Colloid Polym Sci 275:1162–1168 (1997)

ORIGINAL CONTRIBUTION

C.V. Ramesh
R. Jayakumar
R. Puvanakrishnan

Physicochemical characterization of a novel surfactant peptide containing an arginine cation and laurate anion

Received: 14 February 1997
Accepted: 13 August 1997

C.V. Ramesh · Dr. R. Puvanakrishnan (✉)
Department of Biotechnology
Central Leather Research Institute
Adyar, Madras 600 020
India

R. Jayakumar
Bioorganic Laboratory
Central Leather Research Institute
Adyar, Madras 600 020
India

Abstract A novel surfactant peptide consisting of an arginine cation with laurate anion has been synthesized, purified and characterized. The critical micellar concentration (cmc) of peptide in aqueous solutions has been determined using spectroscopic techniques and is found to increase from 0.06 to 0.11 mM with increasing temperature (15–45 °C). Cmc is also determined in the presence of salts like NaCl, KCl and sodium acetate and it is found that these electrolytes hinder aggregation with a significant increase in the case of sodium acetate. The aggregation number of the surfactant peptide has been determined using fluorescence quenching measurements and is observed to decrease from 14 to 6 with increasing temperature (15–45 °C). The standard free energy change (ΔG^0_m) and standard enthalpy change (ΔH^0_m) of the peptide aggregate are found to be negative with a small positive value for standard entropy change (ΔS^0_m). The peptide aggregate seems to undergo phase transition above 50 °C as observed from UV–vis and fluorescence spectroscopy. From pyrene binding studies, it is shown that the interior dielectric constant increases from 5.08 at 34 °C to 8.77 at 50 °C and further decreases with increase in temperature indicating a phase change at 50 °C. Also, the ratio of excimer intensity to monomer intensity, which is a measure of microviscosity of the aggregate, decreases with increase in temperature with a change at 50 °C indicating a phase change.

Key words Surfactant peptide – micelle – aggregation – phase transition

Introduction

Amphiphilic compounds such as surface active agents are known to self-associate into ordered aggregates which play an important role in chemical processes and in biological systems [1, 2]. Various forms of aggregates like micelles, emulsions, monolayers, bilayers and vesicles are used in membrane chemistry, colloidal catalyst, semiconductors and drug transport [3, 4]. Non-covalent interactions between surface active molecules lead to the formation of macro-aggregates which attract a lot of interest in biomimetic and biological chemistry [5]. Elucidation of these interactions forms the basis for new approaches in drug designing [6, 7]. Despite accurate descriptions of organized aggregates in the living systems, a systematic and general understanding of the interactions that is present within the aggregate is still in its infancy.

Surface active peptides and its lipid derivatives play an important role in serving as models to understand peptide–membrane interactions [8–10]. Surface active peptides are designed for better transport across the membrane and also to enhance its half life in the blood stream. The lipid tail that is covalently linked with the

peptide increases its bioavailability and helps the moderate penetration of the drug into the biomembranes [11]. Derivatizing the peptide as a surface-active compound and encapsulating the peptidic site in the aggregated environment will give rise to a better mode of delivery of the peptide drugs [12].

We have developed a new type of surfactant peptide, lauric salt of Boc-L-Arg-L-Pro-lauryl ester that contains an arginine residue cation and laurate anion [13]. The arginine-based peptide is designed in such a way to mask the bigger size of the guanidino group. It is known that the geometry of a surfactant molecule plays a major role in defining the aggregation behavior [14]. Ion paired amphiphiles are known to produce organized aggregates upon sonic dispersion in water. It is suggested that the long chain fatty acid counterion decreases the head group size due to the electrostatic attraction between the counterions and reduces the hydration [15, 16]. In this study, the physicochemical characterization of the dipeptide derivative has been carried out and thermodynamic studies are performed to get a better understanding of the aggregate.

