide)

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ABSTRACT: To study the water-solution properties of a hydrophobically modified poly(*N*-isopropylacrylamide) (PNIPAM) which is temperature-sensitive, the copolymer of *N*-isopropylacrylamide (NIPAM) and octadecyl acrylate (ODA) was synthesized. The aggregation behavior of the copolymer was studied by surface tension and fluorescence probe methods. Simultaneously, the phenomenon of the lower critical solution temperature (LCST) of the copolymer in an aqueous solution with increase of the temperature was also studied using the fluorescence probe method. The results showed that phase separation occurred in an aqueous solution of the copolymer when the temperature was increased to its LCST. The π-*A* isotherms for the copolymer molecules, as an insoluble monolayer on the water–air interface, was determined by the Langmuir–Blodgett (L–B) method. The abnormal phenomenon, by which the monolayer of the copolymer molecules became more and more condensed with increase of the temperature, was observed. It further indicated that phase separation of the copolymer occurred by another method. In addition, to prove the thermosensitive effect of the copolymer on the release behavior of liposomes, small unilamellar vesicles entrapped with 5(6)-carboxyfluorescein [5(6)-CF] were coated with the copolymer. We found that the coating of the copolymer resulted in the reduction of the release below 30°C and enhancement of the release above 30°C, indicating that there are obvious interactions between the copolymer and the liposomes. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 75: 247–255, 2000

Key words: water-soluble polymer; aggregation; phase separation; fluorescence probe

INTRODUCTION

The process of micelle formation of small molecular surface-active agents is usually characterized by surface tension and fluorescence methods. Jiang et al.[1,2] studied the process of micelle formation of surfactants in detail using the fluorescence probe method and discovered that both methods had good sensitivity. But with respect to the amphiphilic polymers which have surface activity, especially for the process of aggregate formation of temperature-sensitive hydrophobically modified poly(*N*-isopropylacrylamide) (PNIPAM), to our best knowledge, there are only a few reports of studies using the fluorescence probe method.[3–6]

The homopolymer of PNIPAM has its lower critical solution temperature (LCST) at 32°C (ref. 7) in aqueous medium. At LCST, PNIPAM exhibits a coil–globule transition. With respect to the LCST behavior of water-soluble polymers, there have recently been many reports. Generally, the

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incorporation of hydrophobic comonomers leads to a lower LCST, and hydrophilic comonomers, to a higher LCST.[8] The LCST behavior of the homopolymer PNIPAM macroscopically exhibits the following phenomenon: It is extremely soluble in water at room temperature, but it precipitates from the solution at 32°C.[7] This phenomenon is attributed to thermoreversible phase separation.

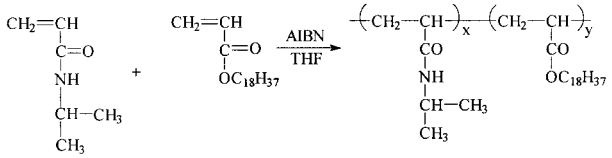
Recent studies have shown that hydrophobically modified PNIPAM has unique applications in drug-delivery systems.[9,10] In brief, the hydrophobic moiety can serve as the anchor of PNIPAM to insert into a model membrane. Once PNIPAM is safely anchored to a liposomal membrane, it can serve not only as a protective layer, but also as a thermosensitive modulator for the liposomes. Ringsdorf et al.[11,12] synthesized a series of hydrophobically modified PNIPAM which have several "anchoring points" in one macromolecule for liposomes; however, regarding hydrophobically modified PNIPAM which has a single anchoring point for liposomes, there are no reports to our best knowledge. By comparison to a polymer which has several anchoring points to insert into liposomes, a polymer with a single anchoring point has the following merits: (1) the number of the polymer segments which diffuse freely can be expected to be identical before and after macromolecular anchoring, and (2) it is expeditious to study the influence of the average molecular weight of a macromolecule on the properties of the protecting layer for liposomes.

