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Pilar Perez-Tejeda*, Rocío Risquez, A. Rosa Perez, Antonia Terriza, M. Pilar Leon.

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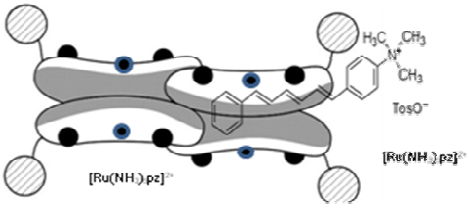
Non-conclusive results on bile salt solutions: the use of TMA-DPH species for CMCs determination

Pilar Perez-Tejeda*, Rocío Risquez, A. Rosa Perez, Antonia Terriza, M. Pilar Leon.
Physical Chemistry Department, Faculty of Chemistry, Seville University, C/ Profesor García González, s/n, 41012, Sevilla, Spain. E-mail: pptejeda@us.es

Graphical Abstract



Scheme showing binding to cholate aggregates

Abstract: A study of aggregation behaviour of cholate and deoxycholate anions (as sodium salts) in aqueous solutions at 298.2K is reported here. Two CMCs (primary and secondary critical micellar concentrations) of both bile salts have been determined using TMA-DPH (NNN-Trimethyl-4-(6-phenyl-1,3,5-hexatriene-1-yl) phenylammonium-p-toluenesulfonate) as a probe molecule in the presence of $[Ru(NH_3)_5pz]^{2+}$ (pz=pyrazine), and in its absence, for comparison of the results**.** These CMCs were obtained from shifts of the TMA-DPH absorption spectrum as a function of bile salt concentration. Although our results suggest the existence of two CMCs, they can also be explained by taking into account a single CMC. Therefore, in this sense, these results cannot be considered as conclusive; the probe molecule (TMA-DPH) does not provide sufficient information on the existence of the secondary aggregates of bile salts.

Keywords: Bile salts, cholate anion, deoxycholate anion, TMA-DPH, critical micellar concentration

1. Introduction

Bile salts are natural amphiphilic compounds that are synthesized in the liver and stored in the gallbladder.[1] They are the most important natural surfactants, being responsible for the solubilisation of lipids, cholesterol, bilirubin, lecithin, and fat-soluble vitamins in living organisms.[2] They also control bile acid and cholesterol biosynthesis by secretory and regulatory properties, and enhance the intestinal absorption of Ca^{2+} and Fe^{2+} by their complexation properties for cations.[2]

Given the relationship between the physicochemical properties and their physiological functions it is not surprising that bile salts have been extensively studied by using different experimental techniques to determine their properties. Such methodologies have been widely employed to understand the biochemistry of bile salts.[2]

In particular, the aggregation number, critical micelle concentrations (CMCs) and the number of counterions bound to the aggregates formed in aqueous solutions are of interest. These quantities are related to the controversy about the aggregation process in bile salt complexes, which can lead to the formation of a CMC as in the common alkyl surfactants or rather to a stepwise self-association.[3,4]

The use of various spectroscopic techniques,[5-10] as well as osmometric[11] and electrochemical methods,[11] revealed that the structure of bile salt aggregates is more complex than those of conventional micelles, for example, those of sodium dodecyl sulphate. Recently, molecular dynamic simulation studies[12] have also shown that the aggregation feature and the shape of the micelles of the bile anions are different from those of common alkyl surfactants. This fact arises because bile salts do not possess the polar head groups and the non-polar

steroid moiety.[13] That is, bile salts exhibit planar polarity with hydroxyl groups generally located on one face and methyl groups on the opposite. A consequence of this planar polarity is that the shape of the bile salt aggregates is different from classical surfactant micelles.

As shown in Fig. 1, NaC is a trihydroxy bile salt; however, there are also dihydroxy salts such as sodium deoxycholate (NaDC) in which the two OH groups are in 3α and 12α positions. It is known that, in general, the dihydroxy salts form aggregates larger than trihydroxy salts, as well as a higher hydrophobic character than those of homologous trihydroxy bile salts.[14]

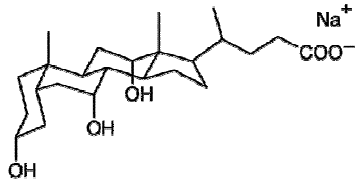


Figure 1. Molecular structure (stereochemical depiction) of sodium cholate (NaC) (3α, 7α, 12α-Trihydroxy-5β-cholan-24-oic acid)

