J Fluoresc (2010) 20:191–196
DOI 10.1007/s10895-009-0537-0

ORIGINAL PAPER

Effective Critical Micellar Concentration of a Zwitterionic Detergent: A Fluorimetric Study on *n*-Dodecyl Phosphocholine

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Received: 29 May 2009 / Accepted: 25 August 2009 / Published online: 16 September 2009

Abstract We have investigated the effect of ionic strength on the aggregation behavior of *n*-dodecyl phosphocholine. On the basis of the classical Corrin–Harkins relation, the critical micellar concentration of this detergent decreases with a biphasic trend on lithium chloride addition. It is nearly constant below 150 mM salt, with a mean value of 0.91 mM, whereas it undergoes a dramatic 80-fold decrease in 7 M LiCl. Such a drop in the critical micellar concentration could be explained by the effect of salting out and the implication of phosphocholine head groups on the organization of surrounding water. Knowledge of the effective critical micellar concentration of *n*-dodecyl phosphocholine could be useful in the purification of membrane proteins in non-denaturing conditions.

Keywords *n*-dodecyl phosphocholine · Critical micellar concentration · Ionic strength · Fluorimetric determination · 1,8-ANS · Effective CMC · Zwitterionic surfactants · Membrane protein solubilization · Membrane mimics

Since pioneering studies on the aggregation behavior of long-chain electrolytes [1–4], it has been known that salts drastically decrease the critical micellar concentration (CMC) of charged surfactants, because they reduce the repulsion between charged head groups, thereby helping micelles to be formed at lower monomer concentrations. This observation is usually interpreted in terms of hydrocarbon and electrostatic contributions to the Gibbs energy change of micelle formation [4]. The latter, in turn, depends on the charge of the micellar system as well as on the degree of counterion binding, and is also affected to some extent by the chemical nature of the counterion [4]. According to this view, the absence of charge interactions causes the effect of salts on the CMC of nonionic surfactants to be less pronounced. Zwitterionic surfactants are electrically neutral, but the charge they carry in the head group does influence their hydrophilicity and causes their properties lie between those of ionic and nonionic surfactants.

Most zwitterionic molecules are employed in structural biology studies because of their ability to solubilize membrane proteins and receptors, usually at pre-micellar concentration [5]. Among these surfactants, *n*-dodecyl phosphocholine (DPC), also known as FC-12, is the most widely used, together with anionic sodium dodecyl sulfate (SDS). DPC belongs to single chain phosphocholines, which are likely to inherit the advantageous features from natural lipids, because their head groups offer unique features in protein containing environment and are expected to retain the main functionality of phosphocholine (PC) groups as observed in biopolymers. Indeed, the PC group is identical with that in phospholipids but the single hydrophobic tail of DPC leads to formation of micelles rather than bilayers. DPC and other single chain PC surfactants display several advantageous features, such as cell membrane mimics in peptide and protein solubilization, antifungal and anti-bacterial activity, aid in the liposomal solubilization of synthetic heme constituting the prosthetic non-polypeptide group within hemoglobin or myoglobin, oxygen transport activation, and alveolar pulmonary surfactant action [6]. Finally, like other surfactants that are largely used to characterize in vitro proteins and/or enzymes that function anchored to a membrane environ-

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ment in vivo [7], DPC has found increasing use in NMR studies on membrane proteins [8–14], because it has been established as an excellent micelle system to obtain high-resolution spectra. It has also been shown to play a crucial role in refolding misfolded membrane proteins, using a procedure referred to as reconstitutive refolding [15].

In this paper, we report the CMC determination of DPC in high salt, which, to our knowledge, has not been investigated to date. To this aim, we took advantage of the extreme sensitivity of the photophysics of 1-anilinonaphtalene-8-sulfonate (1,8-ANS) to changes in the probe environment. Indeed, 1,8-ANS is essentially nonfluorescent in water, only becoming appreciably fluorescent when bound to a nonpolar matrix [16]. Therefore, it has been used as an indicator of protein folding and other processes that modify the exposure of the probe to water, such as detergent aggregation [17, 18]. Our results show that the CMC of DPC occurs at pre-micellar concentrations as compared to water, decreasing as the LiCl concentration increases. This information could be useful in membrane mimics experiments and in studies on membrane proteins or receptors, in which knowledge of the dependence of the CMC on ionic strength is crucial to preserve their structure and function.

