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Micellization and adsorption behaviour of bile salt systems

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Abstract

The interfacial and bulk phase properties of sodium cholate (NaC), sodium deoxycholate (NaDC), sodium taurocholate (NaTC), sodium taurodeoxycholate (NaTDC) and their equimolar binary and ternary combinations in aqueous solution have been investigated using surface tensiometry, conductometry, microcalorimetry, and fluorescence probing. The obtained experimental results are utilized to evaluate critical micellar concentration (*cmc*), counterion binding (*f*), surface excess, minimum area per molecule (A_{min}), thermodynamics of adsorption and micellization, and microenvironment of the bile-salt micelles. The magnitude of counterion binding and its temperature dependence was found to be low. Binary and ternary bile salt mixtures show higher and lower $A_{\min}$ values respectively compared with the $^{\text{ideal}}A_{\min}$ at the experimental temperatures. The low I_1/I_3 values of single and mixed bile salts suggest the microenvironment of the micellar entities to be nonpolar as hydrocarbon. The enthalpy of micellization values obtained following the methods of Van't Hoff and microcalorimetry have been compared. The composition of the mixed micelles have been estimated on the basis of regular solution, Clint, Rubingh and Rubingh-Holland theories along with the activity coefficients and interaction parameters at different temperatures.

Key Words: Sodium cholate (NaC), Sodium deoxycholate (NaDC), Sodium taurocholate (NaTC), Sodium taurodeoxycholate (NaTDC), Micelle, Microcalorimetry.

Introduction

Mixed surfactant systems are extremely important because of fundamental, technological, pharmaceutical and biological considerations. In recent years much attention has been devoted to the study of mixed solutions of natural and synthetic surfactants.[1-2] Combinations of amphiphilic compounds are particularly relevant in biochemistry and biology. Bile salts are biologically important molecules and considered as special class of surfactants due to their characteristic structural features, composed of hydrophobic and hydrophilic moiety and surfactant like properties.[3] Bile salts play crucial roles in a number of physiological processes[4-5] such as lipid digestion, drug absorption, cholesterol solubilization, and so forth. The physiological function of bile salt systems depend on the formation of mixed species involving bile salts and biological lipids. Bile salts form mixed micelles with the lipids[6] by solubilizing them. This phenomenon is of crucial importance in intestinal absorption of the products of fat digestion such as fatty acids, monoglycerides, sterols, and vitamins.[7] In combination with synthetic surfactants, bile salts are used in medicines. For example, mixed bile salt micelles prepared with phospholipids or salts of fatty acids such as sodium oleate, have been shown to be suitable as drug carriers for parenteral administration.[8] In addition, studies of the chemical stability of incorporated drugs have been reported.[9] The behavior of bile salt micelles is quite different from micelles formed by detergents. Bile salt micelles are smaller, more highly charged and of different structure than detergent micelles. Bile salts form mixed micelles with a variety of other soluble and insoluble lipidic substances. It is important to mention that bile salts are advantageous over conventional surfactants in that mixed micelles of bile salts are locally and systematically well tolerated and have proven to be neither embryotoxic nor mutagenic.[10] In the field of enzymology, bile salts have found wide applications as excipients in novel formulation to secure enzyme solubilization.[11]

Considerable efforts have been focused on the study of mixed solutions of bile salts and synthetic detergents as well as lipids from the view point of interfacial and bulk phase properties.[12-14] However investigations dealing with the binary mixtures of bile salts are conspicuously rare [15] while no reports on micelle formation and interfacial adsorption characteristics of ternary bile salt combinations are available to date, though studies dealing with ternary mixtures of a number of synthetic detergents are recorded[16-18]

This communication presents for the first time a comprehensive study on micellization and adsorption of the conjugated and non-conjugated bile salts, for example, sodium cholate (NaC), sodium deoxycholate (NaDC), sodium taurocholate (NaTC), and sodium taurodeoxycholate (NaTDC) and their binary and ternary combinations using conductometry, tensiometry, fluorescence spectroscopy, and microcalorimetry. The composition of the bile salt aggregates and intermicellar interaction parameters have been estimated following the models of Clint,[19] Rubingh,[20] and Rubing-Holland.[18] We believe that the obtained information will help to understand the behavior of bile salt systems *in vivo.*'[21]

Experimental Section

Materials

The sodium salts of Deoxycholic acid, Cholic acid, Taurocholic acid and Taurodeoxycholic acid obtained from Sigma Chemical Co., (purity ≥99%) were used as received. Pyrene, used as micellar probe in the fluorescence measurements, was a Sigma product with purity >98%. It was purified by sublimation in vacuum. Stock solution of pyrene was prepared in absolute ethanol. The quencher, cetylpyridinium chloride (CpCl, Aldrich), was recrystallized twice from the mixed solvent system of acetone and isopropyl alcohol. All the solutions were prepared in doubly distilled water. The stock solutions of the bile salt micelles were prepared by dissolving required amount of the bile salt in double distilled water which were further used to prepare solutions of desired concentrations. **Methods**

Surface tension. Surface tension of the solutions was measured with a Krüss 8 (Germany) tensiometer by the platinum ring detachment method. The measured surface tension values were corrected according to the procedure of Harkins and Jordan.[22] The concentrated surfactant solution was added in installments using a Hamilton microsyringe and measurements were made after thorough mixing and temperature equilibration. Temperature was maintained within ±0.1 ^{o}C by circulating water from a HAAKE GH thermostat. The measurements were made at 10, 20, 30 and 40 ^{o}C. The accuracy of measurements was within ±0.1 dyne cm $^{-1}$.

Fluorescence. Steady-state fluorescence experiments were carried out with a Kontron SFM 25 spectrofluorometer, connected to a PC computer. A 1 cm stoppered silica cell was used for the spectral measurements at four different temperatures as described above. Selecting 335 nm as the excitation wavelength of the pyrene (1x10^{-6} M), the emission spectra were collected from 360 to 420 nm. The micropolarity values were collected as the ratio of intensities of first (I_1) and third (I_3) vibronic peaks of pyrene which appear at 373 and 383 nm respectively. The aggregation number $\overline{N}$ was determined using the fluorescence quenching technique. Cetylpyridinium chloride (CPC) was used as the quencher. The aggregation number was obtained from the slope of the plots of quencher concentration [Q] vs $\ln\frac{I_0}{I}$ according to the equation:[23]

$$\ln\frac{I_0}{I} = \frac{[Q]\overline{N}}{([surf]-CMC)} \qquad (1)$$

The excitation wavelength was taken at 320 nm for the quenching experiments and the solutions were prepared according to Stam et al.[24]

Conductivity. Conductance measurements were taken by employing a Denver (USA) conductometer (Denver, model-50) using the cell with a cell constant of 1.01 cm^{-1}. The measured conductance values were accurate within ±0.5%. Conductivity measurements were taken as described earlier.[16]

Microcalorimetry. The microcalorimetric measurements were taken in an OMEGA Isothermal Titration Calorimeter of Microcal Inc., USA at 298 K by measuring heat change resulting from the injection of equimolar mixtures of bile salts into water. Aliquots of a concentrated solution of bile salts of appropriate

volume were added in several installments in suitable volume of water taken in a cell and the resulting heat flow versus time profiles were measured. The addition of bile salt solution and the measurements of heat were done as programmed. All the experiments were performed in triplicates to check the reproducibility.

