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Fluorescence Spectroscopy Studies on Micellization of Poloxamer 407 Solution

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It has been reported that at low temperature region, poloxamers existed as a monomer. Upon warming, an equilibrium between unimers and micelles was established, and finally micelle aggregates were formed at higher temperature. In this study, the fluorescence spectroscopy was used to study the micelle formation of the poloxamer 407 in aqueous solution. The excitation and emission spectra of pyrene, a fluorescence probe, were measured as a function of the concentration of poloxamer 407 and temperature. A blue shift in the emission spectrum and a red shift in the excitation spectrum were observed as pyrene transferred from an aqueous to a hydrophobic micellar environment. From the I_1/I_3 and I_{339}/I_{333} results, critical micelle concentration (cmc) and critical micelle temperature (cmt) were determined. Also, from the fluorescence spectra of the probe molecules such as 8-anilino-1-naphthalene sulfonic acid and 1-pyrenecarboxaldehyde, the blue shift of the λ_{max} was observed. These results suggest a decrease in the polarity of the microenvironment around probe because of micelle formation. The poloxamer 407 above cmc strongly complexed with hydrophobic fluorescent probes and the binding constant of complex increased with increasing the hydrophobicity of the probe.

Key words: Poloxamer 407, Micelle, CMC, Complexation, Fluorometry

INTRODUCTION

Poloxamer 407 is a polymer that consists of one lipophilic and two hydrophilic blocks. This material is non-toxic and has a high solubilizing capacity. Consequently poloxamer 407 has been widely used as a controlled drug carrier (Shin *et al.*, 2000; Yong *et al.*, 2003).

It is well established that, at low temperature region, poloxamers existed as a monomer. Upon warming, an equilibrium between monomers and micelles was established, and finally aggregates were formed at higher temperature (Alexandridis and Hatton, 1995; Schillen *et al.*, 1993). And these micelles were spherical and consisted of a polyoxypropylene (PPO) core with a polyoxyethylene (PEO) shell. This conformation was attributed to the fact that PPO was poorly water soluble and PEO was highly soluble in aqueous solvent (Mortensen, 1993; Zhou and Chu, 1988). Temperature had a significant influence on micelle formation. In general, poloxamer began to form a micelle at critical micelle temperature (cmt) (Mortensen and Pedersen, 1993). These cmc and cmt values were determined by UV spectroscopy (Gaisford *et al.*, 1998), light scattering (Yu *et al.*, 1992) and surface tension (Cho *et al.*, 1997). Especially, fluorescence spectroscopy has been applied to the determination of cmc, micellar size, micropolarity and microviscosity using fluorescent probes. The fluorescence properties of probes such as intensities, intensity ratios, lifetimes, quenching and polarization vary depending on physical parameters of the micelle and micellar aggregates (Vasilescu *et al.*, 2001; Zana and Lang, 1990).

Fluorescence was sufficiently sensitive and, thus, the formation of micelles at low concentration of poloxamer may be readily detected. In this study, we used fluorescence probe techniques to study the micelle formation of the aqueous poloxamer 407 solution. We were particularly interested in the effects of polymer concentration and temperature on the fluorescence of pyrene on the micellar formation. In addition, the complexation of poloxamer 407 micelle with several fluorescent probes were investigated in order to study the effect of the hydrophobicity of the probe on the association in micelles.

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MATERIALS AND METHODS

Materials

In this study, six different fluorescent probes, viz, pyrene, 8-anilino-1-naphthalenesulfonic acid (ANS), 2-(*p*-toluidino)-naphthalene-6-sulfonic acid (TNS), *N*-phenyl-1- naphthylamine (PNA), [5-dimethylaminonaphthalene-1-sulfonamidoethyl] trimethyl ammoniumperchlorate (DASP) and 1-pyrenecarboxaldehyde (PyCHO) were studied. All probes were obtained from Sigma Chemical Co. (USA) and poloxamer 407 with the molecular weight of 12,000 was received from BASF (Germany). Other reagents were of analytical grade and were used without further purification.