In this work, the synthesis of PNIPAM containing one hydrophobic monomer per polymer chain was attempted. Additionally, to explore the thermosensitivity of the copolymer, the phase-separation behavior of hydrophobically modified PNIPAM in aqueous solution was also studied. There have been some reports that the phase-separation behavior of the homopolymer PNIPAM can be demonstrated by a variety of experimental techniques, including viscosity measurements,[13] osmometry,[7] light scattering,[14] and microcalorimetry.[15] In the present study, the fluorescence probe method and other methods were used to study the phase-separation behavior of the PNIPAM copolymer in aqueous solution. Furthermore, liposomes entrapped with 5(6)-carboxyfluorescein [5(6)-CF], made from soybean phosphatidylcholine (PC), are coated with the copolymer. The interaction between the copolymer and the liposomes was also studied.

EXPERIMENTAL

Synthesis and Characterization of the Copolymer of *N*-Isopropylacrylamide (NIPAM)–Octadecyl Acrylate (ODA)

The monomer of NIPAM was prepared using the method of Plaut and Ritter.[16] ODA was supplied by the Research Center of the Beijing Eastern Chemical Works (Beijing, China). NIPAM (10 mmol) and ODA (0.2 mmol) were dissolved in tetrahydrofuran (THF) (15 mL, freshly distilled from sodium). The concentration of the initiator azobisisobutyronitrile (AIBN) (3×10^{-2} mmol) added was selected to satisfy a suitable molecular weight of the product. The solution was stirred and heated to 60°C for 5 h under a N_2 atmosphere. After cooling to room temperature, the polymer was precipitated by diethyl ether (400 mL). Then, it was dissolved in THF (40 mL) and reprecipitated from diethyl ether (400 mL) several times and a white powder polymer was obtained. The reaction is presented as follows:



The chemical composition of the copolymer was determined from a careful analysis of the ^{1}H-NMR spectra of the $CDCl_3$ solution, which was recorded using a DPX400 nuclear magnetic resonance spectrometer. The ratio of isopropyl to *n*-octadecyl was calculated from the area of the peak at 4.0 ppm, which is assigned to the C-2 proton of the isopropyl groups, and the area of the peak at 0.8 ppm, which is attributed to the terminal methyl proton of the *n*-octadecyl chains[11] (Fig. 1). The monomer ratio of the product calculated from the spectra recorded was 64:1, which exceeds the monomer feed ratio about 20%.

The weight-average molecular weight (M_w), the number-average molecular weight (M_n), and the heterogeneity index, M_w/M_n, were determined to be 5198, 2912, and 1.79, respectively, using a Waters 150C GPC with measurements run in THF and calibrated with polystyrene standards.

According to the calculation from the composition and the average molecular weight of the synthesized copolymer, it can be considered that there was only one hydrophobic comonomer used as an "anchoring point" in the obtained polymer

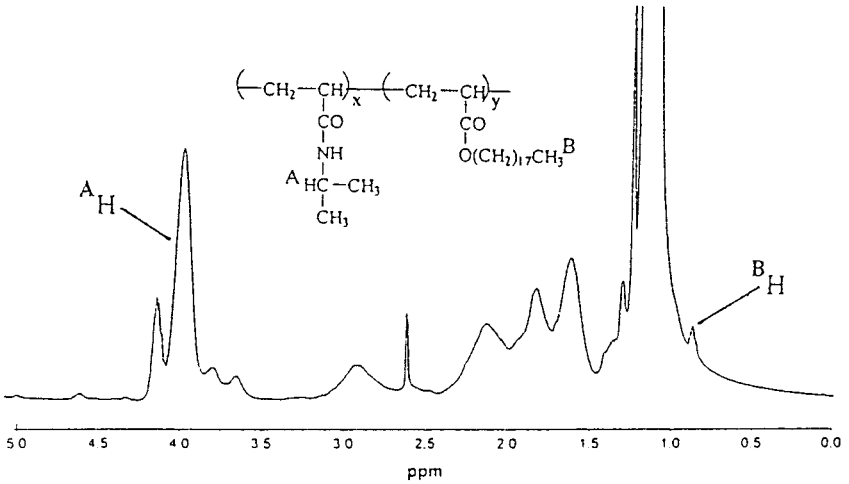


Figure 1 ^{1}H-NMR spectrum of the NIPAM–ODA copolymer in $CDCl_3$.

chain. It can be favorable for the study of a polymer's interaction with liposomes as described above. It was also demonstrated that the amount of initiator used in this work was suitable.