One of the most widely used models for aggregation of bile salts is the primary-secondary micelle model.[14-16] Accordingly: i) the monomers of bile salts are oriented with the hydrophilic faces outwards, at the contact with water; ii) the bile salt aggregates exhibit two critical micellar concentrations, which are referred to as primary (CMC_1) and secondary (CMC_2) critical micellar concentrations. Above CMC_1, bile salts form primary aggregates with a small number of monomers (3-10). These aggregates are constituted by association with the hydrophobic faces of the monomers, which leads to the formation of a hydrophobic binding site. Above CMC_2, the primary aggregates are agglomerated to form larger secondary aggregates. According to recent studies (small-angle X-ray scattering and small angle neutron scattering)[17] the structure of secondary aggregates resembles an elongated rod with a central core filled with water and the ions (hydrophilic binding site or biocavity). The latter structure has also been recently observed by computer simulations.[18]

However, the helical model suggests that, in aqueous solutions, bile salt aggregates are formed by association with the hydrophilic faces of the monomers, which give rise to helices stabilized by polar interaction. In most of the cases, the aggregation numbers in the helix are three or multiples of three. As the concentration of the salt monomer is raised, the helices form oblate and cylindrical aggregates. From the point of view of this model, the concept of CMC is questionable for bile salts, which very probably give rise to a continuous self-aggregation as a function of concentration, pH, ionic strength, and temperature.[10]

Both aggregation models, however, take into account the stepwise nature of the bile salts aggregation.[8] Evidence for stepwise aggregation and polydispersity of aqueous bile salt solutions is the critical micellar concentration (CMC) broadening phenomena, and consequently, sometimes the appearance of two CMCs.[3,4,19]

Nevertheless, whether the polydispersity of the aqueous bile salt solutions is manifested or not depends on factors such as bile salt type and experimental conditions.[20] These events demonstrate the inherent complexity of aqueous solutions of bile salts. Hence, the study of aggregation and its impact continues to be of interest.

A method for determining the CMCs of the cholate and deoxycholate aggregates is reported here, using TMA-DPH (NNN-Trimethyl-4-(6-phenyl-1,3,5-hexatriene-1-yl)phenylammonium-p-toluenesulfonate) (Fig. 2) as a probe molecule. To our knowledge, TMA-DPH has not been used as a probe for obtaining CMC values. Other aromatic probes such as, for example, pyrene and berberine alkaloids were previously used.[4, 21] However, given that, the objective is to obtain two CMCs, one in the hydrophobic region and another in the hydrophilic or biocavity region, it was necessary to use an amphiphilic molecule as TMA-DPH.

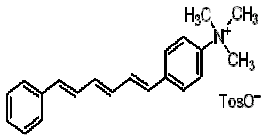


Figure 2. Molecular structure of TMA-DPH

The experiments were performed in the presence of $[Ru(NH_3)_5pz]^{2+}$ (pz=pyrazine), and in its absence, for comparison of the results**.** We have used the $[Ru(NH_3)_5pz]^{2+}$ complex to access the change in CMCs due to the presence of this ruthenium complex (of opposite charge signs to those of cholate and deoxycholate aggregates) in solution. The main reason for this choice is that CMC values are necessary in advance, in order to obtain binding equilibrium constants from kinetic data.[22] That is, our idea was the same as in the case of the study of $[Ru(NH_3)_5pz]^{2+}$ + $[Co(ox)_3]^{3-}$ reaction in the presence of SDS or CTACl micelles.[23]

Therefore, using TMA-DPH as a probe molecule CMCs were obtained in the presence of $[Ru(NH_3)_5pz]^{2+}$, and in its absence. Nevertheless, the results are negative in the sense of that it is impossible distinguish clearly the existence of the secondary aggregates of bile salts, and thus the existence of a second CMC. Consequently, the probe molecule (TMA-DPH) does not provide sufficient information on the existence of the secondary aggregates of bile salts.

2. Experimental Section

2.1 Materials

The complex $[Ru(NH_3)_5pz]^{2+}$ (pz=pyrazine), as perchlorate salt was prepared and purified according to the procedures described in the literature.[24] NaC, 3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid (Fig. 1), NaDC,3α,12α-Dihydroxy-5β-cholan-24-oic acid sodium salts and TMA-DPH (Fig. 2) were obtained from Sigma Ultra and used as purchased. The water used in the preparation of the solutions had a conductivity of about 10^{-6} S m^{-1}.