Experimental

All the chemicals used were of analytical grade. The peptide, Boc-L-Arg-L-Pro-lauryl ester laurate was synthesized by solution phase method, purified by column chromatography and characterized by NMR, IR and UV spectroscopy [13].

Determination of critical micellar concentration (cmc)

UV–vis and fluorescence spectroscopy were used to determine the cmc of the peptide derivative. UV–vis and fluorescence spectra were recorded on Shimadzu UV-260 spectrophotometer and Hitachi model 650-40 Fluorimeter, respectively. Prior to the measurements, all the solutions were thermostated for more than 20 min. The temperature reproducibilities were within $\pm 0.05\,^{\circ}C$. The external probes used for UV–vis and fluorescence spectroscopy were *p*-nitrophenol and resorcinol, respectively. The concentrations of the probe used were sufficiently kept low (1×10^{-5} M for UV–vis, 0.5×10^{-5} M for fluorescence) so that the ratio between the probe and the peptide concentration was significantly lesser than unity. The absorbance at 317 nm (*p*-nitrophenol) and at 197 nm (for peptide) was recorded for different concentrations of the peptide at various temperatures. Absorbance as a function of the peptide concentration was plotted in order to obtain the cmc. The cmc of the peptide derivative at different temperatures was noted from an abrupt change in the slope of the straight lines. The cmc of the peptide in presence of different concentrations of electrolytes like NaCl, KCl and sodium acetate was also monitored using UV–vis spectroscopy. Fluorescence measurements were recorded at 25 °C with the excitation wavelength at 270 nm and emission wavelength at 307 nm, respectively. The emission intensity as a function of peptide concentration was plotted in order to obtain the cmc.

Determination of aggregation number (N)

The aggregation number of the peptide derivative was determined by measuring the quenching of an aggregate bound fluorescent probe with different concentrations of quencher [17, 18]. This technique assumes that the numbers of both probe and quencher molecules per micelle have Poisson distribution which leads to the expression

$$\ln(I_0/I) = \frac{N[Q]}{C_s - \text{cmc}}, \tag{1}$$

where I_0 and I are the emitted light intensities with quencher concentration zero and $[Q]$, respectively. N is the mean peptide aggregation number and C_s the total concentration of peptide. Aggregation number was calculated for different temperatures from the slope of $\ln(I_0/I)$ vs. $[Q]$ for fixed C_s. The fluorescence probe used was the Mg salt of 8-anilino-1-naphthalene sulfonic acid (ANS) and the quencher used was N-cetyl pyridinium chloride (CPC) [18]. The excitation wavelength was 346 nm and the fluorescence emission was observed at 463 nm. Using biphasic micellar model [19] the standard free energy change for aggregate formation, ΔG_m^0 of the dipeptide derivative was calculated from the equation

$$\Delta G_m^0 = RT \ln \text{cmc} = \Delta H_m^0 - T\Delta S_m^0 . \tag{2}$$

The standard enthalpy change of aggregation ΔH_m^0 was calculated from the slope of the plot of ln cmc vs. $1/T$. Using Eq. (2), the standard entropy change ΔS_m^0 could be calculated.

Determination of transition temperature (T_m)

The phase transition of peptide aggregate was observed by noting the absorbance at different temperatures for fixed concentration of the peptide. The fluorescence spectra was recorded on Perkin-Elmer LS 5B fluorimeter using pyrene (1×10^{-6} M) as a probe. The intensity of fluorescence aggregate solubilized ANS probe ($\lambda_{max} = 463$ nm), for fixed concentration of peptide at various temperatures was