Results and discussion

Critical Micelle Concentration (cmc)

The cmc's of individual and mixed bile salt systems have been determined by surface tensiometry, conductometry and microcalorimetry. The concentration corresponding to final break point in the plot of surface tension (γ) or conductivity versus logarithm of total molar surfactant concentration (C_t) is taken as the cmc of the single and mixed surfactant combinations. In the case of mixed bile salt systems, we could hardly locate a break point in the conductivity versus concentration plots probably due to very low counterion binding characteristics. A representative tensiometric profile of all the systems at 20 ^{o}C is shown in Fig. 1. A dip in the tensiometric plot of NaC single surfactant system could be due to the small amount of impurity. The cmc values of single bile salts and their equimolar binary and ternary mixtures at different temperatures are contained respectively in Tables 1s, 2s, and 3s of supporting information. However, the data for some representative bile salt systems are given in Table 1. The agreement among the cmc values obtained by different methodologies is found to be reasonably satisfactory. It is known that the cmc values of the bile salts reported in the literature are not always consistent.[13, 25] It is found that the cmc values of dihydroxy bile salts, NaDC and NaTDC are lower than those of trihydroxy salts, NaC and NaTC at all the temperatures studied. The obtained results are in keeping with the higher hydrophobic character of dihydroxy bile salts compared with that of trihydroxy bile salts. It is interesting to note that the bile salts possessing the same number of OH groups with taurine head groups have lower cmc values in comparison with those having COO^- head groups. This may be attributed to the bulky

taurine head group for taurocholates compared with the simple COO^- head group of the non-conjugated bile salts. Similar observations have been reported in literature.[18, 25] The cmc values of binary surfactant combinations usually fall in between the cmc values of individual components.[20] It is found that the cmc values of the binary mixed systems investigated herein, where at least one of the members is a dihydroxy bile salt, are either less than that of the component with lower cmc or fall in between as is found usually. The cmc value of NaC-NaTC mixture is found to be higher than that of the either components of the mixture. The cmc values of the equimolar ternary combinations are usually found to lie in between those of the component surfactants. However, it is noted that the cmc value of the NaC-NaTC-NaTDC mixture is lower than that of the component NaTDC.

The variation of cmc values of individual bile salts along with their equimolar binary and ternary mixed systems against temperature is shown in Fig. 2. The cmc values are found to decrease on increasing temperature from 10-30^oC beyond which the cmcs of single surfactants increase excepting NaTDC in which case a minimum cmc value is observed around 20 ^oC. Similar variation of cmc values of NaC, [25]NaDC,[25] NaTC,[18] and NaTDC[18] obtained employing different methodologies have been reported. Equimolar binary combinations of bile salts exhibit similar trend excepting NaTDC-NaDC and NaC-NaTC systems where continuous decreasing trend is observed. Recently Hildebrand et al.[25] studied cmc variation of NaC and NaDC and their equimolar mixtures with sodium oleate in the temperature range of 10-70 ^oC. On the basis of calorimetric results the authors attributed the appearance of minimum to entropy-enthalpy compensation phenomenon during micellization. The initial cmc decrease is ascribed to predominant entropic contribution to micelle formation while driving force of aggregation becomes more and more enthalpy driven at higher temperature. Similar behaviors are observed in the case of various bile salt systems [26-27] Small et al.[25] put forward an explanation of the phenomenon by suggesting a balance between the monomer solubility and hydrophobic behavior of NaTC, NaTDC and their equimolar

combinations. The equimolar ternary systems, however, depict a continuous cmc decrease against temperature. This suggests stabilization of micelles at increased temperature (upto 40 ^{o}C).

There is reliable evidence that bile salts self-assemble to form micelles whose size continuously increases with increasing amphiphile concentration.[28-30] Several models have been proposed to describe the unusual self-assembly of bile salts. The most accepted model is a two-step model[31] involving the formation of primary and secondary micelles. In the first step, around cmc, small aggregates known as primary micelles begin to form, where a maximum of ten molecules can be associated.[31] In the second step at higher concentration, large micelles called secondary micelles are formed by mutual association of primary micelles. The primary-secondary micelle scenario has been confirmed by simulations.[32-33] The two step model is also consistent with two cmc values[34] corresponding to two types of micelles. Thus it is concluded that at cmc_1 primary micelles are formed, and at cmc_2 self-assembly of primary micelles occur to form secondary micelles whose size increases with the increase in bile salt concentration. It has been reported in literature[35] that NaDC exhibits two cmc values at 10 mM and 60 mM corresponding to the formation of primary and secondary micelles. Also ^{1}H-NMR study confirmed the existence of primary micelles upto 100 mM concentration, beyond which self-assembly occurs.[36-37] Based on ESR measurements, at least two kinds of micelle were suggested to co-exist in dehydroxy bile salts at 100 mM concentration.[38]. Li et al.[39] have shown increase in micellar polydispersity and average micellar size due to increase in NaTC concentration only after 50 mM. For cholate and deoxycholate micelles, secondary micelles have been reported[40] to form beyond 100 mM concentration. Thus from the literature it may be concluded that self-assembly of bile salt micelles and polydispersity occurs only at higher concentration (>50 mM or 100 mM), which is much larger concentration than the investigated concentration range of our experimental system. We have used concentration of bile salts upto the maximum of 12 mM, indicating absence of secondary micelles and polydispersity in our investigated system.

Degree of Counterion Binding (f)

The fraction of the counterions bound to the micelles (*f*) of ionic surfactant systems can be determined conductometrically following the procedure of Evan[41] by utilizing the plot of specific conductance against surfactant concentration. The results of single bile salt systems in the temperature range 10-40 ^{o}C are presented in Table 1s of supporting information and that of a representative system are contained in Table 1.

The obtained values of *f* are found to be low in accordance with the reported values.[42-43] In addition their temperature dependence was found to be very small. Small et al.[18] showed that in the case of NaTC, the plot of log(cmc) versus log[NaCl] yielded a straight line with approximately zero slope indicating negligible counterion binding on its micelles while for NaTDC slightly negative slope suggested less than one quarter of ionic groups of its micelles bound to counterions. The authors also found even lower counterion binding for their mixed systems which were almost two third of the value for pure NaTDC micelles. In the case of ionic/nonionic[16] as well as ionic/bile salt combinations [13] very low counterion binding values have been reported. We could hardly locate a break point in specific conductance versus surfactant concentration plots in binary and ternary mixtures of bile salt systems indicating very low or negligible counterion binding characteristics. For this reason we have used cmc values obtained by surface tension method for further analysis and interpretation of the results.

Surface excess and minimum area per molecule of the single and mixed bile salt systems

In order to calculate the amount of surfactant adsorbed per unit area at the air/aqueous solution interface for different systems, we followed Gibb's adsorption equation[13] (See supporting information). The maximum adsorption denisty (Γ_{max}) and the minimum area per surface active component (A_{min}) for the

single, binary and ternary mixtures are given in the Tables 1s, 2s and 3s of supporting information respectively. The results of some representative bile salt systems are contained in Table 1. Since dilute surfactant solutions were used, the dlog $\gamma_{\pm}$ term was not considered in calculation. A_{min} (in our case) refers to the average of that contribution of the components assuming equimolar composition of the amphiphiles at the interface. It is not possible to evaluate surface excess values of individual components in a multicomponent system by surface tensiometry measurement, however, an idea of comparative relationship of total surface excess and corresponding minimum area per molecule of the surfactant combinations can be obtained.

