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Effect of urea on self-organization of sodium *N*-(11-acrylamidoundecanoyl)-L-valinate in water

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Abstract

In this paper, the hypotheses proposed for the action of urea on the perturbation of molecular assemblies have been tested through studies of the effects of urea on the aggregation properties of a chiral surfactant, sodium *N*-(11-acrylamidoundecanoyl)-L-valinate in water. Surface tension, fluorescence, and circular dichroism were used to characterize the solution behavior of the amphiphile in the presence of urea. Surface tension measurement indicated decrease of critical aggregation concentration (cac) with the addition of urea in the low concentration range. Fluorescence probe studies using pyrene and 1-anilinonaphthalene indicated solubilization of urea molecules near the aggregate-water interface. Fluorescence anisotropy measurements using 1,6-diphenylhexatriene as probe molecule suggested increase of packing of the hydrocarbon chains of the amphiphiles upon addition of low concentration of urea. Dynamic light scattering measurements showed an increase of the hydrodynamic radius (R_h) in the presence of increased concentration of urea. At higher concentrations of urea, the R_h value decreased. Circular dichroism spectra showed the presence of chiral aggregates even in the presence of high concentration of urea.

Keywords: Urea; Chiral amino acid-derived surfactant; Hydrodynamic radius; Fluorescence anisotropy; Circular dichroism

1. Introduction

The micellar association of an amphiphilic molecule is a result of a delicate balance between "hydrophobic" and "hydrophilic" interactions in a solvent [1]. The micellar aggregates are known to enhance solubility of hydrophobic compounds through concomitant change of their microenvironment such as polarity and viscosity. The structure of the micelles and the physicochemical properties, such as critical micellar concentration (cmc), aggregation number (N_{agg}), thermodynamics of micelle formation, and degree of counterion binding, of micellar solutions depend upon this balance between "hydrophobic" and "hydrophilic" interactions [2–4]. The balance between "hydrophobic" and "hydrophilic" interactions can be perturbed by addition of salts or organic additives. Micellar association in the presence of different organic additives has been extensively examined [5–10] because of their enhanced use in combination with one or more additives simultaneously for their scientific, experimental, industrial, and theoretical applications [11–13]. Shinoda [14] and Harkins et al. [15] presented the first systematic studies on the effect of added solutes, particularly alcohols, on the cmc values of ionic surfactants. A good amount of work has been done on the interaction of organic solubilizates with ionic and nonionic surfactant micelles and also on the effect of the solubilizate on a single, specific property of the surfactant micelle [16–21]. It is reported that short-chain alcohols (methanol to propanol) are solubilized mainly in the aqueous phase and affect the micellization process by modifying solvent properties. Addition of these alcohols has been found to cause reduction of the size of the micelles and also a progressive breakdown of the surfactant aggregate at very high concentration.

The most commonly used additive whose effects on micellization processes have been studied is urea. This is because urea is often used as a denaturating agent for proteins

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and other biopolymers [22–25]. Several studies have been performed in the past using urea as an additive to study the effect of this additive on the denaturation of proteins [26,27]. To elucidate the mechanism of protein denaturation by urea, many researchers have studied its effect on micelles [28–35], as the denaturation of proteins can be taken as equivalent to demicellization in aqueous urea solutions. It has been observed that urea increases cmc and decreases the size of both ionic and nonionic micelles. Urea is also found to perturb structure of molecular self-assemblies. This is thought to be due to the ability of urea to act as a "water structure breaker." In fact, these studies have suggested that the additive can act through either a direct or an indirect mechanism. In the direct mechanism, the additive molecules interact with surfactant molecules and thereby help solvation of hydrophobic molecules by replacing some of the water molecules from the hydration shell of the solute. On the other hand, in the indirect mechanism, the additives act as water structure breakers and thereby promote solvation of the hydrophobic solute. In general, the indirect mechanism is more widely accepted and there are experimental results that seem to support this hypothesis [34,36–38]. However, there are also many theoretical as well as experimental studies that support the direct mechanism for the effect of urea on micellar properties [35,39–42]. It should be noted here that most of these studies have been performed with urea concentrations higher than 1.0 M. Mukherjee had proposed that an additive that is surface-active at the hydrocarbon–water interface would mainly be solubilized at the headgroup region and would promote micellar growth [43]. More recently it has also been shown that urea is a water structure breaker, not a structure maker, and there is no direct correlation between a solute's effect on water structure and its effect on protein or micellar stability [44].