Colloid & Polymer Science, Vol. 275, No. 12 (1997)

also measured. In order to study the aggregation behavior with regard to phase transition, pyrene binding studies with fixed peptide and pyrene concentrations were performed at different temperatures. Studies on the micro polarity and micro-viscosity of the aggregate were carried out by using the vibronic fine structure of the fluorescence emission of pyrene. The spectrum of pyrene consists of structured monomeric emission and non-structured excimer emission. The ratio of the intensities of the first and third vibronic peaks, I_1/I_3 (I_1, at 372.6 nm, I_3, at 383.6 nm) is dependent largely on the solvent polarity [20, 21], and usually it can reflect the polarity of the micro-environment in which pyrene is. A linear relationship between I_1/I_3 and the interior dielectric constant (ε) is expressed by the equation [22]

$$\varepsilon = 86.2(I_1/I_3) - 87.8 \,. \tag{3}$$

Results

The cmc of peptide aggregate was determined by measuring the absorption at 317 nm due to the probe as well as due to the peptidic absorption at 197 nm. The absorption was found to increase with increase in the peptide concentration. The plot of concentration of peptide versus absorption due to probe showed that the cmc increased with increasing temperature (Fig. 1). Similar plot due to the peptidic absorption was also obtained (figure not shown). The cmc obtained with different electrolytes are tabulated in Table 1. While all the electrolytes studied showed an increase in cmc in general, a significant increase in cmc was noticed in case of sodium acetate. The cmc obtained from fluorescence measurements at 25 °C using resorcinol as probe was comparable to the value obtained from UV–vis method (Table 2). From fluorescence quenching studies (Fig. 2) the aggregation number of the peptide aggregate was found to decrease with increasing temperature as observed from the fluorescence emission of ANS at 463 nm and was found to be low (Table 2). From the cmc values of the peptide aggregate, thermodynamic parameters like standard free energy change ΔG_m^0, standard enthalpy change ΔH_m^0 and standard entropy change ΔS_m^0 of aggregation were calculated for the peptide. ΔG_m^0 and ΔH_m^0 of the peptide aggregate were found to be negative with small positive value for ΔS_m^0 (Table 2). From Fig. 1 it could be observed that at 50 °C there was no change of slope indicating the absence of micelle formation. The phase transition was monitored using absorption at 317 and 197 nm due to the probe *p*-nitrophenol and the

Table 1 Effect of various concentrations of electrolytes on critical micellar concentration of the peptide

Concentration of electrolytes (mM)	Critical micellar concentration (mM)		
	NaCl	KCl	NaOAc
5	0.14	0.13	0.25
10	0.16	0.16	ND
20	0.19	0.18	*
50	0.21	0.20	ND

ND – Not determined; * – cmc was not observed.

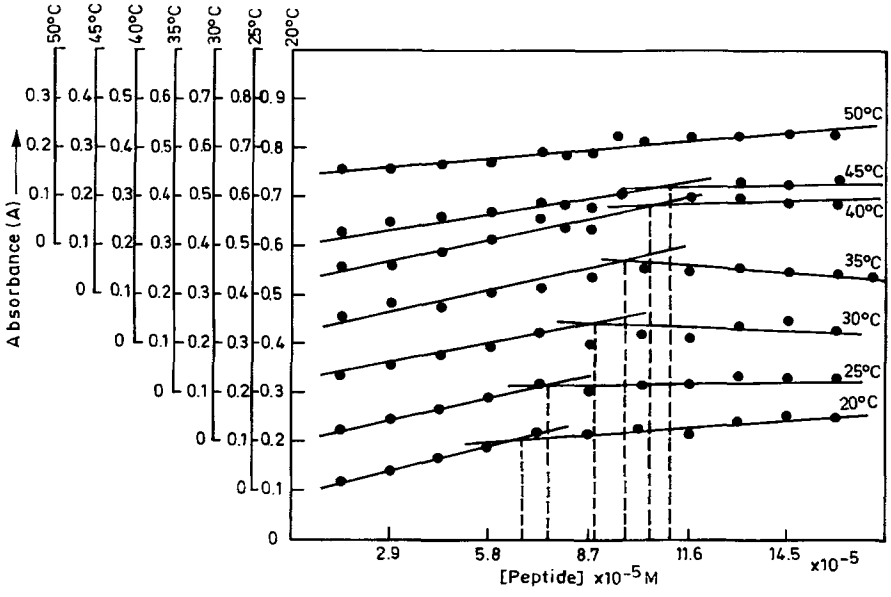

Fig. 1 Plot of absorbance (at 317 nm) as a function of peptide concentration at various temperatures; [*p*-nitrophenol] = 1×10^{-5} M (fixed)