Material and methods

Chemicals and solutions

Ultrapure DPC was purchased from Avanti Polar Lipids, Inc. 1-Anilinonaphthalene-8-sulfonic acid magnesium salt dihydrate was from Fluka. In order to explore a large ionic strength interval, LiCl from Fluka was chosen as the salt because of its very high solubility. Stock solutions of these substances were always prepared using bidistilled water and used without any further treatment throughout experiments, and their concentrations were 20×10^{-3} M (i.e., well above the CMC, which is about 0.9×10^{-3} M in water), 10 M, and 51.4×10^{-6} M, for DPC, LiCl, and 1,8-ANS, respectively. This last concentration was estimated using a molar absorptivity coefficient of 6,800 M^{-1} cm^{-1} at 374 nm [19].

Fluorescence

The fluorescence of 1,8-ANS was excited at 374 nm at room temperature, and emission spectra were recorded from 450 to 600 nm, using 10.0 mm×5.00 mm quartz cells in a Varian Model Cary Eclipse spectrofluorimeter. Five nanometers excitation and emission bandwidths were used throughout the experiments, with a scan speed of 120 nm/min and a time constant of 2 s. The final concentration of 1,8-ANS in each sample was 5.14×10^{-6} M, which was in the linear range of fluorescence intensity changes originated by the interaction of the probe with micelles versus fluorophore concentration. To avoid dilution errors due to the addition of subsequent surfactant aliquots to a given salt solution, fluorescence measurements were carried out exploring several DPC concentrations, for each of which a series of 0–7 M LiCl solutions was prepared. In any case, a DPC-free solution was used for blank correction.

Evaluation of the critical micellar concentration

The CMC of DPC was evaluated by linear least squares fitting of the 1,8-ANS fluorescence intensity at 490 nm versus the surfactant concentration. Points before and after the change of slope were fitted to two straight lines, at the intersection of which the CMC was calculated as the negative ratio of the intercept difference (A) to the slope difference (B), $-\mathrm{CMC} = A/B$, as previously described [18]. As the CMC is a function of two measured independent variables (A and B), the accuracy of measurements, expressed as ΔCMC, was obtained by error propagation, taking the partial derivatives of CMC with respect to each variable, multiplying with the error in that variable (ΔA and ΔB, respectively), and adding these individual terms in quadrature:

$$\Delta\mathrm{CMC} = \sqrt{\left[\left(\frac{\partial \mathrm{CMC}}{\partial A}\right)_B \Delta A\right]^2 + \left[\left(\frac{\partial \mathrm{CMC}}{\partial B}\right)_A \Delta B\right]^2}. \quad (1)$$

It can be easily verified that this procedure is equivalent to the root mean square (RMS) method, as it results in

$$\Delta\mathrm{CMC} = \sqrt{\left(\frac{1}{B}\right)^2 \left[\Delta A^2 + (\mathrm{CMC}\cdot\Delta B)^2\right]} = \mathrm{CMC}\sqrt{\left(\frac{\Delta A}{A}\right)^2 + \left(\frac{\Delta B}{B}\right)^2}, \quad (2)$$

which is the absolute RMS error. Here $\Delta A/A$ and $\Delta B/B$ are the relative errors on A and B, respectively. Data analysis was performed by the program Scientist for Windows version 2.0 by MicroMath Scientific Software.

Results

The principle of any fluorimetric determination of the CMC hinges on sharp changes in the photophysical properties of a ‘reporter’ molecule able to interact with a surfactant aggregate [4]. As a rule, this approach might not work if probe and detergent have opposite charges [5], but the charge of the probe should not be important in assays

involving zwitterionic surfactants. In this regard, 1,8-ANS is an anionic two-ringed probe that can be described as able to assume either a strongly fluorescent coplanar geometry in presence of a nonpolar environment, such as that of a micellar core, or a weakly fluorescent non-coplanar arrangement in water, with emission maxima around 490 and 520 nm, respectively [16]. Preliminary spectra of 1,8-ANS in aqueous solution were obtained in presence and absence of micellar DPC. As can be appreciated from Fig. 1, both the blue shift of the fluorescence emission and the huge increase of the fluorescence emission intensity on DPC addition suggest that this probe can be reliably used to detect DPC aggregation. Indeed, the fluorescence enhancement is known to be caused by incorporation of 1,8-ANS into the lipid-like environment of micelles, which results in a pronounced increase of the quantum yield [16]. The shape of these spectra did not appreciably depend on viscosity and ionic strength.