These apparently conflicting reports led us to investigate the effect of urea, a well-known cheotrope, on the self-assembly properties of a chiral *N*-acyl amino acid surfactant, sodium *N*-(11-acrylamidoundecanoyl)-L-valinate (SAUV), to test the suggested mechanisms. We have recently studied the aggregation behavior of SAUV and other structurally related surfactants in aqueous solution by various techniques including surface tension, fluorescence, light scattering, and transmission electron microscopy. Details have been reported elsewhere [45]. Briefly, the surface tension and fluorescence studies have indicated the initial formation of flat bilayer structures above a low critical aggregation concentration (cac) of the surfactant. With the increase of surfactant concentration above the cac value the bilayer lamellar structures transform into spherical vesicles. The cac value was observed to decrease with the increase in pH of the solution. The bilayer structures were found to form at solution pH above the pK_a (5.0) of the free surfactant molecule. The results of dynamic light scattering and transmission electron microscopic (TEM) studies have confirmed the presence of large spherical vesicles in surfactant solutions above cac. The bilayer structure is stabilized by intermolecular amide hydrogen bonding at the surfactant headgroup as well as at the end of the hydrocarbon tail. The amphiphile has also been shown to form chiral helical aggregates in aqueous solutions. Thus there is a similarity between microstructure of the amphiphile and that of a protein in aqueous solution. The focus of this work is to study the mechanism of how urea influences the aggregation properties of the amphiphile.

2. Experimental section

2.1. Materials

Sodium *N*-(11-acrylamidoundecanoyl)-L-valinate was prepared according to a procedure described elsewhere [45] and was purified by recrystallization from ethanol–acetone mixture. Urea (molecular biology grade) was procured from SRL and was used without further purification. The fluorescence probes, pyrene, 1,6-diphenyl-1,3,5-hexatriene (DPH), and 1-anilinonaphthalene (AN), were all obtained from Aldrich and were purified by repeated recrystallization from ethanol. All the solvents used were reagent grade and were used after distillation. Stock solutions of the amphiphiles were prepared in deionized water (18.2 MΩ, pH 6.0) purified by a Milli-Q water system (Millipore).

2.2. Methods

2.2.1. Surface tension measurement

The surface tension measurements were performed with a torsion balance (Hurdson and Co., India) using the Du Nuoy ring detachment method. The platinum–iridium ring was regularly cleaned with ethanol–HCl solution. A stock solution of SAUV was made either in Milli-Q water (18.2 MΩ) or in aqueous urea solution. An aliquot of this solution was transferred to a beaker containing a known volume of water. The solution was gently stirred magnetically and allowed to stand for about 5 min at room temperature (∼30 °C) and then the surface tension was measured. For each measurement three readings were taken and the mean γ value was recorded. Before each experiment the instrument was calibrated and checked by measuring the surface tension of distilled water.

2.2.2. Fluorescence measurements

The steady-state fluorescence spectra were measured on a SPEX Fluorolog-3 spectrofluorometer. A stock solution of pyrene of strength 0.39 μM was made either in Milli-Q water or in aqueous urea solution. These solutions were used to make stock solution of the surfactant. Appropriate volumes of the surfactant and urea stock solutions were taken in a 5-ml flask and the volume was made up with the same pyrene stock solution. Thus solutions of constant surfactant and probe concentration with varying concentration of urea were obtained. The solutions were excited at 335 nm and emission intensity was measured in the wavelength range of

350 to 550 nm. The excitation and emission slit widths were both set at 1 nm. Each spectrum was blank subtracted and was corrected for lamp intensity variation during measurement.