Table 2 Critical micellar concentration (cmc) aggregation number (N) and some thermodynamic parameters of the peptide derivative at various temperatures

Temperature (°C)	cmc (mM)	cmc (mole fraction)	N	ΔG_m^0 (kJ mol^{-1})	ΔH_m^0 (kJ mol^{-1})	ΔS_m^0 (J K^{-1} mol^{-1})
15	0.060	1.08×10^{-6}	14	− 32.90	− 16.07	58
20	0.068	1.22×10^{-6}	13	− 33.17	− 16.07	58
25	0.076	1.37×10^{-6}	12	− 33.45	− 16.07	58
30	0.089	1.60×10^{-6}	10	− 33.62	− 16.07	58
35	0.098	1.76×10^{-6}	9	− 33.93	− 16.07	58
40	0.105	1.89×10^{-6}	8	− 34.30	− 16.07	58
45	0.110	1.98×10^{-6}	6	− 34.72	− 16.07	58

Note: From fluorescence measurements using resorcinol as probe the cmc was found to be 0.078 mM at 25 °C.

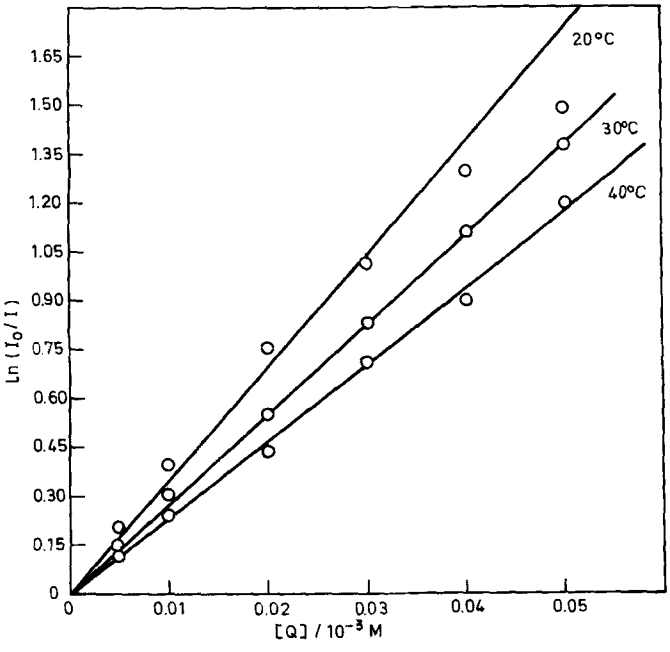

Fig. 2 Plot of ln (I_0/I) vs. quencher concentration at various temperatures, [ANS] = 1×10^{-5} M (fixed); [peptide] = 4.3×10^{-4} M (fixed); λ_{ex} = 346 nm, λ_{cm} = 463 nm