The evaluated Γ_{max} values of individual bile salts (given in Table 1 of manuscript and Table 1s of supporting information) are found to be higher and thus corresponding lower A_{min} values than those reported by Jana et al.[13] The reported trend in A_{min} followed the order NaDC>NaTDC>NaC at 30°C. However, our results at approximately all temperatures maintained the sequence NaDC>NaTC>NaC>NaTDC. This indicates more compact monolayer formed by the bile salt NaTDC. In the case of binary and ternary bile salt combinations, the A_{min} and Γ_{max} values are found to be intermediate in comparison with those of the components (Tables 2s and 3s of supporting information). A_{min} values for NaC-NaTDC are comparatively lower than those of the other binary mixtures while for ternary mixtures NaTC-NaTDC-NaC shows lower values. It is evident from Table 1 that the temperature dependence of the values doesn't show any regular trend. The minimum area per molecule under ideal mixing situation ($^{ideal}A_{min}$) of the mixed entities can be expressed by the relation.

$$^{ideal}A_{min} = \sum \alpha_i A_{i,min} \qquad (2)$$

Where α_i is the bulk mole fraction and $A_{i,min}$ the minimum area per molecule for ith pure component at a given temperature. The $^{ideal}A_{min}$ values obtained by the Eq. (2) are also included in the Tables 2s and 3s (supporting information) and in Table 1 for representative ones. The binary mixtures of bile salts exhibit

higher A_{min} values compared to $^{ideal}A_{min}$ at all the experimental temperatures studied excepting NaTC-NaTDC and NaTDC-NaDC combinations. However, the ternary bile salt mixtures show lower A_{min} values compared to $^{ideal}A_{min}$ except for NaTDC-NaDC-NaC system. The composition of the adsorbed mixed surfactant layer may not be equal to bulk composition.[44] Thus these values may be to some extent erroneous. However increased charge repulsion among the amphiphiles of similar head groups may be responsible for higher A_{min} values. The unexpected surface tension behavior[45] may be attributed to factors other than interfacial composition. The obtained results indicate non-ideal mixing of the bile salt systems. A critical assessment of the results is needed to properly understand the phenomenon.

Thermodynamics of interfacial adsorption and micelle formation

The standard free energy of adsorption[13,46] at the air-saturated monolayer interface, ΔG^{o}_{ad} for single, binary and ternary bile salt systems was obtained from the relationship

$$\Delta G^{o}_{ad} = \Delta G^{o}_{m} - (\pi_{cmc} / \Gamma_{max}) \qquad (3)$$

where π_{cmc} is the surface pressure at cmc ($\pi_{cmc} = \gamma_o - \gamma_{cmc}$) γ_o and γ_{cmc} being surface tension of water and that of a given surfactant system at its cmc respectively) and Γ_{max} is the surface excess at minimum adsorption level.

The free energy of micellization per mole of monomer unit, ΔG^{o}_{m} values[13] can be evaluated by applying the equation

$$\Delta G^{o}_{m} = (1+f)RT \ln cmc \qquad (4)$$

where f is the fraction of counterions bound to the micelles.

Since the obtained values of f were very low for single bile salt systems and negligible in the case of binary and ternary combinations, they were not considered in the evaluation of ΔG^{o}_{ad} and ΔG^{o}_{m} values.

The ΔG^{o}_{ad}, ΔG^{o}_{m} and π_{cmc} values of the representative individual, binary and ternary bile salt systems are presented in Table 2 and those of other bile salt systems are given in Table 4s of supporting information.

The ΔG^{o}_{m} values for the single bile salts are found to be comparable with those reported in literature.[13] The evaluated values do not show much difference in the case of individual and mixed systems at the experimental temperatures. However, the values for NaTDC and NaTC-NaTDC-NaC mixtures show more favourable spontaneity of micellization. Again ΔG^{o}_{ad} values of the single and mixed systems are found to be more negative compared with the ΔG^{o}_{m} values indicating stronger interaction between amphiphiles at air/aqueous solution interface than in the bulk phase. It may be mentioned that ΔG^{o}_{ad} values for the mixed bile salt systems are lower in magnitude (higher) compared to that of bile salt-CTAB mixed combinations reported by Jana et al.[13] The result in our case may be due to repulsion between the similarly charged head groups.

For a complete thermodynamic analysis ΔH^{o}_{m} is required along with that of G^{o}_{m}. The temperature dependence of cmc can be used to estimate ΔH^{o}_{m} and hence ΔS^{o}_{m} assuming the aggregation number as well as counterion binding to be independent of temperature. The detailed procedure is given in supporting information. The data may provide an idea of the general trend found in the thermodynamics of micellization of the single as well as mixed bile salt systems. However, the evaluated thermodynamic properties (Table 4S of supporting information and Table 2 of manuscript) are not accurate enough compared with those obtained from calorimetric determinations. The trend observed in the case of NaTC and NaTDC are more or less comparable with the data reported by Small et al.[18] It is found that in the case of (a) NaC, NaTC, NaDC, NaC-NaTDC, (b) NaDC-NaTC, NaTC-NaTDC, NaC-NaDC (c) NaTDC, negative ΔH^{o}_{m} values are obtained beyond 30 ^{o}C, 20 ^{o}C and 10 ^{o}C for the systems (a), (b) and (c) respectively. The ΔS^{o}_{m} values for all the investigated systems are fairly positive excepting in a few cases at

higher temperatures. The positive ΔS^{o}_{m} values are attributed to the randomness resulting from the melting of "icebergs" around the non-polar moiety[47] of the surfactant monomers during micellization and location of non-polar end in similar or like environments in the micelle interior. The process of micellization is found to be entropy driven for the studied systems at the experimental temperatures since $T\Delta S^{o}_{m} > \Delta H^{o}_{m}$. Interestingly Small et al.[13] reported enthalpy directed micellization of bile salts at temperatures higher than 50^{o}C.

The behavior of hydrophobic groups of amphiphilic molecules in water is considered as a case of entropy-enthalpy compensation phenomenon.[48] The compensation between ΔH^{o}_{m} and ΔS^{o}_{m} is depicted in Fig. 3 where the lines have the same slope but different intercepts at $\Delta S^{o}_{m} = 0$. The different intercepts reflect varied hydrophobicities of the systems. The constant slope (=0.29) of the compensation line represents T_c, compensation temperature, which characterizes the solvation phenomena of the process.[48] It may be said that during the process of micellization, both the hydrophobic hydration of the amphiphilic monomers and the hydrophilic hydration of ionic head groups (due to their mutual association) decrease, leading to the transfer of bile salts from their hydrophobically hydrated state in aqueous medium to the oily core of the micelles.

Microcalorimetric studies

Microcalorimetry is the most reliable method to explore the thermodynamics of self-aggregation of amphiphilic molecules. The cmc and enthalpy of micellization, $\Delta H^{o}_{m.}$ can be obtained in a single run using the methodology. The cmc values of the bile salts and their mixtures at equimolar combination were determined using Isothetmal Titration Calorimetry (ITC) at 298 K. The resulting heat flow versus time profiles of one of the investigated systems is exhibited in Fig. 4a.

The bile salt concentration in the injector was maintained appreciably high above the cmc, so that the injector contained a mixture of micelles and monomers. Initially, a series of relatively large endothermic peaks were observed when the bile salt solutions were injected into the reaction cell. The enthalpy changes occur due to micelle dissociation since the concentration in the reaction cell was below cmc.[49] The endothermic nature of these peaks ($\Delta H>0$) indicate that demicellization must lead to an overall entropy increase of the systems, since micelle dissociation is thermodynamically favorable below cmc ($\Delta G<0$); thus $T\Delta S>\Delta H$. This entropy increase may be attributed partly to the release of the counterions associated with bile salt surfactants when micelles break down to monomers[49] and partly to the loss of orientation or packing arrangement of amphiphiles within micelles. It is pertinent to mention that the positive entropy change during micellization is not contradictory to the positive entropy change during demicellization. This is explained as: During micellization, change in entropy is a competition between two competitive factors, the orientation effect (packing of amphiphile monomers into micelles) and dehydration effect (melting/release of water molecules around the hydrophobic tail of the monomer).[50] It is reported that the dehydration effect is accompanied by a positive entropy change[51-52] and orientation effect is accompanied by a negative entropy change.[52-53] Further positive contribution to entropy due to dehydration effect dominates the negative contribution to entropy due to orientation effect during micellization[50] making over all entropy of micellization positive. A quantitative estimation of individual contribution of these two effects is difficult. In contrast during demicellization, the collective effect of breaking of micelle into monomers and release of counterions may lead to positive entropy change. The two statements are not contradictory, as the negative entropy change due to packing of monomers during micellization is analogous to the positive entropy change due to breaking of micelle into monomers during demicellization. Positive values of entropy during micellization and demicellization are frequently reported in literature.[54-57]