The fluorescence anisotropy measurements were performed with a Perkin–Elmer LS-55 equipped with a polarization accessory that uses the L-format configuration and thermostated cell holder. The temperature of the sample was controlled (within ±0.1 °C) by use of a Thermo Neslab (Model RTE 7) circulating bath. The sample was excited at 350 nm and the emission intensity was followed at 430 nm using excitation and emission slits with bandpass of 2.5 and 5 nm, respectively. The fluorescence anisotropy values (r) were calculated employing the equation

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}), \quad (1)$$

where I_{VV} and I_{VH} are the fluorescence intensities polarized parallel and perpendicular to the excitation light, and G ($=I_{VV}/I_{VH}$) is the instrumental grating factor. All fluorescence measurements were carried out at 30 ± 0.1 °C. The measurements started 2–3 hr after sample preparation.

2.2.3. Circular dichroism spectra

The circular dichroism (CD) spectra were recorded on Jasco J-810 spectropolarimeter using quartz cells of path length 2 or 10 mm. Solutions containing 2.5 mM SAUV were prepared in water and in aqueous urea solution. The spectra were corrected for solvent blank.

2.2.4. Dynamic light scattering

The dynamic light scattering (DLS) measurements were performed with a Photal DLS-7000 (Otsuka Electronics Co. Ltd., Osaka, Japan) optical system equipped with an Ar^+ ion laser (75 mW) operated at 16 mW at $\lambda_0 = 488$ nm, a digital correlator, and a computer-controlled and stepping-motor-driven variable-angle detection system. A 5 mM solution of the amphiphile was prepared in Milli-Q water containing different amounts of urea. The solution was filtered directly into the scattering cell through a Millipore Millex syringe filter (Triton free, 0.22 μm). Before measurement, the scattering cell was rinsed several times with the filtered solution. The DLS measurements started 5–10 min after the sample solutions were placed in the DLS optical system to allow the sample to equilibrate at the bath temperature. For all light-scattering measurements, the temperature was 25 ± 0.5 °C. The scattering intensity was measured at a 90° angle to the incident beam. The data acquisition was carried out for 10 min and each experiment was repeated two or three times. The time decay of the autocorrelation function of the concentration fluctuations has a characteristic decay rate, Γ, which is proportional to q^2 ($\Gamma = Dq^2$), which characterizes a translational diffusion with the mutual diffusion constant D. The scattering vector, q, is given by the equation

$$q = 4\pi n/\lambda_0 \sin(\theta/2), \quad (2)$$

where n is the refractive index of the solvent, and θ is the scattering angle. For data analysis we adopted the second-order cumulant method [46]. The measured translational diffusion constant was related to the average hydrodynamic radius R_h of the particles through the equation

$$D = kT/6\pi\eta R_h, \quad (3)$$

where k is the Boltzmann constant, η the solvent viscosity, and T the absolute temperature.

3. Results and discussion

3.1. Effect of urea on cac

The cac values of the SAUV aggregates in the presence of urea were determined by surface tension method. The concentration at the break point of the $\gamma - \log C$ plot (not shown) was taken as the cac. The cac values have been plotted as a function of mole fraction urea in Fig. 1, which shows a decrease of cac value with the addition of urea in the concentration range studied. The surface tension method could not be used for the determination of cac at higher concentrations of the additive because of its adsorption on the air/water interface. However, fluorescence probe studies with solutions containing urea greater than 1.0 M (mole fraction 0.0177) indicated an increase of the cac of SAUV (results not included). This indicates a decrease of the ionic repulsion among the polar headgroups that facilitates aggregation. It has been recently proposed that upon addition of urea there is an increase of the hydrophilic (dielectric) property of water, which results in more strongly solvated polar headgroups of amphiphilic molecules [47]. If this were true then one would observe a continuous decrease of the cac value of an amphiphilic molecule in aqueous solution. The results suggest that there is a direct interaction of urea molecules with the amphiphiles forming the bilayer. At low concentrations the urea molecules replace the water molecules from the hydration shell of the amphiphile. The large