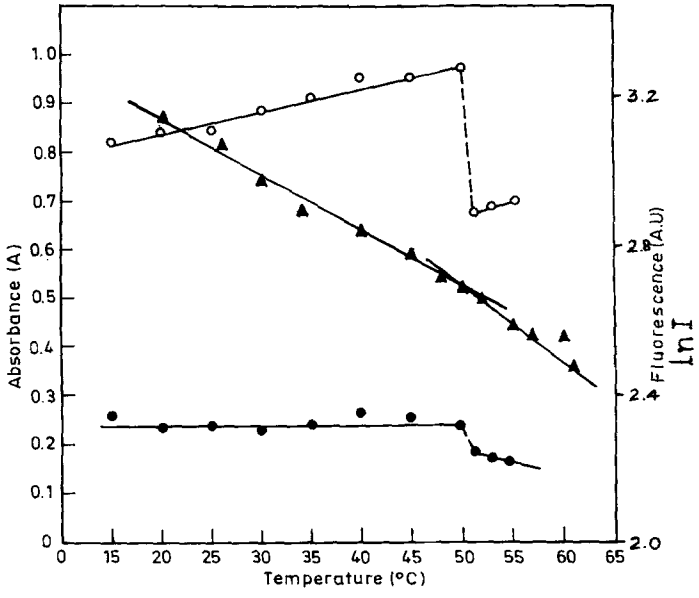

Fig. 3 Plot of absorbance of p-nitrophenol at 317 nm (●), peptide absorption at 197 nm (○) and fluorescence emission (ln I) of ANS (▲) versus temperature, [peptide] = 1.6×10^{-4} M (fixed); [p-nitrophenol] = 1×10^{-5} M (fixed); [ANS] = 1×10^{-5} M (fixed); λ_{ex} = 346 nm

peptidic absorption at different temperatures for fixed concentration of the peptide derivative, which showed a discontinuous jump at 50 °C (Fig. 3). ANS spectra at various temperatures for fixed concentration of the peptide derivative was recorded and the emission intensity of ANS was found to decrease with increasing temperature. Plot of ln I (emission intensity; λ_{max} = 463 nm) as a function of temperature showed a change in slope at 50 °C indicating phase transition (Fig. 3).

The pyrene spectra at various temperatures are depicted in Fig. 4. From the I_1/I_3 ratio, interior dielectric constant was calculated at different temperatures using Eq. (3). The computed dielectric constant at 34 °C was 5.08. On increasing the temperature, the hydrophobicity of the pyrene binding site decreased which in turn increased the dielectric constant to a maximum of 8.77 at 50 °C. Above 50 °C, the computed dielectric constant decreased with the temperature indicating phase change (Fig. 5). From the pyrene spectrum, the intensity of the excimer (I_e) and the intensity of the monomer (I_m) was monitored at 470 and 392.5 nm respectively [23, 24]. From the plot of I_e/I_m versus temperature (Fig. 5), the ratio

Colloid & Polymer Science, Vol. 275, No. 12 (1997)

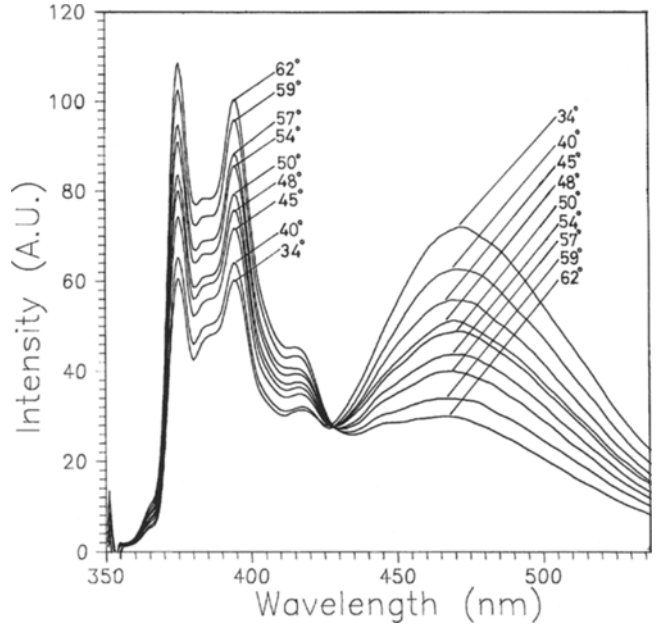

Fig. 4 Fluorescence of pyrene probe in the presence of peptide derivative at different temperatures, [peptide] = 1.6×10^{-4} M (fixed); [pyrene] = 1×10^{-6} M (fixed); λ_{ex} = 335 nm