Preparation of sample solutions

Poloxamer 407 was added into water at about 4°C and stirred gently. For water soluble guest molecules such as ANS, TNS and DASP, 0.01 mL of the stock solution of probe molecule was mixed with 0.99 mL of the poloxamer solution. Pyrene, PNA and PyCHO were dissolved in acetone because of the poor solubility in water. An aliquot (0.01 mL) of pyrene (6.0×10^{-5} M), PNA (1.0×10^{-4} M) or PyCHO (1.0×10^{-4} M) in acetone was added to a vial and the solvents were evaporated to form a thin film at the bottom of the vial. Poloxamer stock solution was added and the resulting mixture was kept at 40°C for 1 h.

Fluorescence measurements

Complexation between the poloxamer 407 and fluorescent probe molecules was investigated. The fluorescent intensity was determined as a function of the poloxamer concentration or temperature. Fluorescence spectra of pyrene were obtained at the excitation wavelength λ_{ex}= 339 nm and the emission wavelength λ_{em}=390 nm using a spectrofluorometer (Jasco FP 777, Japan). Emission spectra were obtained at λ_{ex}=365.5 nm for PyCHO and λ_{ex}=380 nm for ANS. The cmc and the cmt values of the poloxamer were estimated from the spectra. To obtain the binding constant (*K*) between a fluorescent probe molecule and the poloxamer 407, fluorescence spectra were measured by excitation at 340 nm for PNA, at 380 nm for ANS, at 330 nm for TNS, and at 350 nm for DASP on fluorometer equipped with a thermoregulated cell compartment. The binding constant was calculated by the Benesi-Hildebrand relationship such as

$$\frac{A_0}{A}=\frac{1}{\varepsilon}+\frac{1}{K\varepsilon[Poloxamer]},$$

where A and A_0 are the relative fluorescence intensity in the presence (A) and absence (A_0) of a given amount of poloxamer, respectively. K is the binding constant and ε is the fluorescent intensity when it is considered that all fluorescent probes are complexed with the poloxamer. The plot of A_0/A vs 1/[Poloxamer] yielded a straight line. From the slope of the straight line, K was obtained, while ε was obtained from the intercept on the vertical axis.

RESULTS AND DISCUSSION

Fluorescence spectroscopy of pyrene in poloxamer 407 solutions

In the literature, it has been indicated that pyrene was an effective fluorescence probe because of the long life time of pyrene monomers and its ability to form efficient eximers (Kalyanasundaram and Thomas, 1977). The fluorescence spectrum of the pyrene molecule yielded information about the polarity sensed by pyrene in its solubilization site. In particular, the fluorescent intensity of the third vibrational peak of pyrene was quite sensitive to the solvent polarity.

Fig. 1 showed the emission spectra of pyrene in the presence of varying concentrations of poloxamer 407. These spectra indicated that the intensity increased with increasing poloxamer concentration.

Fig. 2 showed the excitation spectra of pyrene in the presence of varying concentrations of poloxamer 407. A red shift was observed in the excitation spectrum with increasing concentrations of poloxamer 407. Specifically, the (0,0) band for pyrene, which was at 333 nm in water, has been shown to shift to 339 nm upon the addition of poloxamer 407 block copolymer. As shown in Fig. 2, pyrene in water had only a very small absorption at 339 nm, while it increased substantially upon transfer to the less polar micelle domain, similar to that described in the literature (Wilhelm *et al.*, 1991).

Intensity ratio and total fluorescent intensity as a function of concentration

In general, the vibrational fine structure may change if

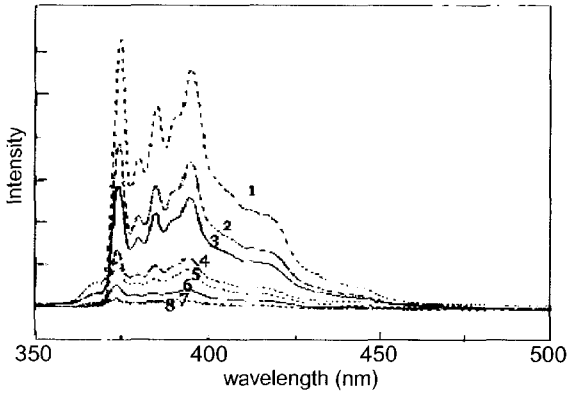

Fig. 1. Emission spectra of pyrene as a function of poloxamer 407 concentration in water. Concentration of poloxamer 407 was 1) 1.66×10^{-2} M, 2) 1.45×10^{-2} M, 3) 8.32×10^{-3} M, 4) 8.32×10^{-4} M, 5) 4.17×10^{-4} M, 6) 5.89×10^{-5} M, 7) 8.31×10^{-7} M and 8) 0 M.