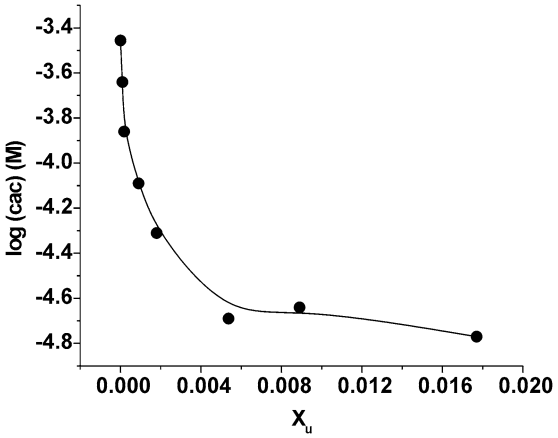

Fig. 1. Plot of log(cac) vs mole fraction (x) of urea.

size (2.5 times that of water) of the urea molecule compared to that of a water molecule reduces the headgroup repulsion, facilitating aggregation of the amphiphile. This results in a decrease of cac. Recently, molecular dynamics simulation [48–50] and NMR studies (NOESY) [51,52] on interaction between ethanol and phospholipid bilayers containing palmitoyloleoyl–phosphatidylcholine lipids have suggested that ethanol molecules interact strongly with the lipid headgroups and accumulate near the membrane–water interface. However, if this direct mechanism for the action of urea were considered to be true, then at higher concentrations, the urea molecules would penetrate into the hydrocarbon core and would increase the volume of the micellar core, which is equivalent to increasing the volume of the hydrocarbon portion of the amphiphilic molecule. This in turn will result in an increase in the packing parameter [2], which is responsible for micellar growth. This means an increase in size of the aggregate with the increase of urea concentration. Further, recently it has been shown that penetration of urea molecules into the micellar core results in an increase of local viscosity [35]. Therefore, we have investigated the microenvironment of the bilayer self-assemblies of SAUV to find the location of urea molecules.

3.2. *Influence of urea on the microenvironment of the bilayer self-assemblies*

Any structural change or disruption of molecular assemblies should be indicated by a change of the microenvironment of a suitable probe molecule. The local environments of the molecular assemblies including micellar-type aggregates can be determined by introducing hydrophobic molecular probes. The extremely low solubility of pyrene in water [53] is known to cause its partition almost completely into the hydrophobic region of the aggregates. The aggregates formation is thus easily detected with the change of the local polarity sensed by pyrene molecule and therefore pyrene is widely used as a polarity probe [54]. It is well established that the ratio of the first and third vibronic bands (I_1/I_3) of the fluorescence emission spectrum of pyrene is sensitive to solvent polarity [55]. Therefore, we have measured the I_1/I_3 ratio of pyrene fluorescence in the presence of urea. Fig. 2 shows the dependence of the I_1/I_3 ratio on the urea concentration in 5 mM SAUV solution. It is evident that the polarity does not change significantly (see inset of Fig. 2) until the urea concentration is greater than 0.3 M (mole fraction 0.0054). Since pyrene molecules are solubilized in the core of the bilayer aggregates, low concentrations of the additive cannot perturb their environment. At higher concentrations, the polarity parameter increases only slightly. This could be due either to displacement of the probe molecule toward the aggregate surface or increased solvation of the hydrophobic tail of the amphiphile that breaks the bilayer structure.