Figure 2 shows some representative fluorescence titration profiles of 1,8-ANS in various conditions. In a typical plot, the fluorescence intensity of 1,8-ANS remains weak on DPC addition, until a rapid rise occurs after a breakpoint. This provides evidence that 1,8-ANS is able to interact with micellar DPC, showing the effect of the close vicinity with a nonpolar environment, even if it might not be embedded in the micellar core. On further addition of DPC, the number of micelles increases with a concomitant increase in the amount of bound 1,8-ANS causing an increase in fluorescence. Thus, assuming that the different slopes reflect the interaction of the probe with free and micellar surfactant, respectively, the surfactant concentration that is taken as the CMC corresponds to the breakpoint, and can be calculated as the intersection between the linear regressions through the fluorescence intensities before and after the breakpoint, as described under the experimental section.

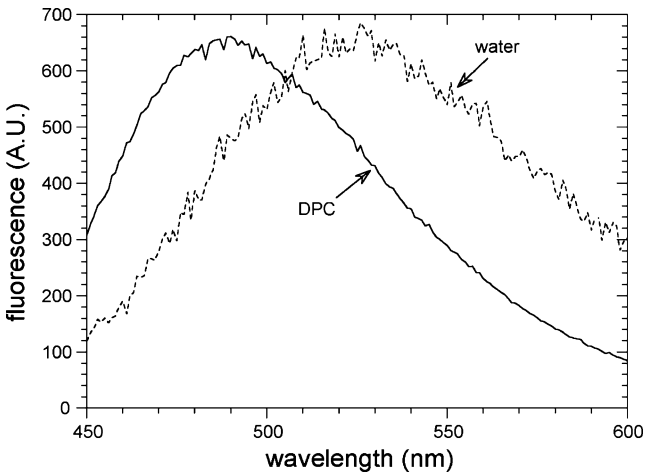

Fig. 1 Fluorescence spectra of 1,8-ANS. The spectrum in water has been amplified 60-fold as compared to the actual one

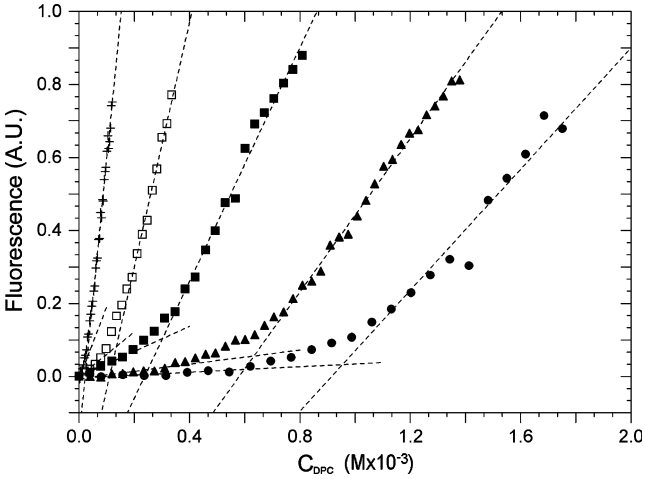

Fig. 2 Critical micellar concentration of DPC in various media. (+) 5.0 M LiCl; (□) 3.0 M LiCl; (■) 2.0 M LiCl; (▲) 0.5 M LiCl; (●), H_2O

The whole set of experimental data is listed in Table 1. Error analysis results in a relative error of 25.8 and 4.5% in the worst and in the best case, respectively, and of 9.8% on the average. It can be appreciated that the CMC in pure water is in good agreement with values between 0.9 and 1.1 mM from light scattering [8, 20], surface tension [6, 21], ^{31}P NMR [22], fluorescence [23], and MD simulation [24], but significantly lower than 1.3 mM from fluorescence [25] and contact angle [26], and 1.5 mM, as reported by Anatrace Inc. [27]. As for the ionic strength dependence, the drop of the CMC from (0.96±0.08) mM in pure water to (0.88±0.08) mM in 100 mM LiCl can be compared with a recent study by surface tension measurements [6], where the CMC of DPC has been found to drop to (0.75±0.05) mM and (0.71±0.05) mM by effect of 0.1 M NaCl and $CaCl_2$, respectively, as compared to (0.91±0.05) mM in pure water. Thus, within the accuracy of our measurements, our data overlap values in water and in 0.1 M NaCl from surface tension, while the effect of ionic strength is obviously larger in 0.1 M $CaCl_2$. In the light of this agreement, the value of 0.12 mM (at 20–25 °C and ~50 mM Na^+) reported by a handbook covering integral membrane proteins, multiprotein complexes, and inclusion bodies [28] appears largely underestimated.