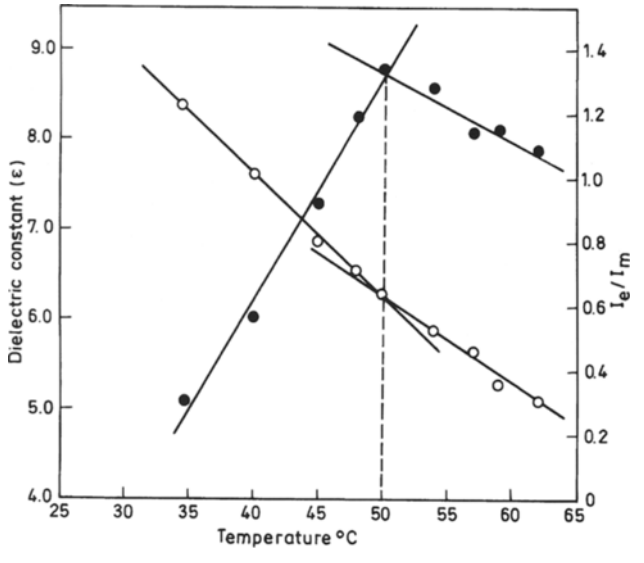

Fig. 5 Plot of interior dielectric constant (●) and I_e/I_m (○) versus temperature, [peptide] = 1.6×10^{-4} M (fixed); [pyrene] = 1×10^{-6} M (fixed); λ_{ex} = 335 nm

(I_e/I_m) which corresponds to the microviscosity of the aggregate decreased with increasing temperature. At 50 °C, a change in microviscosity of the aggregate was observed.

Discussion

In this peptide surfactant, two lauryl chains are present, one at the carboxyl terminal end which is covalently linked as an ester and the other as laurate counter ion at the guanidino end of arginine. The cmc is found to increase with increase in temperature as observed from Fig. 1. The absorbance is found to increase with increase in peptide surfactant concentration even at premicellar state indicating low equilibrium complex formation between the peptide surfactant and the probe. The ratio of probe and peptide surfactant concentration is much lesser than unity favoring aggregate formation rather than complex formation. The low concentration of probe might form multiple complex with the peptide surfactant resulting in low equilibrium complex.

Hall and Pethica [19] have applied the small system thermodynamics to micelle formation and they have discussed the actual intrinsic thermodynamic functions of the aggregates. Analysis of the thermodynamic parameters indicate that the aggregation is governed by both the enthalpy factor as well as the entropy factor. The enthalpy drive for aggregation might be due to the electrostatic interactions which play an important role in ion pair aggregation [25]. The entropy drive for the aggregation results from the interaction among the long hydrophobic lauryl groups. The negative enthalpy and small positive entropy values generally indicate ionic interactions in a medium of low dielectric constant i.e. association of charged groups (salt-bridge formation) excluded from the polar solvent [26–28].

Numerous studies have been reported on the effect of added electrolytes on the micellar properties of ionic surfactants [1]. Addition of electrolytes to surfactant micelles stabilized by hydrophobic interactions will cause a reduction in the thickness of the ionic atmosphere surrounding the polar head groups and a consequent decreased repulsion among them resulting in the reduction of cmc. In this study, micellization as mentioned earlier is attributed to the ionic interaction between the surfactants which will ultimately get weakened in the presence of added electrolytes. The ion-pair formation between carboxylate and guanidino groups should result in uniform charge distribution on the surface of the micelle. On addition of electrolytes there will be an uneven accumulation of counter ions of both the positive and negative charges on the surface of the micelle. The uneven counterion distribution will be levelled out partially by the dissociation of the ion pairs from the micellar aggregates which ultimately leads to an increase in cmc. In the case of sodium acetate there is a appreciable additive effect. At 20 mM concentration there is no organized micelle formation. This could be because of the specific interaction of acetate ion which

effectively competes with lauryl carboxylate ions thereby hindering aggregation. Thus the electrostatic model proposed for the given micellar aggregate could be better rationalized by these results.