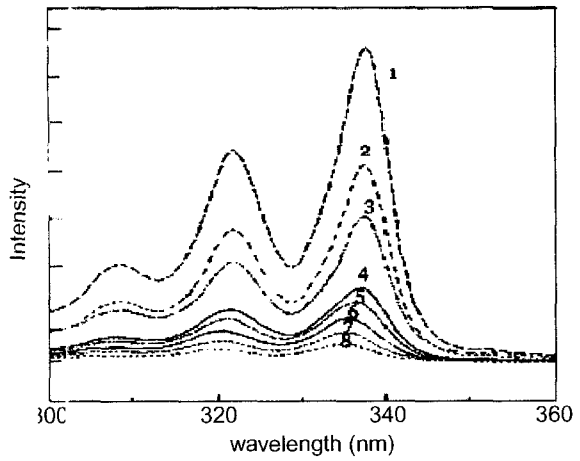

Fig. 2. Excitation spectra of pyrene as a function of poloxamer 407 concentration in water. Concentration of poloxamer 407 was 1) 1.66×10^{-2} M 2) 1.45×10^{-2} M, 3) 8.32×10^{-3} M, 4) 8.32×10^{-4} M, 5) 4.17×10^{-4} M, 6) 5.89×10^{-5} M, 7) 8.31×10^{-7} M and 8) 0 M.

the environment of pyrene is changed from polar to nonpolar states (Kalyanasundaram and Thomas, 1977). This change was described in terms of the ratio I_1/I_3, the intensities of the first and third bands in the pyrene fluorescence spectrum, respectively. Since both the fluorescence life time and the I_1/I_3 ratio in the vibronic band intensities were the function of the environment around the probe, both τ_f and the I_1/I_3 ratio showed sharp breaks at the cmc (Kalyanasundaram and Thomas, 1977). This indicated the onset of micellization at this concentration. Fig. 3 showed that the ratio of I_1/I_3 peak height decreased sharply in a sigmoidal manner over the certain concentration range at various temperatures and remained constant thereafter. The I_1/I_3 ratio was very helpful for determining the location of the pyrene probe in the micelles and local polarity. The ratio I_1/I_3 was high in polar media, for example, I_1/I_3= 1.81 in water. The ratio decreased with decreasing polarity as I_1/I_3=1.12 for pyrene solubilized in sodium dodecylsulfate micelles (Kalyanasundaram and Thomas, 1977). Generally, in the case of poloxamer, monomolecular micelles formed initially, and polymolecular micelles formed at higher concentrations. As the concentration increased further, the PPO segments continued to dehydrate, causing a progressive decrease in the polarity around pyrene. Eventually, the dehydration was sufficient to form micelles at the inflection point in the curve, which represents the cmc. The further decrease in I_1/I_3 could be attributed to the continued expulsion of water from the micelle interior, making it more nonpolar (Bohorquez *et al.*, 1999).

Another parameters by which cmc values could be measured were the intensity ratio of the excitation spectra and total fluorescent intensity. Fig. 4 illustrated a plot of red shift in the excitation spectrum, i.e., $I_{339/333}$ ratio. At low concentrations of poloxamer 407, negligible changes in the shifts in the excitation spectrum and total fluorescent intensity were observed. As the concentration of poloxamer 407 was increased at a certain polymer concentration (i.e., cmc), the total fluorescent intensity and red shift in the excitation spectrum increased dramatically in a sigmoidal manner. From the Figs. 3 and 4, the estimated cmc values were 5×10^{-4} M at 20°C and 2×10^{-6} M at 37°C.