An appreciable decrease in peak height was observed after a few injections because the bile salt concentration in the reaction cell exceeded the cmc and hence the micelle in reaction cell was no longer dissociated. The enthalpy change is thus solely the result of micelle dilution effects[58] above the cmc. Fig. 4b depicts the dependence of enthalpy change (ΔH) per mole of bile salt injected into the reaction cell, calculated by integration of heat flow versus time profiles. The cmc of the bile salt solutions obtained from the inflexion point in ΔH versus bile salt concentration are presented in Table 3. The cmc values of the bile salt solutions obtained by surface tensiometry show fairly reasonable agreement with those determined calorimetrically excepting a few cases. The reported cmc values of some of the bile salts are not always consistent.[13, 18,25] The cmc value of NaTDC, for example, is found to be in the range of 2-70 mM, depending on the methodology used.[13] The enthalpy change due to micellization is obtained from the difference in enthalpy between the final and initial plateau region at the points of discontinuity in sigmoidal enthalpograms (Fig. 4b) for dilution of concentrated bile salt solution in water (a prototype shown in Fig 4c). The ΔH^{o}_{m} values obtained following the procedure of Kreshek and Hargraves method[59] are presented in Table 3.

The observed large negative ΔH^{o}_{m} values for the more hydrophobic binary (NaDC-NaTDC, both are dihydroxy bile-salts) and ternary (NaTC-NaDC-NaTDC containing two dihydroxy bile-salts) systems probably resulted due to the unfavorable interaction involving water and hydrophobic mixtures of the bile salts. When hydrophobic molecules enter into water the mutual phobicity of the hydrophobic moieties and water leads to the exclusion of water molecules by a hydrophobic molecule resulting in aggregation (or so called ice berg formation)[47] of such water molecules. Hence during micellization, heat is released due to release of such structures. The higher negative ΔH^{o}_{m} values of the more hydrophobic NaDC-NaTDC system are found compared with the low negative values for less hydrophobic NaC-NaTC combination. Therefore microenvironment of micelles containing dihydroxy bile salts seems to be more hydrophobic

than those containing trihydroxy ones. This is also reflected from Fluorescence measurements, discussed in later section of manuscript. The calorimetric study of the bile salts may help a comprehensive understanding of micelle formation process. However the associated factors like hydration, orientation, microenvironment and packing of bile salt molecules influence the thermodynamic parameters and a quantitative interpretation of the phenomenon seems to be a very difficult proposition at this stage.

Correlation between calorimetric and Van't Hoff enthalpies

In order to evaluate thermodynamic parameters of the micellization of bile salts, the enthalpy of micelle formation is obtained directly using microcalorimetry, as well as by estimating the property utilizing the tensiometric cmc values at different temperatures following Van't Hoff equation. In terms of mass action principle, the standard free energy of micellization, ΔG^{o}_{m} is given by the following equation

$$\Delta G_m^o = \left(1 + \frac{m}{n}\right) RT \ln X_{cmc} + \frac{RT}{n} \ln\left[2n(n+m)\right] \qquad (5)$$

Where m and n are the number of counterions bound per micelle and the aggregation number of a micelle respectively. The term m/n is thus equal to the fraction of the counterions (f) bound to micelle. X_{cmc} is the cmc in mole fractional unit. The enthalpy of micellization, ΔH^{o}_{m} can be calculated by using Eqs. (7-8) given in supporting information.

It is observed that although the cmc of bile salts determined by the calorimetry and other methods show fair agreement, the ΔH^{o}_{m} values obtained by the direct calorimetric method appreciably differ from those calculated by Van't Hoff equation (Table 3). Literature[60] reports support the observation. It should be mentioned that such differences are often found and have been pointed out by Chatterjee et al.[61] The changing aggregation number, shape, and the counterion binding of micelles not accommodated in the

Van't Hoff rationale, are considered to affect the ΔH^{o}_{m} value of the process. The contributions of the above mentioned factors, on the other hand, are inherent in the direct determination of ΔH^{o}_{m} by calorimetry. Thus, the observed discrepancy of the results by the two approaches is not unusual. Corkill[62-63] proposed a relationship for the evaluation of ΔH^{o}_{m} of nonionic surfactants accounting for the contribution of aggregation number and the effect of changing aggregation number on the micellar surface charge. Holtzer and Holtzer[64] provided an explanation of the electrostatic free energy of micelle formation process of ionic surfactants (on the basis of similar consideration) and pointed out the inefficiency of the Van't Hoff approach. The calorimetrically obtained ΔH^{o}_{m} values are found to be lower in magnitude compared with those estimated by Van't Hoff method.[65] This is reflected in the results of the present study. The lower values of ΔH^{o}_{m} obtained by calorimetry may be due to hydrophobic interaction and breaking of ice bergs with associated exothermic heat change during micellization. This makes the resultant ΔH^{o}_{m} more exothermic compared with Van't Hoff result where the second exothermic process does not influence the enthalpy of n(monomer)↔micelle formation equilibrium obtained from the temperature dependence of cmc. It may be mentioned that the two methods have a basic difference; while the calorimetry provides integral heat of micellization, the Van't Hoff method deals with differential heat treatment.[66] Thus, direct method of calorimetry registers all sorts of heat changes, the sum of which is compounded as the heat of micellization. This may include the contribution from: a) dissociation of micelles b) changes in the bile salt-bile salt interactions c) changes in the hydration or counterion binding d) molecular rearrangement, mixing etc. Although calorimetry is the most accurate method, a major limitation is that the method measures only the overall enthalpy change of a system and it is not possible to directly isolate the contribution of different mechanisms. Ideally the methodology should be used in combination with other methods providing complementary information regarding the system. A quantitative accounting incorporating pragmatic concepts and additional information are thus needed to resolve the issue.

Micropolarity of the bile salts micelles

The ratio of intensity of first to the third fluorescence vibronic peaks (I_1/I_3) in a monomeric pyrene fluorescence emission spectrum yields information about the solubilization site sensed by pyrene in surfactant micelles. The ratio is known to be a sensitive index of the polarity in the microenvironment.[37] A low value of I_1/I_3 indicates nonpolar environment while its high value suggests a polar environment. Characteristic values of I_1/I_3 are 0.6, 1.04, 1.23, 1.33, and 1.84 in cyclohaxane, tolvene, ethanol, methanol, and water respectively.[67] These micropolarity values of the representative single, binary and ternary bile salt systems are presented in Table 1 (For rest, see Tables 1s, 2s and 3s of supporting information). The low I_1/I_3 values of individual as well as their combinations indicate the microenvironment of the micelles to be nearly non-polar like hydrocarbon solvents.[67] Thus it may be considered that pyrene is solubilized in the core of bile salt micelles. [68]In contrast in the case of ordinary surfactants, pyrene is considered to reside in the palisade layer of micelles. The micropolarity of the systems (at all temperatures) follow the order: NaTC>NaC>NaTDC>NaDC. Pyrene is exposed to the more hydrophilic environment of trihydroxy bile salts than dihydroxy ones. Fluorescence and Spin label studies[69] revealed that trihydroxy bile salts form smaller micelles compared with those of dihydroxy systems and thus pyrene monitors relatively polar environment in trihydroxy bile salt solutions. For a given number of hydroxy groups on the hyrdophobic surface of bile salts, the micropolarity in the core of the micelles with bulky taurine terminal group is more hydrophilic than in the bile salts having COO^- terminal group.