Fluorescence emission of ANS in aqueous medium takes place at 520 nm upon excitation at 346 nm [29]. However, in presence of peptide aggregate, the emission peak of ANS shifts to 463 nm. Such a blue shift of ANS might be due to the increased microviscosity of ANS environment [29]. The decrease in aggregation number with increasing temperature indicates that the process of aggregation might be due to the attractive forces between the monomeric systems (as seen in the $-\Delta H_m^0$ values). The aggregation number is small and such low aggregation number has been reported for some micellar systems [30]. In the micellar systems, the translational entropy always operates in favor of the disintegration of molecular clusters. If the average electrostatic energy of a molecule in an aggregate is independent of the aggregate size, then, there is no energetic advantage in adding the molecule to a larger aggregate rather than to a small one, no matter how strong the forces involved and the total entropy of the system will favor smaller clusters as the temperature increases [31]. In the present system increase in temperature results in the decrease of aggregation number rationalizing the ion pair model proposed. Above 50 °C, there is no change of slope indicating the absence of any peptide aggregate with a definite boundary. Discontinuous jump around 50 °C as in Fig. 3, indicates drastic change in the micro-environment of the probe. The plot of $\ln I$ of ANS with different temperatures implies a phase transition taking place in the aggregate around 50 °C. The increase in the slope on increasing the temperature indicates that ANS senses less hydrophobic interior above transition temperature (Fig. 3) [29].

The process of phase change involves cooperative transitions of the aggregate resulting in simultaneous reorientation of a large number of hydrogen bonds or in deorientation of ionic moieties to favor better electrostatic interactions. This in turn results in large negative values of enthalpy changes associated with phase change of the aggregate. Even at low concentration of pyrene (1×10^{-6} M), relatively high excimer fluorescence quantum yield indicates that a substantial number of micelles accommodate more than one pyrene molecule (thus allowing excimer formation). This is because of low concentration of micelles in the solution which increases the possibility of excimer emission. However, when the temperature is increased, the excimer fluorescence quantum yield gets decreased favoring the monomer fluorescence (Fig. 4). The decrease of the excimer fluorescence quantum yield corresponding to the decrease in the number of encounters between excited and non-excited pyrene molecule could be due to the increase in microviscosity, and thus I_e/I_m (the excimer intensity to monomer intensity) ratio could be taken as a good indicator of microviscosity changes. Abrupt change in the slope of I_e/I_m around 50 °C indicates very slight microviscosity changes with temperature implying the phase change around that temperature (Fig. 5).

The phase change at 50 °C is also implied from the following observations. The increase in UV absorption indicates the *p*-nitrophenol binding to the hydrophobic interior of peptide aggregate [32]. Above 50 °C, there is no abrupt change in the absorbance of the *p*-nitrophenolate probe (Fig. 1) indicating the absence of any distinct cmc at which the micellization could begin. Even above 50 °C, absorbance of the probe (fixed concentration) increases on increasing the surfactant concentration implying the probe *p*-nitrophenol getting bound to the peptide aggregate. The pyrene fluorescence studies show that the interior dielectric constant (calculated) above 50 °C decreases with increasing temperature indicating that the pyrene still binds to surfactant molecules. The presence of an excimer band above 50 °C, substantiates the presence of aggregates at higher temperature. An increase in temperature will lead to a decrease in the quantum yield depending on the binding site of the ANS molecule. It is worth noting that the ANS fluorescence studies also indicate the existence of aggregates. The decrease is more pronounced in the more non-polar environment. In the given system the 50 °C transition change indicates that even above 50 °C, nonmiceller aggregates (without any definite boundary) are present.