Surface Tension Determination

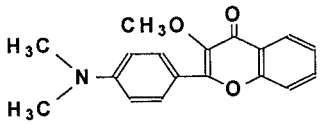
The copolymer was dissolved in deionized water to prepare solutions with different concentrations. The surface tension of the copolymer aqueous solution was measured by the sessile drop method at the room temperature of 25°C. The drop numbers for the standard solvent of deionized water and the aqueous solutions of the copolymer passing through the given graduation were both measured. The drop rate was controlled at 5 s per drop in all cases. The formula for the calculation of the surface tension is the following[17]:

$$\sigma_1/\sigma_2 = N_2 d_1/d_2 N_1 \quad (1)$$

where σ_1 and σ_2 are the surface tension of water and the copolymer aqueous solutions, respectively; N_1 and N_2, the drop numbers of water and the copolymer aqueous solutions in a definite volume, respectively; and d_1 and d_2, the specific gravity of water and the copolymer aqueous solutions, respectively. Because dilute solutions of the copolymer were determined, d_1 and d_2 are considered to be equal. The surface tension of the copolymer aqueous solution was calculated with the given value of the surface tension of water (71.97 mN/m) at room temperature.

Fluorescence Probe Compound

3-Methoxy-4′-*N*,*N*-dimethylaminoflavone derivatives (DMMF) was used as the fluorescence probe in this work. Its molecular structure is expressed as follows:



(DMMF)

DMMF was synthesized according to the method of Wang.[18] It has the following feature: There is a very good linear relationship between the relative emission intensity of the fluorescence spectra of DMMF and the polarity parameter—Dimroth E_T (30)[19] with different solvents, which indicates that the fluorescence spectra of DMMF can change sensitively with the change of solvent polarity. Therefore, DMMF can be used as a fluorescence probe to detect the media's polarity and then to deduce the change of macromolecular conformation in the solution.

Fluorescence Spectra Determination

DMMF was dissolved in methanol at the concentration of 2.5×10^{-3} mol/L. The copolymer was dissolved in water to prepare solutions with concentrations ranging from 0.01 to 1 mg/mL. Then, DMMF was added to the aqueous solution of the copolymer to give a final concentration of 2.5

$\times 10^{-6}$ mol/L in all cases. The two components were mixed under ultrasonic vibrations for 5 min. DMMF's emission spectrum was obtained using a Hitachi MPF-4 fluorescence spectrophotometer at the room temperature of 25°C. The probe was excited at 380 nm and the emission spectrum was recorded over the range of 400–640 nm. The excitation and the emission slit openings were 12 nm without exception.

Determination of π-A Isotherms

The isotherms of the surface pressure (π) versus area (A) for the copolymer of NIPAM–ODA were measured on a Langmuir balance, Lauda FW2(FRG). (The area of the bath was 248 cm^2.) The copolymer was dissolved in chloroform, which was freshly distilled, to prepare the solution whose concentration was 0.025 mg/mL. Then, 0.3 mL of the copolymer solution was introduced at the water–air interface. After chloroform evaporation, the monolayer was compressed up to 30 cm^2 of the bath area at the compression rate of 0.1 cm^2/min. The π-A isotherms at different temperatures could be achieved by changing the temperature of the system.