2.2 pH Measurements

The pH of the bile salt solutions was measured in a micropH2000 from Crison at 298.2 ± 0.1K. The pH of NaC and NaDC solutions does not change significantly when the concentration increases; their values being 7.1, 7.2 and 7.7 for $6.2x10^{-3}$, 11.0 $x10^{-3}$ and 0.3 mol dm^{-3} of bile salts, respectively, in good agreement with previously reported data,[21] therefore all the experiments were realized in the absence of a buffer.

2.3 Spectroscopic Measurements

The critical micellar concentrations were determined from shifts of UV-visible absorption spectra of a sensitive probe, TMA-DPH, whose concentration was $2.0x10^{-5}$ mol dm^{-3}, as a function of the NaC or NaDC concentration. The spectra of TMA-DPH were obtained in both the absence and presence of $[Ru(NH_3)_5pz]^{2+}$ species. All spectra were corrected from little absorption of NaC or NaDC and those in the presence of the ruthenium complex from the tail of the absorption band of this complex (molar absorption coefficients from 300nm to 400nm about 6250 to12500 mol^{-1} dm^3 cm^{-1}, respectively). According to the preliminary experiments, the spectra of TMA-DPH, NaC and NaDC did not change in the presence of the ruthenium complex. All spectra were recorded on a Cary 500 Scan spectrophotometer at a fixed temperature of 298.2 ± 0.1K. The resolution of that spectrum was registered as ± 0.1nm.

3. Results and Discussion

Generally, the presence of ions bearing opposite charge signs from those of head groups of the surfactants facilitates the micellization process. That is to say, the CMC values are smaller in the presence than in the absence of these types of ions. In order to get these values for NaC and NaDC aggregates, TMA-PDH was used as a sensitive probe. Fig. 3 shows the absorption spectrum of TMA-DPH in the presence of NaC, as an example (a figure containing TMA-DPH spectra in the presence of NaDC is included into the Supplementary Material).

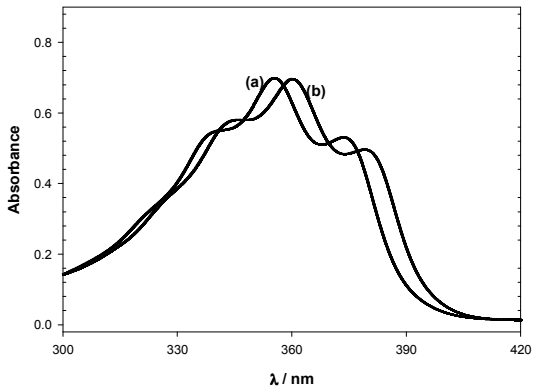


Figure 3. Shift of TMA-DPH absorption spectrum in the presence of NaC solutions of concentrations: a) $2.0x10^{-3}$ and b) $4.0x10^{-2}$ mol dm^{-3} at 298.2 K.

TMA-DPH spectrum shifts towards a longer wavelength (see Fig. 3) upon the addition of gradually increasing amounts of NaC and NaDC. Spectral variations appear at approximately $6x10^{-3}$ and $2x10^{-3}$ mol dm^{-3} for NaC and NaDC, respectively, and levels off at about $11x10^{-3}$ and $6x10^{-3}$ (or $4x10^{-3}$) mol dm^{-3} for NaC and NaDC, respectively. The observed bathochromic shifts in the presence of the cholate and deoxycholate aggregates indicate an association (inclusion) between the molecule probe and the bile salt aggregates. That is, TMA-DPH interacts favourably with primary aggregates of NaC and NaDC, which have a hydrophobic character.[17] The levelling of the spectrum could be explained considering that TMA-DPH, with the ability to bind to primary sites, remains in this site even when secondary aggregates are present.[25]

Accordingly, theses different interactions can be used to measure the critical micellar concentrations, CMC_1 and CMC_2, as is depicted in Figs. 4 and 5 for NaC and NaDC, respectively. The intersection point of the straight lines gives the values of the CMC_1 and CMC_2.