Acknowledgments The authors thank Dr. T. Ramasami, Director, Central Leather Research Institute, for his kind permission to publish this work. The Research Fellowship awarded to one of us (CVR) by the Council of Scientific and Industrial Research, New Delhi is gratefully acknowledged.

References

1. Attwood D, Florence AT (1983) In: Surfactant Systems – Their Chemistry, Pharmacy and Biology. Chapman and Hall, New York
2. Robertson RN (1983) In: The Lively Membranes. Cambridge University Press, London
3. Fendler JH (1984) Chem Br 20:1098–1103
4. Knight CG (1981) In: Lipsomes: From Physical Structures to Therapeutic Applications. Elsevier, Cambridge

5. Tadros Th F (1984) In: Tadros Th F (ed) Surfactants. Academic Press, London, pp 323–335
6. Mutter M, Vuilleumier S (1989) Angew Chem Int Ed Engl 28:535–554
7. Hruby VJ, Al-Obeidi F, Kazmierski W (1990) Biochem J 268:249–262
8. Schwyzer R (1992) Brazilian J Med Biol Res 25:1077–1089
9. Brugge JS (1993) Science 260:918–919
10. Wallace BM, Lasker JS (1993) Science 260:912–913
11. Poznansky MJ, Juliano RL (1984) Pharmacol Rev 36:277–335
12. Eppstein DA, Longenecker JP (1988) CRC Critical Reviews in Therapeutic Drug Carrier Systems 5:99–139
13. Ramesh CV, Jayakumar R, Puvanakrishnan R (1995) Int J Peptide and Protein Res 45:386–390
14. (a) Evans DF (1988) Langmuir 4:3–12; (b) Israelachvili JN, Marcelja S, Horn RG (1980) Q Rev Biophys 13:121–200
15. Fukuda H, Kawata K, Okuda H, Regen SL (1990) J Am Chem Soc 112: 1635–1637
16. Kaler EW, Murthy AK, Rodriguez BE, Zasadzinski JAN (1989) Science 245: 1371–1374
17. Luo H, Boens N, Van der Auweraer M, De Schryver FC, Malliaris A (1989) J Phys Chem 93:3244–3250
18. Turro NJ, Yekta A (1978) J Am Chem Soc 100:5951–5952
19. Hall DG, Pethica BA (1967) In: Vol 1. Schick MJ (ed) Nonionic Surfactants, Marcel Dekker, New York, pp 516–557
20. Kalyanasundaram KD, Thomas JK (1977) J Phys Chem 81:2176–2180
21. Lanos PL, Lang J, Straziulle C, Zana R (1982) J Phys Chem 86:1019–1025
22. Turro NJ, Kuo PL (1986) J Phys Chem 90:837–841
23. Zana R (1987) In: Surfactant Solutions: New Methods of Investigation. Marcel Dekker, New York and Basel, pp 241–294
24. (a) Pownall HJ, Smith LC (1973) J Am Chem Soc 95:3136–3140; (b) Emert J, Behrens C, Goldenberg M (1979) J Am Chem Soc 101:771–772
25. Bashford D, Case DA, Dalvit C, Tennant L, Wright PE (1993) Biochemistry 32:8045–8056
26. Klotz IM (1973) Ann NY Acad Sci 226:18–35
27. StCharles R, Watz DA, Ednonds BFP (1989) J Mol Biol 187:341–348
28. Mayo KH, Chen MJ (1989) Biochemistry 28:9469–9478
29. Slavik J (1982) Biochim Biophys Acta 694:1–25
30. Moro ME, Rodriguez LJ (1991) Langmuir 7:2017–2020
31. Ruckenstein E, Nagarajan R (1975) J Phys Chem 79:2622–2626
32. Cramer F, Saenger W, Spatz H (1967) J Am Chem Soc 89:14–20