The fluorescent molecule AN is also an excellent probe because of its very low fluorescence quantum yield as well as poor solubility in water. Upon transfer to a less polar environment, it fluoresces strongly and exhibits a high degree of sensitivity to environmental changes by the large blue shift of the emission spectrum. The AN probe is known to be solubilized in the palisade layer of a micellar aggregate. Therefore, we have used AN to study the change of the microenvironment of its solubilization site in aid to the results obtained using pyrene as probe molecule. In 5 mM SAUV solution, in the absence of urea the fluorescence spectrum (not shown here) of AN is blue-shifted relative to that in water, indicating that the probe molecules are solubilized in an environment that is less polar than water. The spectrum exhibits a continuous red shift upon addition of urea even in the low concentration range. The plot of emission maxima versus mole fraction of urea is shown in Fig. 3. This suggests that the probe molecule is solubilized in the palisade layer of the bilayer aggregate and that the addition of urea increases the local polarity of the probe. This confirms our earlier conclusion that at low concentrations, the urea molecules replace the water molecules in the hydration shell facilitating the aggregation process. The fluorescence spectrum of AN in the presence of 6.0 M (mole fraction 0.097) urea is still blue-shifted relative to that in pure water. This suggests that the bilayer structure of the amphiphile is stable even at high urea concentrations.

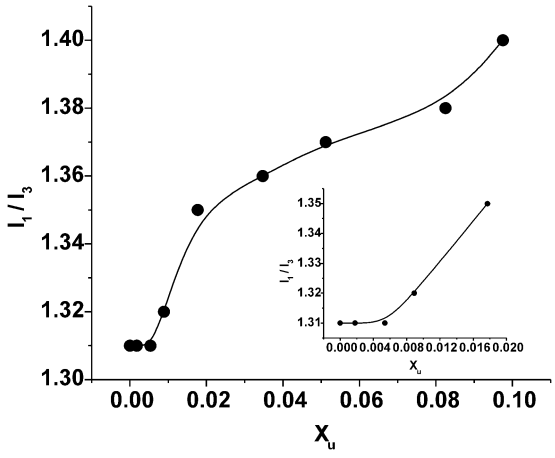

Fig. 2. Plot of I_1/I_3 vs mole fraction of urea; inset: plot of I_1/I_3 vs mole fraction of urea in the low concentration range.

To explore changes in the interior of the organized self-assemblies of SAUV, we have studied the effect of urea on the fluorescence anisotropy of the DPH probe, which partitions into the deeper regions of the aggregates. The fluorescence anisotropy is a measure of the viscosity of the local environment. Therefore, the steady-state fluorescence anisotropy of DPH in 5 mM SAUV solution was measured as a function of urea concentration. The results are plotted in Fig. 4. The plot shows an initial increase in anisotropy value upon addition of urea at low concentrations and a decrease at higher concentrations. The dependence of r upon urea concentration in the low concentration range has been separately shown in the inset of Fig. 4. At low urea concentration, replacement of the water molecules from the hydration shell

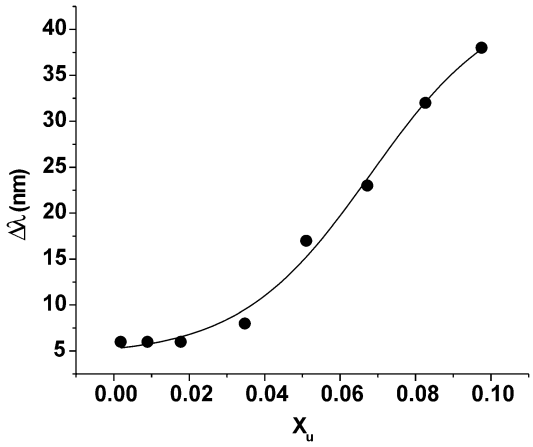

Fig. 3. Shift of emission maximum ($\Delta\lambda$) of AN as a function of urea concentration (x).

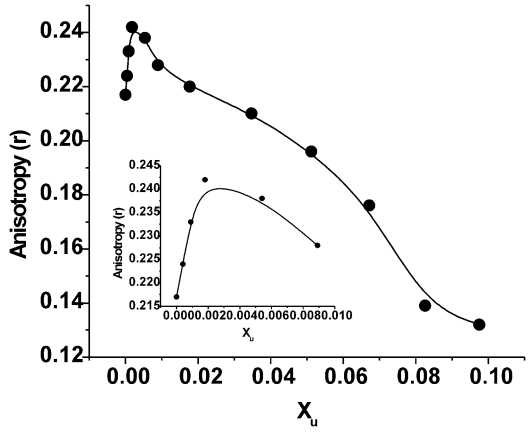

Fig. 4. Plot of fluorescence anisotropy (r) vs urea concentration (x); inset: plot of r vs mole fraction of urea in the low concentration range.