Binary combinations of trihydroxy bile salts NaTC-NaC show more hydrophilic microenvironment compared with those of the dihydroxy NaDC-NaTDC systems. Other mixed solutions of di- and trihydroxy bile salts exhibit intermediate or nearly similar micropolarities. The micropolarity values are found to be closely comparable with those of the component dihydroxy bile salts suggesting their

predominance in mixed micelles. The data in Table 4 (Table 5s of supporting information) support this observation indicating higher mole fraction of dihydroxy bile salts in mixed micelles. In the case of ternary mixtures, the micropolarities are more or less comparable with one another having values in between those of NaTC and NaDC.

Temperature dependence shows an increase in I_1/I_3 values with temperature for all pure and mixed systems. This may be pointing to slightly increased water penetration into the micelles due to loosening of their structure and exposing pyrene to more hydrophilic microenvironment.

Interactions in bile salt mixed micelles

In a multicomponent mixed micellar system, the cmc values of mixtures are related to individual cmc values of the components by Clint't equation.[19] The evaluated ideal cmc values ($^{ideal}C_{mix}$) for some equimolar binary and ternary systems at different temperatures along with the experimentally determined cmc values are contained in Table 1 (See Tables 2s and 3s of supporting information). The observed differences between the ideal theoretical and experimental values indicate the degrees of non-ideality of the systems investigated. In the case of NaTC-NaTDC, NaC-NaTDC and NaC-NaDC binary mixtures negative deviations of C_{mix} from $^{ideal}C_{mix}$ at the experimental temperatures excepting NaC-NaDC system at 40 ^{o}C are observed. The deviation is found to be positive for NaTDC-NaDC at low temperatures and negative at higher temperatures. Hildebrand et al. [25] observed similar trend for bile salt-sodium oleate mixed system. All the ternary systems except NaTC-NaDC-NaC show negative deviation of experimental cmc values from those of ideal ones at different temperatures. Conspicuously high negative deviation of C_{mix} from C_{mix}^{ideal} is observed in the case of ternary mixrure of NaTC-NaTDC-NaC. Interestingly the bile salt combinations comprising NaTDC show negative deviation.

Rubingh[20] provided a theoretical treatment to estimate the micellar mole fraction X_i, activity coefficients g_i, of the components of the binary surfactant systems and intermolecular interaction parameter β, on the basis of experimental cmc values. Table 4 depicts the micellar composition X_1 (the mole fraction of first component), activity coefficients, g_1 and g_2 of one bile salt and the β values of one binary bile salt mixtures (for all bile salt systems, see supporting information). The evaluated β values show similar trend as found in the deviation of C_{mix} from $^{\text{ideal}}C_{mix}$ described above. The average value of β for the systems NaTC-NaTDC, NaC-NaDC and NaC-NaTDC at studied temperatures exhibit negative values indicating synergistic behavior. On the contrary positive β values of NaDC-NaTC and NaC-NaTC combinations suggest antagonistic interaction. Probably the positive β value of NaDC-NaTC is a manifestation of stearic repulsion between the bulky head groups. The antagonistic behavior of the NaC-NaTC mixture may be attributed to the less favorable interaction involving trihydroxy bile salts and reflected in their large cmc values and higher aqueous solubilities. The deviation of activity coefficients from unity indicates non-ideality of the systems.

Jana et al[13] reported antagonistic interaction between cetyltrimethylammonium bromide CTAB and a few bile salt mixtures and on the contrary synergism between sodium dodecylsulphate SDS and the bile salts at higher proportions. Similar trends were observed for sodium oleate-nonionic surfactant mixtures.[70] Synergistic behavior of NaC and NaDC with alkyl sulphates has been reported in literature,[71] which, however seems illogical in view of similar charges on the head groups.

The mixed micellar composition X, activity coefficients g_{i}, and the interaction parameter β of ternary combinations have been evaluated using pseudobinary Rubingh's treatment[18, 16,72] where two surfactants are paired and treated as one component and third as the other. The cmc of the paired component has been taken as that in their equimolar binary mixtures. In the case of ternary bile salt mixtures, twelve sets of

results stand for three possible pairings and the results are given in Table 6s of supporting information to reduce the length of the manuscript. The results show that, X_1 and β obviously depend on the way of selection for the pairing of the three components. The β values are found to be positive or negative at different temperatures. Interestingly NaTDC-NaTC-NaC combination depicts negative β at all temperatures irrespective of pairing selection of the components thereby suggesting synergism. A_{min} values also show unusual behavior. The calculated activity coefficients are found to be more or less of normal magnitude excepting in a few cases. The deviation of the activity coefficient values from unity and that of X_1 value from 0.5 suggest non-ideality.

Holland and Rubingh[17] proposed a generalized muticomponent nonideal mixed micellar model based on pseudo-phase separation approach. The model has been successfully applied to a number of micellar composition[16-17] and activity coefficients. It involves an effective utilization of net interaction parameters obtained experimentally from the cmc values of binary systems. In accordance with this treatment the activity coefficients g_i, g_j, …. of micelle forming surfactant species i, j, ….. in an n-component mixture are presented on a general basis by the equation

$$\ln g_i = \sum_{\substack{i=1 \\ (j\neq i)}}^{n} \beta_{ij} X_j^2 + \sum_{\substack{j=1 \\ (i\neq j\neq k)}}^{n} \sum_{k=1}^{j-1} \left(\beta_{ij} + \beta_{ik} - \beta_{jk}\right) X_j X_k \qquad (6)$$

where β_{ij} represents the net (pairwise) interaction between component i and j, and X_j is the mole fraction of jth component in the micelles. At cmc, the relation

$$X_i = \frac{\alpha_i C_j g_j X_j}{C_i \alpha_j g_i} \qquad (7)$$

holds where terms C_i and C_j are the cmc values of the ith and jth components in their pure state respectively. Interaction parameter, β_{ij} can be obtained independently from binary mixtures using Rubingh's method. The activity coefficients of a three component system i.e., g_1, g_2 and g_3 at the mixed

cmc can be evaluated by the above equations utilizing the method of successive substitutions and maintaining the constraint that sum of X_i's equals to unity. We have used Livenberg-Marquardt[73] method, an efficient numerical technique for function minimization and evaluation of multiple unknowns from multiple equations. The values of g_i thus obtained can be used to evaluate ${}^{RH}C_{mix}$, the mixed micellar cmc of ternary systems following the equation of

$$\frac{1}{{}^{RH}C_{mix}} = \sum_{i=1}^{3} \frac{\alpha_i}{g_i C_i} \qquad (8)$$

where α_i, g_i and C_i are the bulk mole fraction, activity coefficients and cmc of pure ith component respectively.

The mole fraction values of each component in one of the ternary mixed micelles viz., X_1, X_2 and X_3, their activity coefficients g_1, g_2 and g_3 along with the predicted cmc following Rubingh-Holland method, ${}^{RH}C_{mix}$, are presented in Table 4 (See Table 7s of supporting information for all ternary systems).

The activity coefficient values are found to be within normal range although deviations from unity suggest non-ideal behavior. It is found that the micellar mole fraction of NaTDC is higher in all the ternary mixtures studied while in the case of NaTC-NaDC-NaC combinations predominance of NaDC is observed. In general at least one of the dihydroxy component is seen to predominate in mixed micelles. This behavior in ternary mixtures is also shown in pseudobinary Rubingh treatment for ternary systems as discussed above.

Conclusions

Bile salts, a special group of biosurfactants, have been of interest to colloid and interface scientists.[5] Because, bile salt systems are physiologically important, extensive studies have been carried out on the physicochemical properties of single bile salt systems.[4-7] Considerable attention has been given in recent years towards the study of mixed micelle formation of bile salts and lipids to explore the nature of

interaction both in bulk and at air-water interface.[12-14] Although physicochemical studies of binary bile salt systems are rare,[15] the ternary bile salt systems are still unexplored. The present detailed investigation of the interaction of bile salts in solution with reference to mixed micelle formation, the thermodynamics of micellization, the behaviors at air-water interface and in bulk, and the micropolarity of micelle would be worthwhile.