Further increase of the salt concentration up to 7 M causes an 80-fold decrease in the CMC. Such a drop can reasonably be ascribed to reduced free water left for hydration of the DPC head group. It resembles that usually observed for charged surfactants at low ionic strength, but a double-logarithmic analysis of the whole set of data results in a hyperbolic plot (not shown). This is different from the CMC of charged surfactants, which is known to follow the

Table 1 Effect of ionic strength on the critical micellar concentration of DPC

C_{LiCl} (mM)	–A	ΔA/A	B $(mM)^{-1}$	ΔB/B	CMC (mM)	RMS error (mM)
0	0.756	0.067	0.792	0.057	0.955	0.084
10	0.686	0.074	0.754	0.060	0.910	0.086
20	0.718	0.070	0.790	0.057	0.909	0.082
30	0.757	0.067	0.842	0.054	0.899	0.077
40	0.768	0.066	0.828	0.054	0.927	0.079
50	0.792	0.064	0.850	0.053	0.932	0.077
60	0.742	0.068	0.822	0.055	0.902	0.079
70	0.805	0.063	0.866	0.052	0.930	0.076
80	0.785	0.064	0.866	0.052	0.907	0.075
90	0.746	0.068	0.852	0.053	0.876	0.075
100	0.729	0.069	0.825	0.055	0.884	0.078
150	0.715	0.071	0.808	0.056	0.885	0.080
200	0.518	0.098	0.718	0.063	0.722	0.084
250	0.657	0.034	0.904	0.029	0.726	0.033
300	0.700	0.040	0.958	0.032	0.731	0.037
400	0.710	0.037	1.061	0.028	0.670	0.031
500	0.604	0.043	0.954	0.037	0.634	0.036
1,000	0.594	0.052	1.273	0.059	0.466	0.036
2,000	0.380	0.102	1.257	0.060	0.302	0.036
3,000	0.369	0.085	2.731	0.063	0.135	0.014
4,000	0.319	0.118	4.282	0.128	0.074	0.013
5,000	0.167	0.173	5.722	0.192	0.029	0.008
6,000	0.297	0.103	12.581	0.078	0.024	0.003
7,000	0.227	0.110	19.420	0.063	0.012	0.001

linear double-logarithmic Corrin–Harkins relation. Indeed, Fig. 3 shows that satisfactory linearization of data can be achieved only plotting the logarithm of the CMC versus the salt concentration, as previously reported for other neutral detergents [5, 29, 30].

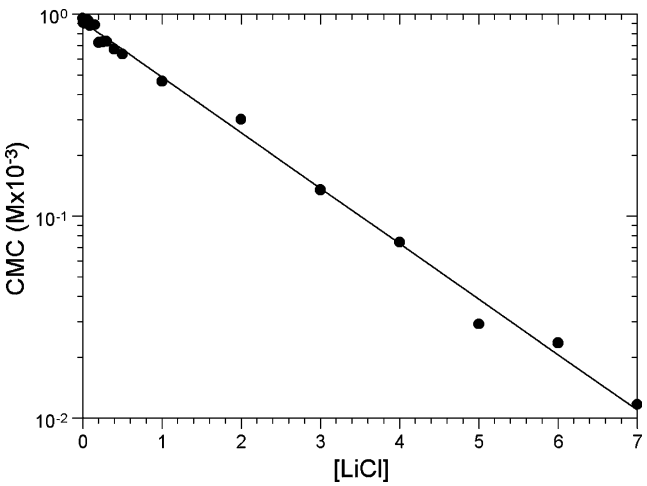

Fig. 3 Semi-logarithmic plot of CMC versus salt concentration. Linear regression of data, with logCMC = constant–k_sC_s, gave: constant = −0.037; k_s=−0.275 L mol^{-1} (r^2=0.996)