Preparation of PC Liposomes Coated with NIPAM–ODA Copolymers

PC was purchased from the Microorganism Culture Medium Products Refinery of the Haidian District, Beijing City, China. It was purified according to the method described by Singleton et al.[20] 5(6)-CF was a kind gift from Professor F. M. Li (Faculty of the College of Chemistry and Molecular Engineering, Peking University, Beijing, China). A solution of PC in chloroform was put into a 50-mL round-bottomed flask. Then, the flask was transferred to a rotary evaporator and the solvent was removed under a reduced pressure at 30°C. The resultant thin film of PC on the walls of the flask was dried under the reduced pressure at room temperature for at least 2 h. The dry PC film was dispersed in 50 mmol/L 5(6)-CF solution, which was prepared using phosphorous buffer saline (PBS) at pH 7.4. Then, the system was sonicated for 30 min using a CQ250 water-bath-type sonicator (from Shanghai Ultrasonic Wave Instrument Works, China, 220 V, 50 Hz) to obtain the small unilamellar vesicles (SUV).[21] The copolymer was dissolved in PBS to prepare an aqueous solution with a concentration of 2 mg/mL. According to the weight ratio of the copolymer to PC at 1 : 1, the copolymer solution was added to the liposome suspension and incubated at 5°C for 2 h with stirring. Free 5(6)-CF and the free copolymer were removed by gel permeation chromatography on a Sephadex G-50 column at room temperature using PBS as an eluting agent.

5 (6)-CF Release from Liposomes

5(6)-CF was used as an aqueous marker entrapped in the liposomes in this present study. It has the following property[22]: In a dilute solution, its fluorescence intensity is in direct proportion to its concentration; however, when its concentration is increased to 10 mmol/L or above, such that it was entrapped in liposomes, its fluorescence self-quenches. This property can be used to determine the permeability of the liposomal membrane. Generally, the percent release of 5(6)-CF from the liposomes is defined as

$$\% \text{ release} = 100[I_f - I_0]/[I_\infty - I_0] \qquad (2)$$

where I_f and I_0 are the initial and intermediate fluorescence intensities of the liposome suspension, respectively. I_∞ is the fluorescence intensity after the addition of Triton X-100 (final concentration 0.3%) to destroy the liposomal membrane completely. The fluorescence intensity of the 5(6)-CF release from the liposomes was determined using a Hitachi MPF-4 fluorescence spectrophotometer. The excitation and monitoring wavelengths were 490 and 520 nm, respectively, and the exitation and emission slit openings were 6 nm without exception.

RESULTS AND DISCUSSION

Aggregation Behavior of the Copolymer

The NIPAM–ODA copolymer is estimated to have the property of amphiphilic molecules according to its structure. So, it is necessary to study its surface activity in aqueous solution. Figure 2 shows the isotherm of the surface tension of the copolymer aqueous solution versus its concentration. According to Figure 2, the surface tension decreased with increase of the copolymer concentration. When its concentration was increased to 0.093 mg/mL, the surface tension was kept at 46.7 mN/m. This indicates that the copolymer in aqueous solution has surface activity and copolymer aggregates at or above the concentration of 0.093

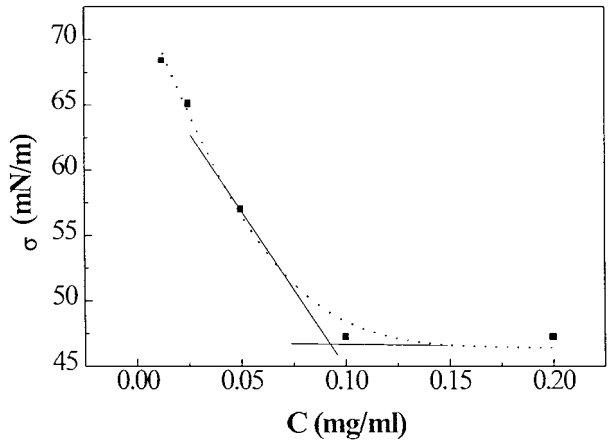


Figure 2 Change of surface tension of the NIPA–ODA copolymer in aqueous solution as a function of its concentration.

mg/mL, which can be thought of as a critical concentration of aggregation (CAC). It is analogous with the formation of micelles.