The cmcs of NaC, NaDC, NaTC, NaTDC and their equimolar binary and ternary mixtures obtained by tensiometry, conductometry, and microcalorimetry show reasonably fair agreement. The degree of counterion binding of the bile salt micelles is found to be rather low consistent with the previous findings,[42-43] while it is almost absent in the mixed micellar system. In comparison with the $^{ideal}A_{min}$ values, the A_{min} of the binary and ternary bile salt mixtures are found to be higher and lower respectively. The ΔG^{o}_{ad} values of the bile salt mixtures are observed to be more spontaneous than the ΔG^{o}_{m} values. The enthalpy of micellization (ΔH^{o}_{m}) process reflects the entropy-enthalpy compensation phenomenon.[48] The microcalorimetrically determined enthalpy values are observed to be more exothermic than those by Vant Hoff enthalpies in tune with the earlier reports for other micellar systems.[65] Fluorescence probing studies indicate that the microenvironment of the bile salt micelles is nearly non-polar like that of a hydrocarbon solvent. The cmc, activity coefficient, composition of the bile salt micelles and intermicellar interaction parameters evaluated following the treatments of Clint,[19] Rubingh[20] and Rubingh-Holland[17] predict nonideal behavior of the bile salt mixtures. The article will be helpful to study the ability of these mixed bile salt systems to act as suitable drug carriers.[8] In addition, the study may act as a preliminary investigation for solubilization of drugs by mixed bile salt systems.[9] Further the application of these mixed bile salt systems can be tested in future in the field of enzymology as excipients in novel formulations to stabilize enzymes.[11]

Supporting information

The information provides brief thermodynamic overview to calculate surface excess and minimum area per molecule of single and mixed bile salt systems. Further, theory behind thermodynamics of micelle formation and adsorption at air/aqueous solution interface has also been discussed. Critical micelle concentration, C_{mix} (experimental) and $^{ideal}C_{mix}$ (for mixed system), maximum surface excess, degree of counterion binding, f (for pure bile salts), Γ_{Max}, minimum area per molecule, A_{min} (experimental), $^{ideal}A_{min}$ (for mixed system) and I_1/I_3 values for single bile salt systems and their equimolar binary and ternary combination are contained in Table 1s, 2s and 3s respectively at different temperatures. Table 4s contains various thermodynamic parameters of different bile salt systems at different temperatures. Table 5s and 6s contains micellar mole fraction, X_1, interaction parameter, β, activity coefficients, g_1 and g_2 of two components for equimolar binary (Rubingh's formulation) and ternary bile salt systems (according to Rubingh's pseudobinary treatment) at different temperatures respectively. Rubingh-Holland treatment parameters for equimolar ternary bile salt systems at different temperatures are contained in Table 7s.**References**

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Tables and Figures

Table 1 Critical micelle concentration, cmc, degree of counterion binding, f, maximum surface excess, Γ_{Max}, minimum area per molecule, A_{min}, I_1/I_3 values, $^{ideal}C_{mix}$ and $^{ideal}A_{min}$ for different bile salt systems at different temperatures.

System	T (°C)	f	cmc (mM) (S.T)	cmc (mM) (Cond)	Γ_{Max} /10^{-6} (mol m^{-2})	A_{min} (nm^2)	I_1/I_3
NaC	10	0.06	10.8	12.5	2.45	0.68	0.86
	20	0.08	7.78	8.25	2.58	0.64	0.88
	30	0.09	5.89	7.35	2.27	0.73	0.92
	40	0.08	6.10	7.50	2.45	0.68	0.96
	T (°C)	C_{mix} (mM)	$^{ideal}C_{mix}$ (mM)	Γ_{Max} /10^{-6} (mol m^{-2})	A_{min} (nm^2)	$^{ideal}A_{min}$ (nm^2)	I_1/I_3
NaDC-NaTC	10	5.95	5.52	1.26	1.32	1.20	0.72
	20	3.92	4.84	1.37	1.21	1.20	0.75
	30	4.75	4.05	1.56	1.06	1.23	0.76
	40	4.83	4.22	1.16	1.43	1.11	0.79
NaTC-NaDC-NaTDC	10	3.85	3.31	1.58	1.05	0.97	0.72
	20	3.32	3.56	1.83	0.91	0.97	0.75
	30	3.20	3.36	1.85	0.90	0.99	0.76
	40	2.65	3.53	1.83	0.91	0.93	0.77

Table 2 ΔG^{o}_{m}, ΔH^{o}_{m}, ΔS^{o}_{m}, ΔG^{o}_{ad} and π_{cmc} values for NaC and equimolar NaDC-NaTC and NaTC-NaDC-NaTDC bile salt systems at different temperatures.

System	T (°C)	ΔG^{o}_{m} (kJ mol^{-1})	ΔH^{o}_{m} (kJ mol^{-1})	ΔS^{o}_{m} (JK^{-1}mol^{-1})	π_{cmc} (mN/m)	ΔG^{o}_{ad} (kJ mol^{-1})
NaC	10	-10.7	21.6	114.1	29.4	-22.7
	20	-11.8	21.6	113.9	30.6	-23.7
	30	-12.9	8.3	73.4	31.8	-27.0
	40	-13.3	-2.9	33.2	33.9	-27.1
NaDC-NaTC	10	-12.1	27.8	140.9	28.8	-34.9
	20	-13.5	8.0	73.5	28.4	-34.2
	30	-13.5	-8.0	18.1	27.7	-31.2
	40	-13.9	-1.4	10.0	27.4	-37.5
NaTC-NaDC-NaTDC	10	-13.1	10.0	81.5	29.6	-31.9
	20	-13.9	6.7	70.2	28.5	-29.5
	30	-14.5	8.5	75.9	27.7	-29.4
	40	-15.4	15.2	97.9	26.9	-30.1

Table 3 Calorimetric determination of ΔH_m^0 (kJmol^{-1}) and CMC (mM) for various equimolar mixtures of bile salts at 25 ^{o}C, Van't-Hoff enthalpy ΔH^o_m (kJmol^{-1}) at 20 ^{o}C.

System	ΔH_m^0 (kJmol^{-1})	CMC (mM)	Vant-Hoff Enthalpy ΔH^o_m (kJmol^{-1})
NaTC-NaC	-0.85	9.69	5.1
NaTC-NaDC	-1.63	4.20	8.0
NaTC-NaTDC	-2.70	3.42	19.9
NaC-NaTDC	-2.94	2.63	14.6
NaDC-NaTDC	-3.16	3.61	4.3
NaC-NaDC	-1.16	7.05	3.7
NaC-NaDC-NaTDC	-1.85	2.72	8.0
NaTC-NaDC-NaC	-1.42	6.13	0.6
NaTC-NaDC-NaTDC	-2.34	3.32	6.7
NaTC-NaC-NaTDC	-2.02	5.17	6.6

Table 4 Critical micelle concentration: C_{mix} (experimental), $^{ideal}C_{mix}$ and $^{RH}C_{mix}$, micellar mole fraction, X_i, activity coefficients, g_i, for equimolar binary and ternary bile salt systems according to Rubingh's formulation and Rubingh-Holland treatment respectively at different temperatures.