The cmt determination by fluorescence intensity ratio

Poloxamer solution exhibited three regions such as monomer, transition and micellar region as temperature changed (Zhou and Chu, 1988). The cmt is the critical solution temperature at which micelles form. This was attributed to a temperature induced change in the solution micropolarity (Alexandridis and Hatton, 1995). As the temperature was raised, I_1/I_3 decreased abruptly, signaling transfer of the probe into the precipitated polymer phase (Fig. 5). At temperatures higher than the cmt, the I_1/I_3 ratios decreased

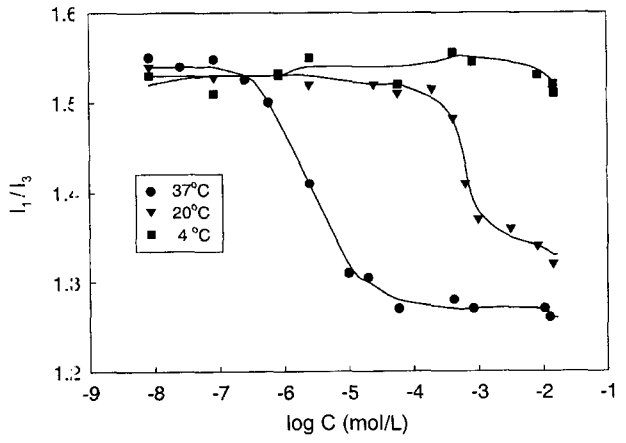

Fig. 3. Intensity ratio I_1/I_3 of the vibrational bands in the emission spectrum as a function of poloxamer 407 concentration.

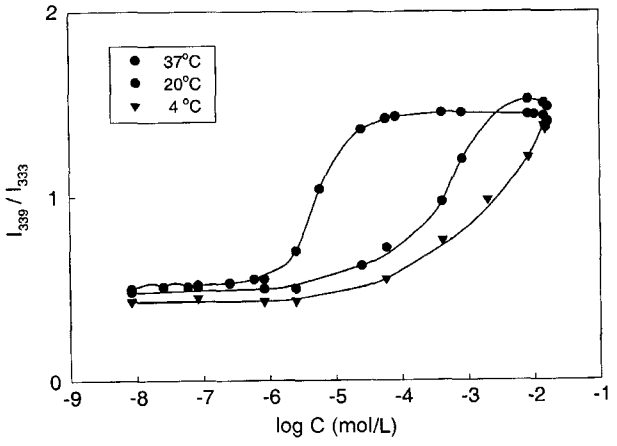

Fig. 4. Intensity ratio I_{339}/I_{333} from the excitation spectrum of pyrene as a function of poloxamer 407 concentration.

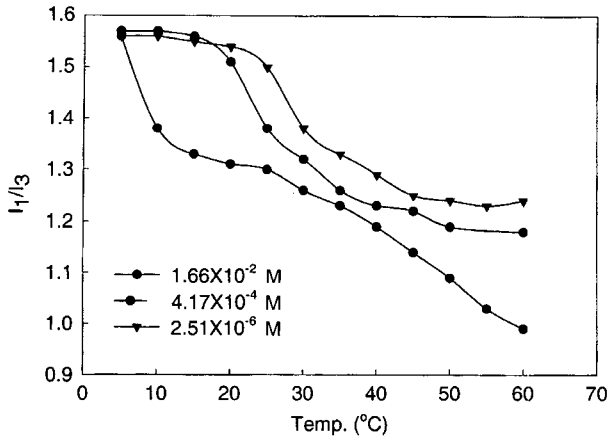

Fig. 5. Intensity ratio I_1/I_3 of the vibrational bands in the emission spectrum as a function of temperature.