Discussion

The main purpose of the present study was to investigate the effect of ionic strength on the CMC of zwitterionic DPC, which, to our knowledge, has not been investigated in depth to date. We have found that, on the basis of the double-logarithmic Corrin–Harkins relation, the CMC of this detergent changes with a biphasic trend on LiCl addition. In fact, it is nearly constant below 150 mM salt, where it starts undergoing a dramatic 80-fold decrease up to 7 M LiCl. A change in the structure of DPC micelles with increasing LiCl content could likely occur, but this matter is outside the aim of the present study. An extensive analysis, including comparison with other salts, such as sodium, potassium and calcium halides, will be presented elsewhere. Meanwhile, the satisfactory semi-logarithmic inverse linear dependence with salt concentration suggests that the mechanism for DPC behavior can be regarded as originating from salting out of the nonpolar moiety and the implication of PC head groups on the organization of surrounding water [6], consistently with the observation from other zwitterionic surfactants [31–33]. Thus, it can be interpreted in terms of a salt-effect constant, based on the application of the principles of salting-out of nonelectrolytes by electrolytes [34]. This theoretical approach has

recently been expanded by incorporating an additional electrolyte effect on the interfacial tension of the micelle-water interface, providing a nearly quantitative explanation of the experimental CMC data up to concentrations of LiCl and NaCl comparable with those used in this study [30].

The second point that is worth discussing is the relevance of our data for the structural proteomics of membrane proteins. The structural characterization of such a molecule begins with its detergent solubilization from the lipid bilayer and its purification within a functionally stable protein-detergent complex. Then, crystallization may be achieved changing the solution environment to promote interactions between exposed hydrophilic surface residues and decrease the solubility. In this regard, membrane proteins have been observed to form crystals close to the phase separation boundaries of the detergent used to form the protein-detergent complex, and knowledge of these boundaries under different chemical conditions provides a foundation to rationally design crystallization screens. Most recent efforts are devoted to detergent phase partitioning studies utilizing different combinations of polyethylene glycols, salts (among which LiCl), and detergents (among which DPC) to generate a significant amount of chemically diverse phase boundary data [35]. Thus, surfactants are extremely important in preventing membrane proteins and/or receptors from forming insoluble aggregates due to their hydrophobic nature, and are common components of the solutions used to crystallize these molecules.

Our measurements could be useful in this field, considering that the use of DPC is widespread in the optimization of purification procedures thanks to its non-denaturing properties [36–40], and that 0.05–7.2 M LiCl has been employed in most recent design of crystallization screens [35]. Since earlier observations, the CMC is considered an important physicochemical characteristic of a given detergent [41, 42]. However, knowledge of the mechanism by which surfactants display their highest efficiency in preserving the integrity of membrane proteins below the CMC in water, but cause loss of function above the CMC, is poor. This has led to the concept of effective critical micelle concentration (CMC_{eff}), which has been defined as the concentration of surfactant monomer that is able to preserve the membrane protein's structure-function integrity in the specific system under study [41, 43]. Indeed, our data look almost linear at high LiCl concentration, which may strongly affect the solubilization of membrane proteins in DPC-containing mediums, and explain why the CMC_{eff} is generally lower than the CMC in water. In their studies on a number of membrane-bound receptors from the central and peripheral nervous systems [5], other authors have shown that the solubilizing efficiency of CHAPS, another zwitterionic detergent largely employed in the reconstitution of membrane proteins, increases with increasing the salt concentration, thus inferring that this was actually to be ascribed to significant lowering of the CMC due to the presence of salt. This suggests that it is highly desirable to extend this kind of studies to other zwitterionic detergents.

Summary

We have investigated the effect of LiCl on the CMC of DPC. Presumably, the aggregation behavior of DPC at low ionic strength can be ascribed to the fact that its PC head group can efficiently counterbalance salting out effects. Indeed, our results agree with constancy of the CMC, as commonly reported at ionic strengths below the physiological limit. However, on increasing ionic strength, DPC aggregation displays a non-linear Corrin–Harkins behavior, as observed for other zwitterionic surfactants, with a 80-fold CMC decrease in 7 M LiCl as compared to water. This seems to offer new insights into the ability of DPC to solubilize membrane proteins and receptors under conditions that preserve their structure and function.

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