System	T (°C)	C_{mix} (mM) (S.T)	$^{ideal}C_{mix}$ (mM)	$^{RH}C_{mix}$ (mM)	X_1	X_2	X_3	g_1	g_2	g_3
NaDC-NaTC	10	-	-	-	0.610	-	-	1.049	1.124	-
	20	-	-	-	0.596	-	-	0.864	0.726	-
	30	-	-	-	0.749	-	-	1.050	1.544	-
	40	-	-	-	0.729	-	-	1.048	1.405	-
NaTC(1):NaDC(2):NaTDC(3)	10	3.85	3.31	3.46	0.225	0.183	0.591	0.986	1.355	0.97
	20	3.32	3.56	2.24	0.342	0.146	0.511	0.325	1.343	0.627
	30	3.20	3.36	2.16	0.362	0.102	0.536	0.325	2.334	0.539
	40	2.65	3.53	2.59	0.248	0.269	0.483	0.651	1.017	0.621

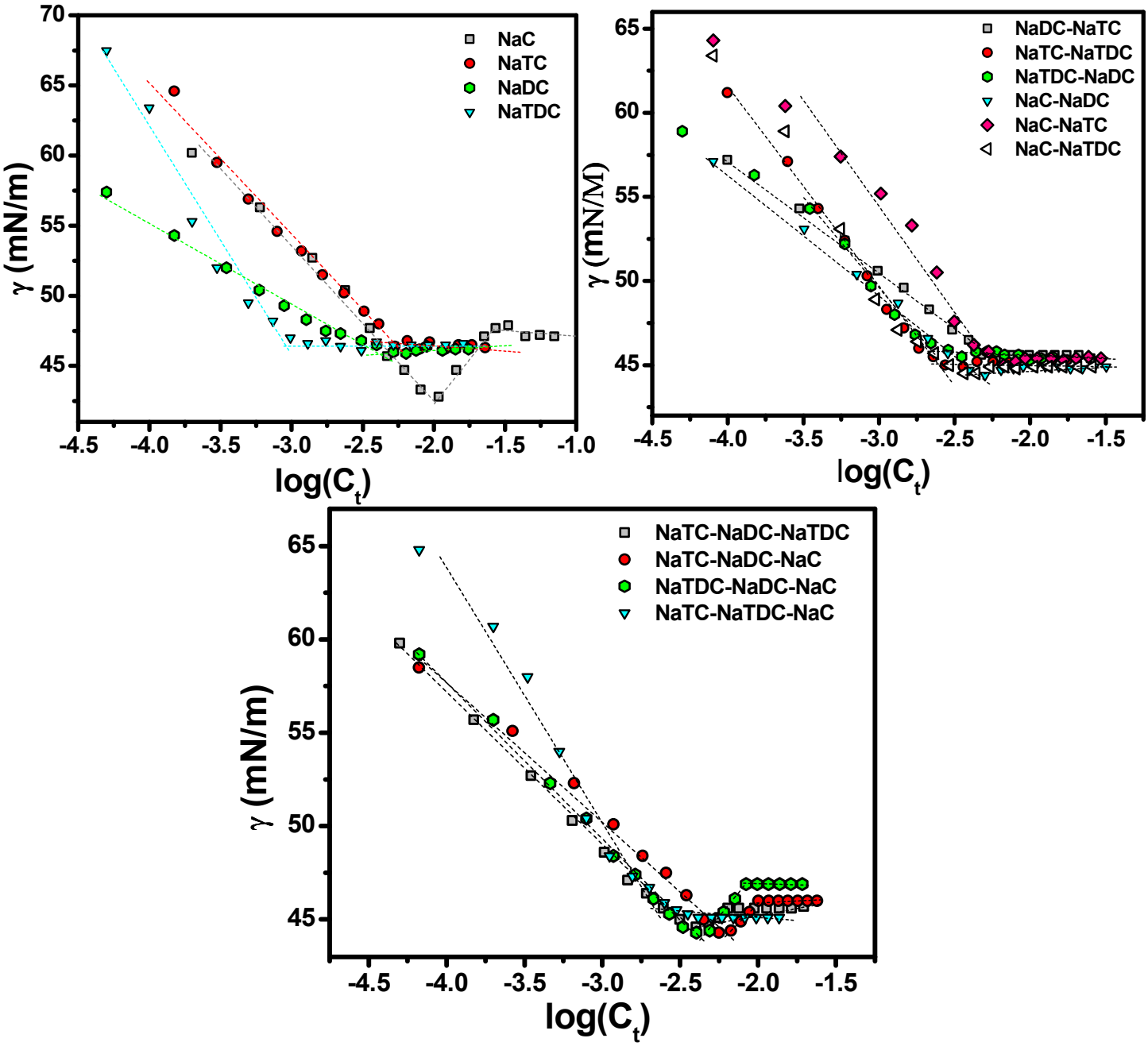

Fig. 1 Plots of the surface tension, γ, of single, binary and ternary bile salt systems vs the total surfactant concentration, C_t (M) at 20 ^{o}C.

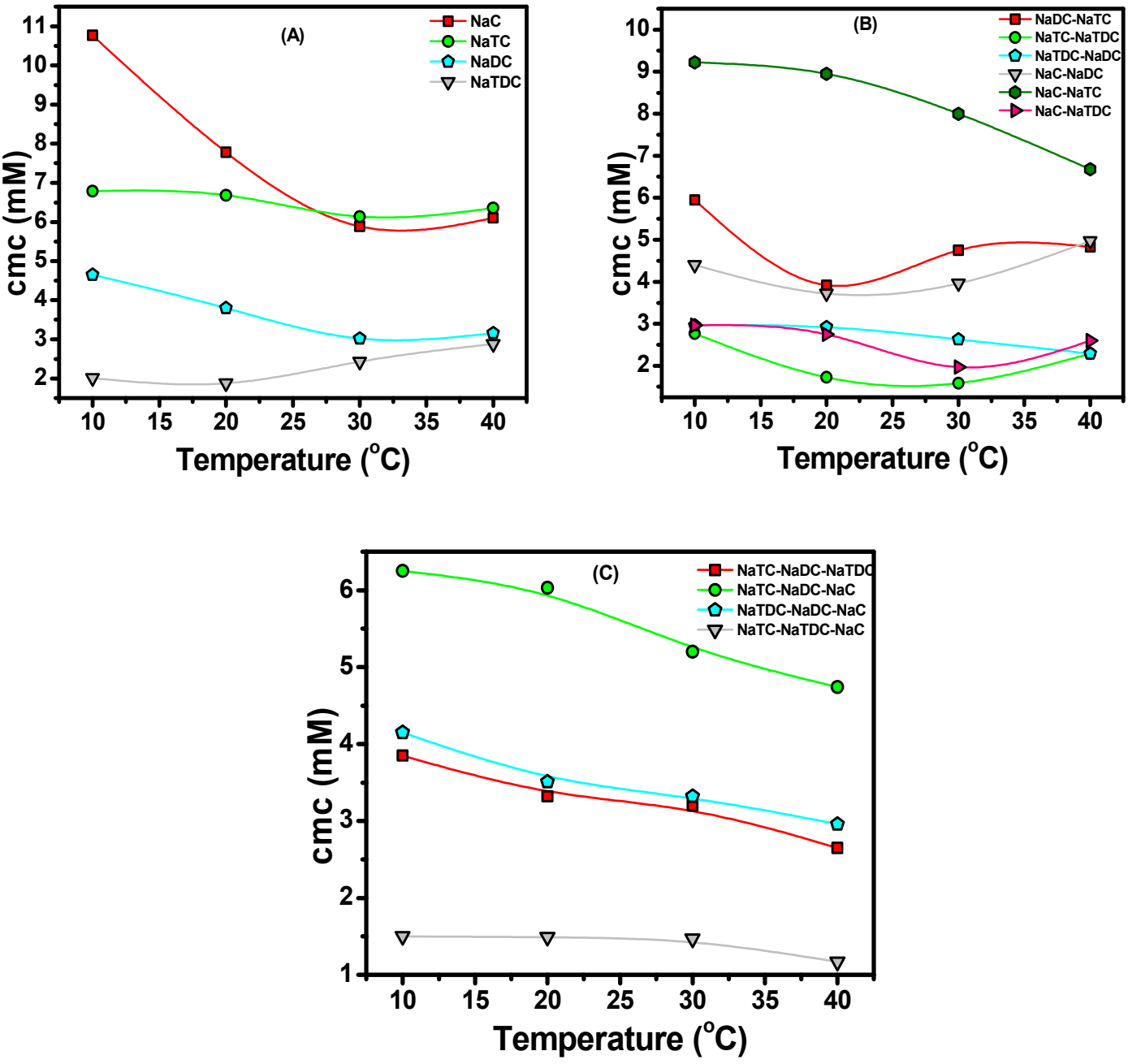

Fig. 2 Plots of the critical micelle concentration, cmc, versus temperature for (A) single, (B) binary and (C) ternary bile salt systems.