Figure 3 shows the fluorescence spectra of DMMF in aqueous solutions of the NIPAM–ODA copolymer of different concentrations. It can easily be seen that the fluorescence intensity (I_f) of DMMF at 485 nm increased gradually with increase of the copolymer concentration. If the plot was made using the changes of the I_f of DMMF as a function of the copolymer concentration (Fig. 4), it was found that the I_f of DMMF at 485 nm increased gradually with increase of the copolymer concentration. But one needs to be aware that the rate of increase for I_f was different at different concentration ranges: The increasing tendency at lower concentrations was higher than that at higher concentrations. So, there is an inflexion point observed at the concentration of 0.108 mg/mL from Figure 4. The concentration corresponding to the inflexion point is very close to the CAC value measured by the surface tension method above.

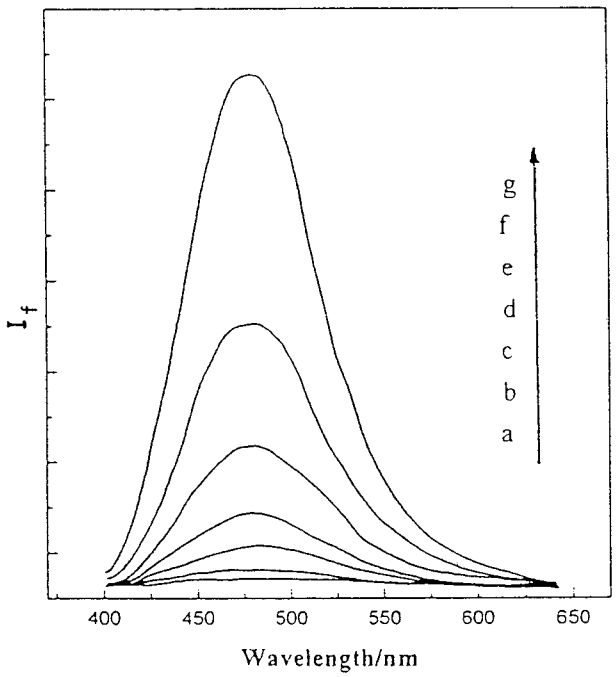


Figure 3 Fluorescence spectra of DMMF in the aqueous solution of the NIPAM–ODA copolymer with concentrations of (a) 0.0125 mg/mL, (b) 0.025 mg/mL, (3) 0.05 mg/mL, (d) 0.1 mg/mL, (e) 0.2 mg/mL, (f) 0.4 mg/mL, and (g) 0.8 mg/mL.

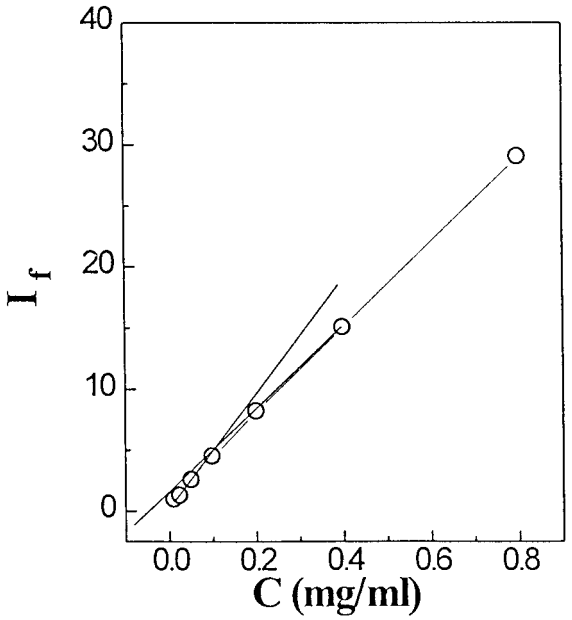


Figure 4 Change of I_f of DMMF at 485 nm with concentration of the NIPAM–ODA copolymer in aqueous solution.