of the amphiphile results in tighter packing of the hydrocarbon chains. This means an increase in viscosity of local environment that increases the rotational restriction of the DPH molecule. This is consistent with the decrease of cac value with the increase in urea concentration. The decrease of anisotropy at urea concentrations above 0.2 M (mole fraction 0.0036) indicates decrease of the local viscosity of the aggregate. This can result from the slow disruption of the bilayer structure. As indicated above for complete disruption of the bilayer assemblies it requires a very high concentration of urea. The stability of the bilayer vesicle structure of SAUV is further suggested by the CD spectra of SAUV as discussed below.

3.3. Effect of urea on the size of the bilayer self-assemblies

The increased packing of the surfactant tails in the bilayer assembly should result in an increase in the size of the vesicles. In order to study the effect of urea on the size of the vesicles, we have performed DLS measurements to obtain the hydrodynamic radius, R_h, of a 5 mM SAUV solution containing different concentrations of urea. The average

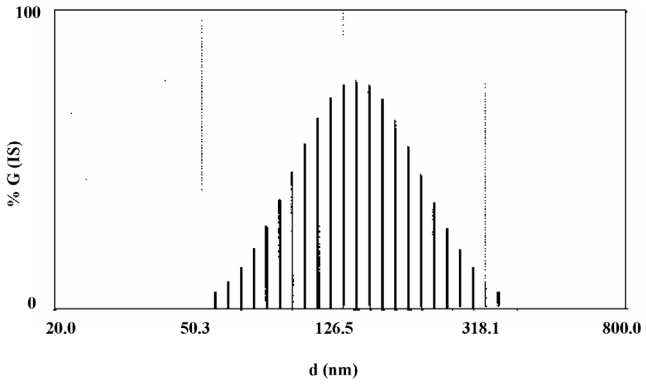

Fig. 5. Size distribution of SAUV vesicles in pure water (pH 8.0).

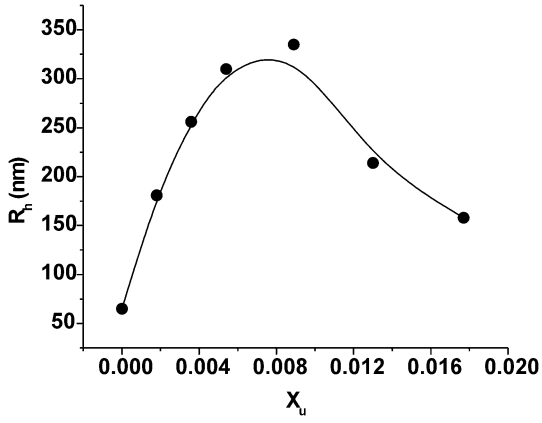

Fig. 6. Plot of R_h against urea concentration (x).

R_h values thus obtained are large, which excludes formation of micellar aggregates. A representative size distribution of the vesicles in pure aqueous solution (pH 8.0) is shown in Fig. 5. As observed, the distribution is very broad. In fact, the TEM picture also showed spherical vesicles of broad size distribution [45]. The dependence of the R_h value upon urea concentration is depicted in Fig. 6. The R_h value increases first and then decreases, passing through a maximum corresponding to a urea concentration of 0.4 M (mole fraction 0.0071). This is consistent with the decrease of cac in this concentration range. This means that the size of the bilayer aggregates of SAUV grow in the presence of low concentration of urea. However, at concentrations higher than 0.4 M (mole fraction 0.0071), it starts to break down. Thus it appears that when urea concentration becomes higher the solvent properties play an important role. As the concentration is increased the enhanced solvation of the hydrocarbon tail of the amphiphile reduces the hydrophobic interactions between amphiphilic molecules. This means an increase of cac value and a decrease of size of the self-assembly.