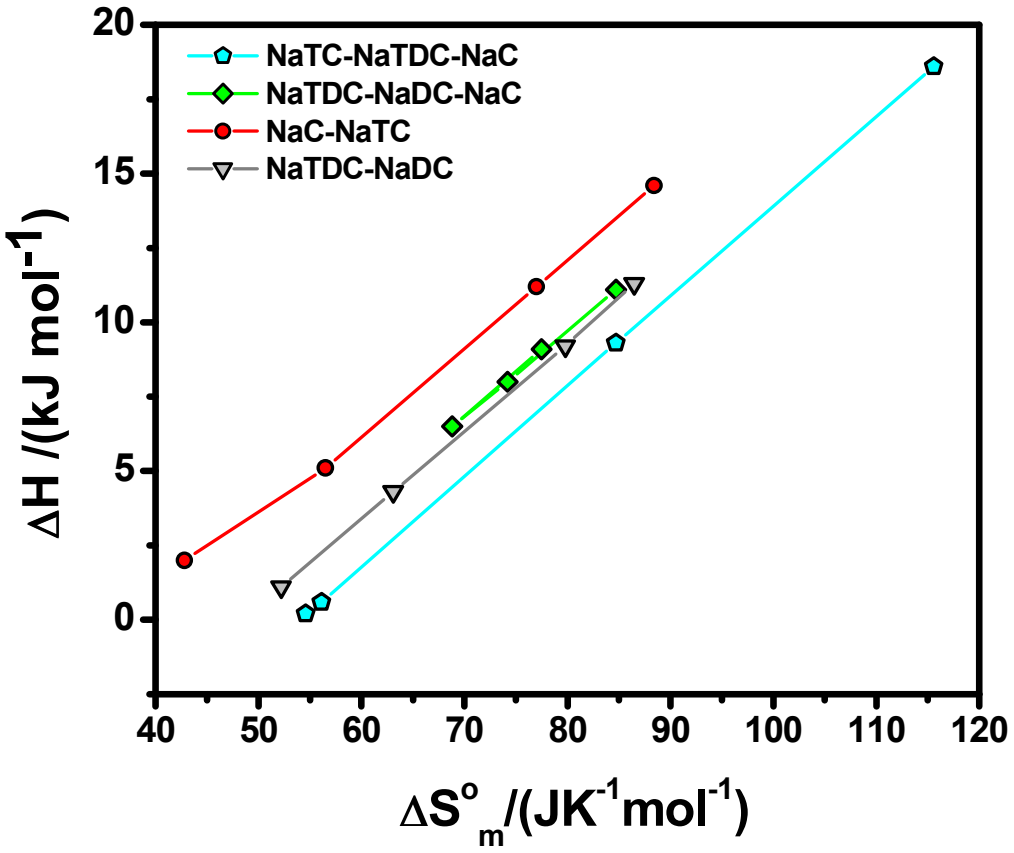

Fig. 3. Compensation plots of ΔH^{o}_{m} and ΔS^{o}_{m} for different bile-salt systems.

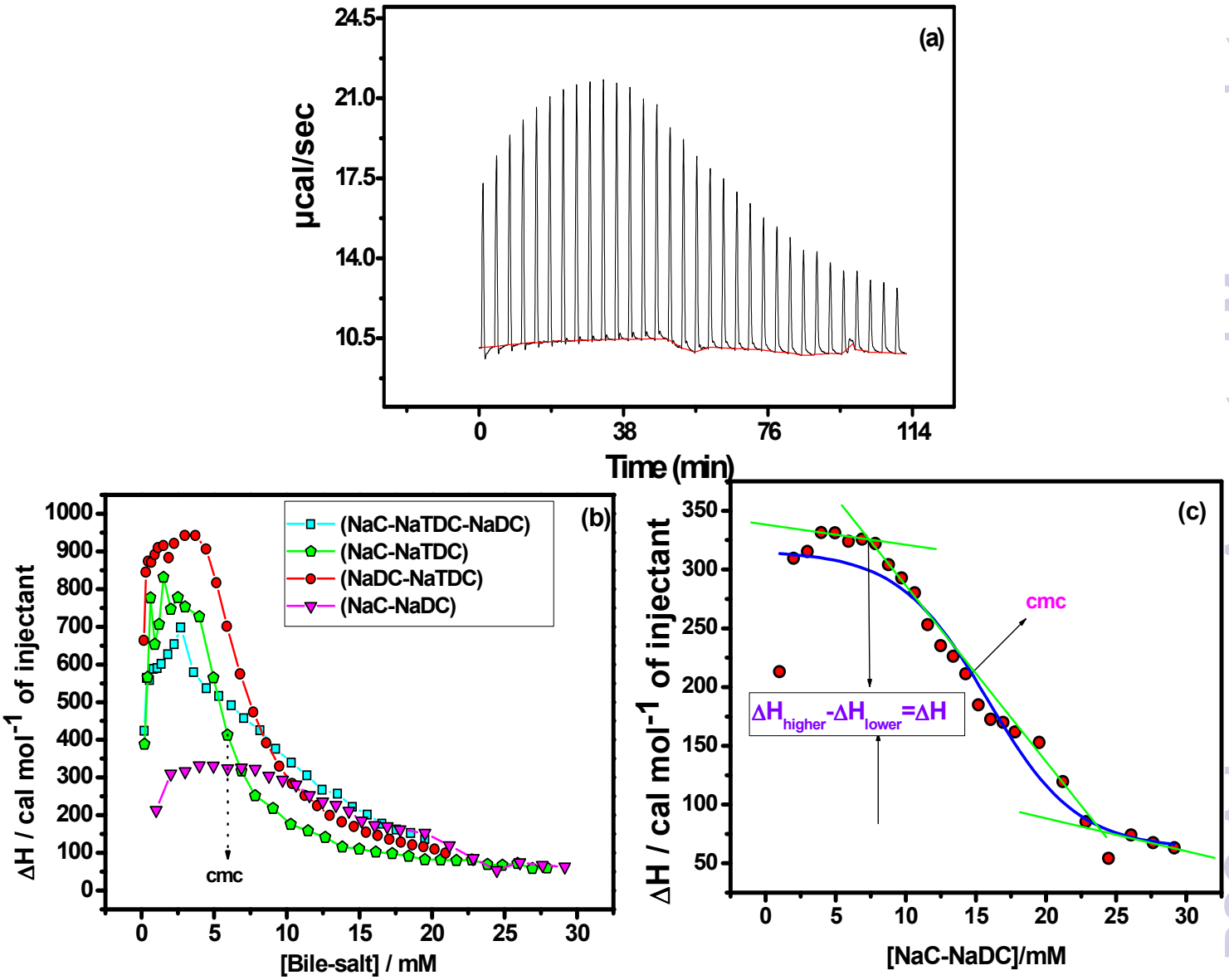

Fig. 4 (a) Calorimetric traces (heat flow versus time) (b) Reaction enthalpy versus total bile-salt concentration in mM (c) Determination of ΔH^{o}_{m} and cmc at 25°C.

Graphical abstract