From the results shown above, it is thus clear that the amphiphilic polymer's CAC value measured by the fluorescence probe method is no clearer than that measured by the surface tension method. It could be considered that using the fluorescence probe method to determine the critical concentration value of micelle formation is suitable for small molecular surface active agents; however, it is not very sensitive to determine the CAC value of macromolecular amphiphiles. This result can be interpreted as follows: The probe selected for this study is very sensitive

to the polarity of the surrounding media. This is because the hydrophobic probe used in this work prefers to embed in the aggregates rather than in the aqueous media. Thus, it is evident that, while the aggregates form, the environment polarity of the probe will change dramatically; concurrently, the I_f of the probe will also change. At much lower copolymer concentrations, the probe can be attached to the copolymer chain, and the environment of the probe becomes more and more hydrophobic with increase of the copolymer concentration before the CAC. On the other hand, after the CAC is reached, the structure of the aggregates might not be considered to be fixed; its compactness will be improved gradually with increase of the copolymer concentration. All this leads to the gradual increase of its I_f with increase of the copolymer concentration and makes the sharpness at the inflexion point unclear.

It is well known that the homopolymer PNIPAM does not have amphiphilic properties. However, from the results in this present study, it is very interesting that the introduction of the very low level hydrophobic moiety of ODA to the polymer backbone will lead to a very dramatic effect: It shows an obvious amphiphilic property and can self-assemble with increase of its concentration.

As is well known, the homopolymer PNIPAM tends to be more hydrophobic than is polyacrylamide (PAM). Thus, PNIPAM in aqueous solution can release bound water at a higher temperature, exhibit self-assembly behavior due to hydrophobic interaction of each molecular chain, and present an LCST phenomenon that will be discussed in the following section. However, PAM does not have the LCST phenomenon because of its much stronger hydrophilicity, so that, if a hydrophobic long-chain alkyl is introduced into PNIPAM chain (although the added amount is quite limited), the copolymer will shift to be so hydrophobic that it is able to behave well as an insoluble film in the Langmuir–Blodgett (L–B) experiment (see Determination of π-A Isotherms section).

Phase-separation Behavior of the Copolymer Studied by Fluorescence Probe

Figure 5 shows the profile of the I_f changes of DMMF at 485 nm as a function of temperature of the copolymer water solution. It is obviously found that the I_f of DMMF at 485 nm increases suddenly at 30°C, indicating that the phase separation occurred at this temperature, which can be thought of as the LCST of the copolymer.

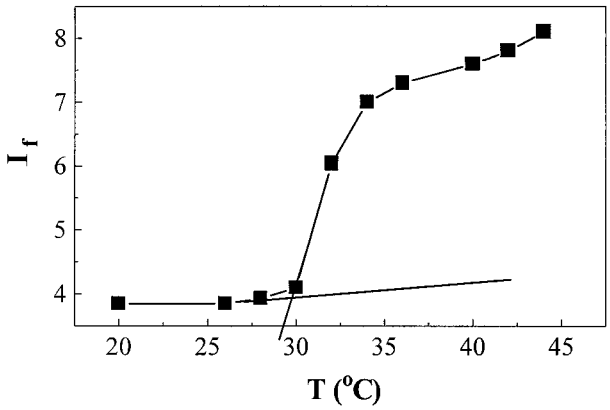


Figure 5 Change of I_f of DMMF at 485 nm with temperature of the NIPAM–ODA copolymer solution.