linearly with increasing temperature. For aqueous poloxamer solutions, it is generally believed that the dehydration and subsequent aggregation of the less polar PPO block with increasing temperature are primarily responsible for micelle formation. As the temperature raised further, the more polar PEO also progressively dehydrated, ultimately resulting in phase separation at a lower critical solution temperature (Pandit *et al.*, 2000). At low temperatures, below the cmt, micelles were not present and the I_1/I_3 ratio was high, indicating an aqueous environment around pyrene. As the temperature was increased, the I_1/I_3 ratio drops abruptly as micelles are formed and the pyrene partitions into the more hydrophobic interior of the micelles (Pandit *et al.*, 2000). I_{339}/I_{333} intensity ratio increased as the temperature increased (Fig. 6). From Figs. 5 and 6, cmt was determined. The cmt decreased as the concentration of poloxamer 407 solution increased. The cmt was 38°C at 2.51×10^{-6} M, 24°C at 4.17×10^{-4} M and 7°C at 1.66×10^{-2} M. These results were similar with other result i.e., 25.8°C at 5 mg/mL (=4.17×10^{-4} M) by light scattering method (Pandit *et al.*, 2000).

The changes of emission maximum wavelength of fluorescent probe were indicative of changes in the polymer environment around probe. The cmt values were also determined from λ_{max} of fluorescent probe molecules such as ANS and PyCHO. ANS fluoresced weakly in polar media and strongly in nonpolar solvents. Transfer of the probe from polar to nonpolar media was also accompanied by a pronounced blue shift. We observed a marked blue shift, suggesting a decrease in the polarity of the microenviornment of the probe (Fig. 7). Fluorescence in poloxamer 407 solutions above cmc (8.33×10^{-3} M/L) was initially weak at low temperature, but increased rapidly to a peak at 18°C (cmt), and decreased with increasing temperature thereafter. The position of the PyCHO emission maximum, λ_{max} varies linearly with the dielectirc constant greater than 10, and that the red shift with increasing solvent polarity is accompanied by an increase in the fluorescence quantum

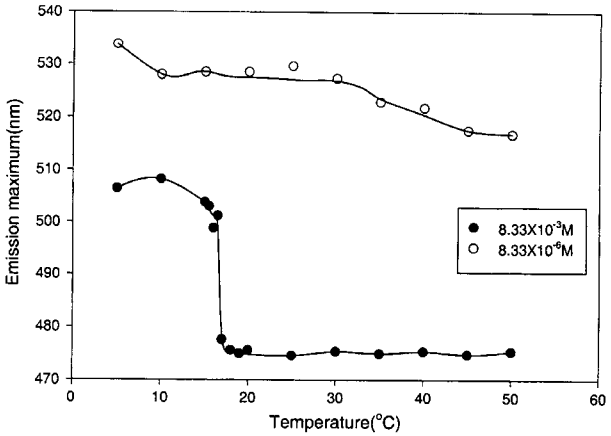

Fig. 7. Emission maxima of ANS in poloxamer 407 as a function of temperature.

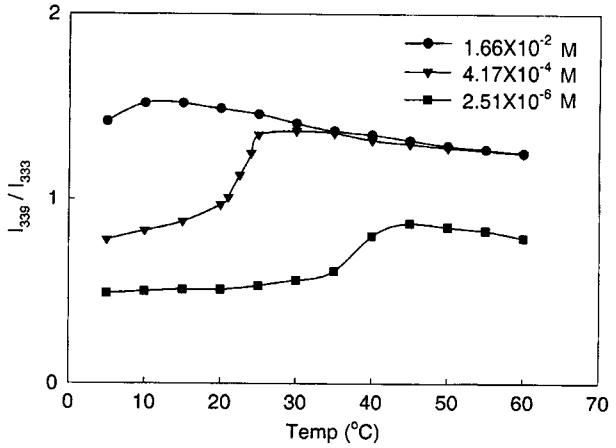

Fig. 6. Intensity ratio I_{339}/I_{333} from the excitation spectrum of pyrene as a function of temperature.