3.4. Circular dichroism spectra

In a recent report [45], we have demonstrated the formation of chiral bilayer aggregates through hydrogen bonding in an aqueous solution of SAUV. Therefore, to investigate

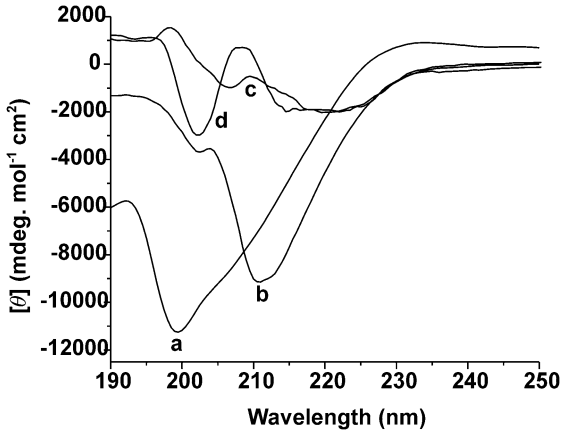

Fig. 7. Circular dichroism spectra of SAUV in water: (a) below cac, (b) above cac, and in the presence of (c) 3.0 M (mole fraction 0.051) and (d) 6.0 M (mole fraction 0.097) urea.

how the morphology of the bilayer aggregates changes we have measured CD spectra of the amphiphile in the presence of urea. The CD spectra (Fig. 7) of a 2.5 mM aqueous solution of SAUV containing 3.0 (mole fraction 0.051) and 6.0 M (mole fraction 0.097) urea, where, in general, the surfactant molecules are not expected to form any aggregate, were recorded. The spectra in the presence of urea are red-shifted relative to that in pure water. Though the molar ellipticity of the red-shifted band at around 220 nm is less than that in pure water, it clearly indicates the presence of chiral helical aggregates. We have also shown that in aqueous methanol solution there is a CD band at ∼215 nm corresponding to the formation of chiral helical structure through aggregation. Formation of similar type of chiral aggregates has been reported for other optically active *N*-acyl amino acid surfactants in methanol solution [56]. It is well known that the hydrogen bonds might break under certain conditions. Fasman [57] has reported that urea breaks hydrogen bonds. Therefore, it can be concluded that the decrease of the intensity of the CD band is due to the partial breaking of the intermolecular amide hydrogen bonds causing disruption of the bilayer self-assembly.

4. Conclusion

The effects of addition of urea on the bilayer self-assembly of SAUV in water were investigated. At low concentrations (<0.3 M, mole fraction 0.0054), the urea molecules were found to be located in the surfactant bilayer–water interface. The replacement of water molecules from the hydration shell of the amphiphiles by urea molecules leads to the decrease of the cac and increase of the size (R_h) of the vesicles. That is, formation of bilayer-type self-assemblies is facilitated in the presence of low concentrations of urea. However, higher concentrations (>0.3 M, mole fraction 0.0054) of the additive result in disruption of the organized assemblies as indicated by the decrease in R_h value. Moderately high concentration of urea is unable to break the intermolecular amide–amide hydrogen bonds in the bilayer self-assemblies of SAUV. The results of fluorescence anisotropy and light-scattering studies data eliminates the hypothesis of direct mechanism of action of urea. Therefore, it appears that the perturbation of the organization of the molecular assemblies by urea is due to its ability to enhance solvation of the hydrophilic surfactant headgroup through localization at the bilayer–water interface at low concentrations. However, at higher concentrations, the urea molecules act as “water structure breaker” resulting in a disruption of the bilayer spherical vesicles of SAUV. It has been shown that the bilayer structures of SAUV are stable even in the presence of 6.0 M (mole fraction 0.097) urea at which most protein structures get denatured in water.

Looking at the results reported in the literature we have a feeling that the apparently conflicting results of various surfactant systems might be due to the difference in microstructures of their molecular self-assemblies and the chosen concentration range employed for the study. Further systematic studies on the effects of organic additives on well-known bilayer-membrane-forming lipids are currently being carried out in our laboratory.

Acknowledgments

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