With respect to the phase-separation phenomenon for the copolymer aqueous solution, the following discussion is given: At temperatures below its LCST, the molecules of the copolymer in water are extended, presumably as a result of hydrogen bonding between the amide groups of the copolymer and the surrounding water molecules.[15] When the solution temperature is increased to or above its LCST, hydrogen bonds are broken and bound water is released. These changes result, on a macroscopic scale, in the separation of a polymer-rich phase, concomitant, on the molecular level, with a collapse of the polymer chains from expanded coils to a more compact conformation of a globule. Because the probe selected for this study is very sensitive to the polarity of the surrounding media as discussed above, at temperatures below the LCST, the probe molecules can be attached to the expanded coil structure of the copolymer containing water molecules linked by hydrogen bonds. This leads to their much lower I_f. Furthermore, their I_f show no evident change. But when the temperature is increased to or above its LCST, a more compact conformation of the copolymer forms. DMMF is inclined to get into the compact structure of the copolymer that has very weak polarity, due to its strong lipophilicity. This induces a sudden increase of the I_f of DMMF. With continuous increase of the temperature, the polymer chains become much more compact, which causes a continuous increase of the I_f of DMMF. In summary, using the fluorescence probe method to measure the CAC of the copolymer is not more sensitive than is using surface tension method, but it can directly reflect the phase-separation course of the copolymer in aqueous solution.

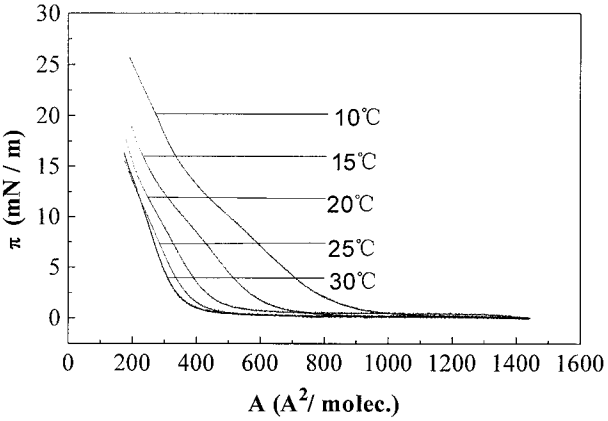


Figure 6 π-*A* isotherms of the NIPAM–ODA copolymer at different temperatures.

π-A Isotherms of the Copolymer Monolayer

Amphiphilic molecules, which are insoluble or have weak solubility, can form an insoluble monolayer at the water–air interface with the aid of a certain external force. Using the L–B technique[23] to measure the change of the force as a function of the area of the monolayer, one can obtain the curve of the surface pressure of the monolayer as a function of the area of an amphiphilic molecule, namely, the π-*A* isotherm. It gives the relationship between the pressure and area of two-dimensional space, which are the most important data for a property study of the insoluble monolayer. The NIPAM–ODA copolymer has good surface activity and a certain insolubility, so the phase-separation course can be studied by determining the π-*A* isotherm of the insoluble monolayer of the copolymer.

As is well known, the state, property, and structure of the insoluble monolayer is affected by the temperature. Generally, with increase of the temperature, the degree of molecular movement becomes greater and the surface vapor pressure of the monolayer increases, which results in the formation of the more expanded monolayer.[24] But it is very interesting that the monolayer formed by the NIPAM–ODA copolymer is contrary to the normal observation. Figure 6 shows the π-*A* isotherms of the monolayer of the NIPAM–ODA copolymer as a function of temperature. At 10°C, the monolayer appears more expanded, and there is a transition region between the expanded region and the condensed region. When the temperature is increased to 25°C, the transition region disappears and the monolayer becomes more condensed.

To our best knowledge, there have been no reports relative to this abnormal phenomenon, but in view of the phase-separation behavior of the amphiphilic polymer at higher temperature in this present study, the abnormal phenomenon can be explained reasonably. At lower temperature, hydrogen bonds between the amide groups of NIPAM–ODA copolymer and surrounding water molecules form, which results in increase of the hydrophilic head groups' volume of the copolymer molecules at the water–air interface. Thus, the average area occupied per copolymer molecule increases, and the interaction of the copolymer molecules at the water–air interface in the lateral direction decreases; therefore, the monolayer appears more expanded. With increase of the temperature, the hydrogen bonds between the amides groups of the copolymer and surrounding water molecules are broken gradually. The binding ability between the hydrophilic head groups of the copolymer and water molecules becomes weaker, whereas the hydrophobic section of the copolymer at the water–air interface increases gradually. Thus, the average area occupied per copolymer molecule decreases, and the interaction between the copolymer molecules at the water–air interface in the lateral direction is strengthened. In this way, the monolayer of the copolymer becomes more condensed. It demonstrates that the L–B technique can also provide evidence for the phase-separation phenomenon of the NIPAM–ODA copolymer at higher temperature.