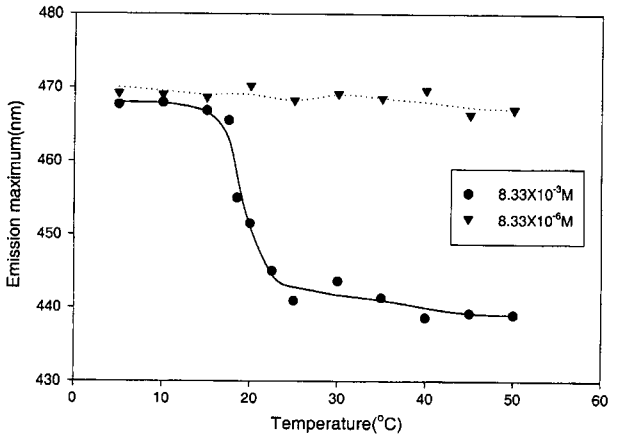

Fig. 8. Emission maxima of PyCHO in poloxamer 407 as a function of temperature.

yield (Schild and Tirell, 1991). Fig 8 showed the dependence of λ_{max} of PyCHO on temperature in aqueous solutions of poloxamer 407. Below cmc (8.33×10^{-6} M), we found λ_{max} to be essentially constant at 530 nm for ANS and 478 nm for PyCHO, independent of temperature over the range investigated. In contrast, above cmc (8.33×10^{-3} M), we observed a marked blue shift at 18°C (cmt), suggesting a decrease in the polarity of the microenvironment of the probe.

Complexation with various fluorescent substances

Complex characteristics of poloxamer 407 micelles were examined by fluorescence probe experiments. By using four different probes, the microenvironmental polarity of the copolymer micelle was determined. Binding of various fluorescent probes such as hydrophobic PNA, less hydrophilic TNS and ANS, and hydrophilic DASP to the poloxamer above cmc was studied, and their binding constants (*K*) were obtained by the Benesi-Hildebrand relationship. Results were summarized in Fig. 9 and Table I. The micropolarity around these probes in the poloxamer 407

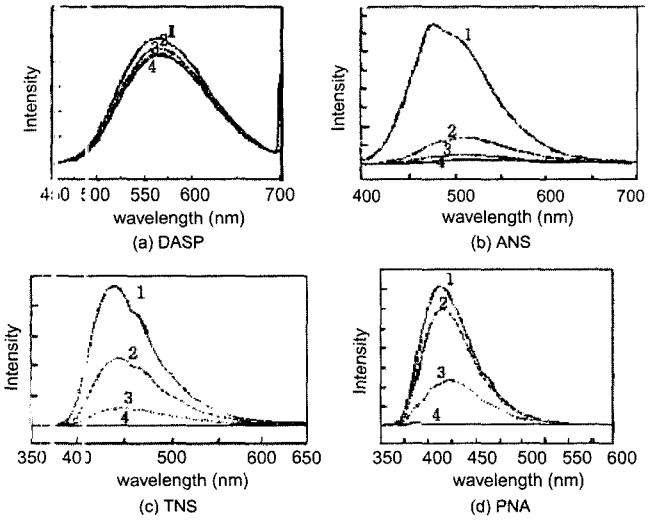

Fig. 9. Fluorescence spectra of probes in poloxamer micelles. (a) DASP, (b) ANS, (c) TNS and (d) PNA. Concentration of poloxamer was 1) 10 mg/mL, 2) 2.5 mg/mL, 3) 1 mg/mL and 4) without poloxamer 407.

Table I. Emission maxima of four fluorescent probes in water with or without poloxamer 407 and the binding constant of them to the poloxamer 407 at 20°C

Fluorescent probe	λ_{max} (nm in water)		K (M^{-1})
	with poloxamer 407	without poloxamer 407	
PNA	412	470	3.28×10^3
ANS	476	527	6.33×10^2
TNS	440	479	4.48×10^2
DASP	563	567	–

could be estimated from their emission maxima of probes. λ_{max} of probes changed shift to lower values when hydrophobicity of the probes increased. This result suggests that the hydrophobic interaction became stronger with increasing nonpolar character of the probe. ANS, TNS, and PNA complexed rather strongly with the poloxamer 407, while DASP did not complex with it at all. The binding constant increased with an increase in the hydrophobicity of the probe. This implied that the main driving force for the complexation was primarily a hydrophobic interaction.

ACKNOWLEDGEMENT

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