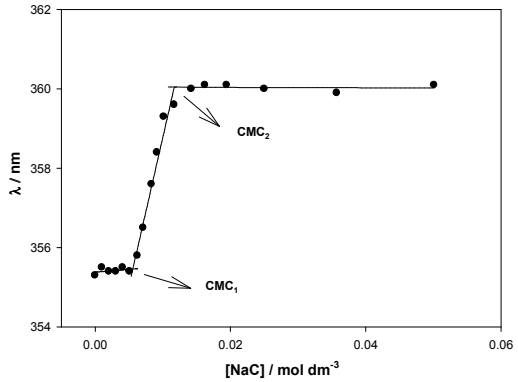


Figure 4. Maximum of TMA-DPH absorption spectrum positions as a function of NaC concentration in order to obtaining CMC values.

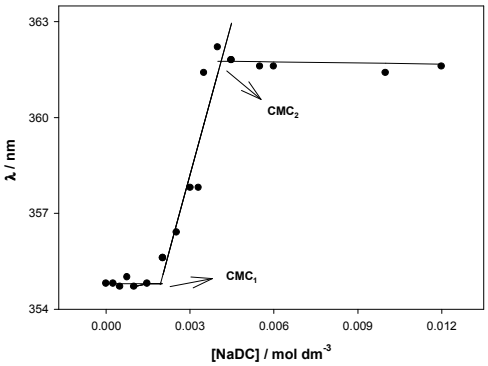


Figure 5. Maximum of TMA-DPH absorption spectrum positions as a function of NaDC concentration in order to obtaining CMC values.

In Table 1 CMC values are collected in the absence and presence of the $[Ru(NH_3)_5pz]^{2+}$ complex (see Introduction Section). Those in its absence are in agreement with previously published data corresponding to non-invasive methods. [3, 26, 27] For the case of deoxycholate aggregates, both in the presence and absence of ruthenium species, CMC values are smaller than those for cholate aggregates, suggesting that the aggregation number of deoxycholate aggregates is greater than for cholate aggregates, which is a result of increased hydrophobic character of deoxycholate anion in relation to its homologous cholate species.[14]

Table 1. Values of CMCs for NaC and NaDC aggregates at 298.2K in the absence and presence of the $[Ru(NH_3)_5pz]^{2+}$ complex.

Bile Salt	10^3xCMC$_1$/ mol dm^{-3}	10^3x CMC$_2$/ mol dm^{-3}
NaC (absence of complex)	6.9	11.8
NaC (presence of complex)	6.2	11.2
[a] NaC	6.2	12.8
NaDC (absence of complex)	2.2	6.3
NaDC (presence of complex)	2.0	4.1
[a] NaDC	2.4	6.5

[a] *From refs. (3) and (4)*

Besides, as can be seen from Table 1, CMC values in the presence of the ruthenium complex and in its absence are not particularly different for the case of cholate aggregates. Curiously, the data of CMCs in the presence of NaCl (0.10 mol dm^{-3})[26, 27] are almost the same as those of this work. In the case of NaDC aggregates, however, the CMC$_2$ values are smaller in the presence of the ruthenium complex than in its absence. Thus, the results in the presence of the ruthenium complex show that the NaC aggregates are less sensitive to the addition of the electrolytes than those of NaDC. In conclusion, it should be noted that the addition of ions carrying a charge of opposite signs to that of the bile salt aggregates has a minor influence on the CMC. However, this is not what happens in the case of alkyl common surfactant micelles, such as those of SDS.[28] This behaviour suggests that the Stern layer,[29] either does not exist or it is not well defined, as in conventional surfactants according to Coello *et al.*[11] and Zana *et al.*[7]

Finally, although the above discussion has been based on the existence of two CMCs, the results shown here can also be explained taking into account a single CMC. That is, the two concentrations of NaC and NaDC that cause abrupt changes in the positions (wavelengths) of the TMA-DPH spectrum can also be taken as the beginning and the end of the aggregation process rather than as two CMCs. In fact, the sigmoid curves shown in Figs. 4 and 5 are characteristic of common alkyl surfactant micelles, in which only a single CMC instead of two is considered. [30] In this regard, these results cannot be considered as conclusive. The probe molecule (TMA-DPH) therefore, does not provide sufficient information on the existence of secondary aggregates of bile salts.

4. Acknowledgements

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22. Usually, from the rate constant variation when the concentration of a receptor changes, one can obtain a binding constant between a receptor (DNA, micelles, cyclodextrins, bile salt aggregates,...) and one or both reactants (ligands). For this purpose, the two states model (free ligand and associated ligand to the receptor) can be used as a starting point. However, to carry out such a purpose for the cases of alkyl surfactant micelles and bile salt aggregates it is necessary to know CMC values and if these concentrations change in the presence of the reactants (ligands).
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