Interaction Between the Copolymer and Liposomes

In this work, the temperature-dependence of the release of 5(6)-CF from copolymer-modified PC liposomes was investigated. The copolymer-modified liposomes and the unmodified liposomes containing 5(6)-CF released essentially the same amount of 5(6)-CF after the addition of Triton X-100. These facts demonstrated that the coating of the liposomes with the copolymer does not affect the encapsulation of 5(6)-CF in the liposomes.

Figure 7 represents the percent release of 5(6)-CF from the copolymer-modified and the unmodified PC liposomes after 30 s as a function of temperature. It is apparent that coating of the copolymer on the liposome surface results in reduction of the release below 30°C and enhancement of the release above 30°C. This temperature agrees well with the LCST of the copolymer.

Regarding the above fact, the following discussion is given: At temperatures below the LCST of

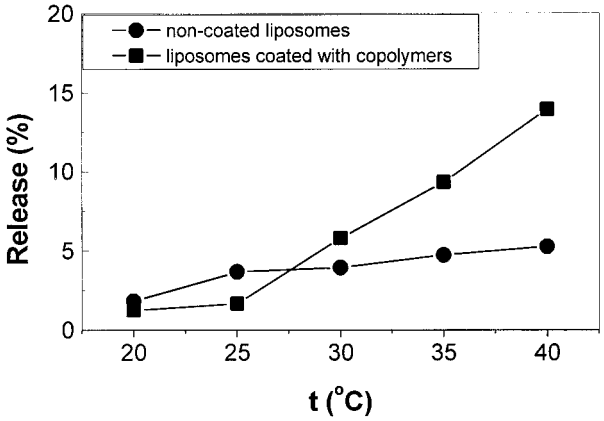


Figure 7 Percent release of 5(6)-CF from the copolymer-coated PC liposomes and the noncoated liposomes as a function of temperature in PBS at pH 7.4. Percent release after 30 s is shown.

the copolymer, the octadecyl chains of the copolymer interact strongly with the liposomal membrane by hydrophobical interactions, which induces the close attachment of the copolymer on the liposome surface. This then results in the stabilization of the liposomes. However, when the temperature is increased to or above the LCST of the copolymer, the copolymer exhibits a coil–globule transition, which produces the contraction of the copolymer. The giant contraction stress induces the disruption of the liposomes, so, then, 5(6)-CF can be released from the liposomes rapidly. Also, the coating of the hydrophobically modified PNIPAM containing only one anchoring point in about one polymeric molecule has enough ability not only to play a protective role for liposomes below 30°C, but also to improve the release rate of the water-soluble marker from the liposomes when the temperature was increased to above 30°C.

CONCLUSIONS

From the above results, the following conclusions can be drawn:

1. The water-solution properties of the amphiphilic copolymer NIPAM–ODA were studied by both surface tension and fluorescence probe methods. The results of both methods show that the copolymer in aqueous solution has obvious aggregation behavior with increase of its concentration. It is evident that the introduction of a very low level of ODA to the polymer backbone will lead to a very dramatic effect on the amphiphilic properties of the polymer studied.
2. The phase-separation behavior of the copolymer in aqueous solution with increase of the temperature was studied using the fluorescence probe and L–B methods. The results demonstrated that phase separation occurred in the aqueous solution of the copolymer when the temperature was increased to 30°C. The result from the L–B determination further indicated that phase separation of the copolymer occurred with increase of the temperature from another angle.
3. Liposomes coated with the copolymer have obvious thermosensitive release behavior. It indicates that the copolymer containing approximately one hydrophobic monomer per polymer chain has strong interaction with liposomes and can serve as a thermosensitive modulator to control their release behavior.

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