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ORIGINAL CONTRIBUTION

# Aggregation behavior of sodium deoxycholate and its interaction with cetyltrimethylammonium bromide in aqueous solution studied by NMR spectroscopy

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**Abstract** In pure sodium deoxycholate (NaDC) dilute solution, the transverse relaxation times ($T_2$) of nearly all proton signals of NaDC obey single-exponential decay, with exception for proton at 3-position (H3) that decays in a two-exponential manner. The two components with different mobility of H3 of NaDC indicate that the molecules are probably associated in head-to-tail pairs via hydrogen bonds in dilute solution. In the mixed NaDC/cetyltrimethylammonium bromide (CTAB) solutions, the $T_2$ values of interested NaDC protons are far less than those in pure NaDC solution, especially when the concentrations of the two components are close to equal-molar. The results of self-diffusion coefficients and the chemical shifts confirm further that the strong interaction occurs between the two components in mixed solutions, especially for equal-molar condition. The arrangement of the mixed aggregates can be speculated for the cross-peaks of proton pairs occurring between NaDC and CTAB molecules in two-dimensional and rotating frame nuclear Overhauser enhancement spectroscopy spectra.

**Keywords** NMR · Sodium deoxycholate · CTAB · Aggregation

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## Introduction

Sodium deoxycholate (NaDC) is one of the most studied bile salts. It forms molecular aggregates in aqueous solution capable of solubilizing many water-insoluble compounds [1–6], some of which are biologically important, such as cholesterol [1, 2], phospholipids [3], and sequential copolypeptides [5]. Unlike the typical surfactants with a polar head and an alkyl chain, the common bile salts have a characteristic steroid structure (see Fig. 1 for NaDC) with the convex side (β-plane or back) and the concave side (α-plane or face) of one having a few hydrophilic hydroxyl groups. This difference in the structure leads bile salts to an atypical aggregation behavior. Small and Carey [7, 8] proposed that, at low concentrations, the bile salts form dimers by hydrophobic back-to-back interactions. In this model, the hydrophobic plane (β-plane) point inward and the hydrophilic α-plane point outward toward the aqueous medium [9–11]. Oakenfull considered that the face-to-face linkage with the hydrogen bonds is the major force associated with the formation of dimers [12, 13]. Some others proposed a helical model for micelles of bile salts that the nonpolar face is oriented toward the aqueous medium and polar inside [14–17]. The helix is characterized by an interior filled with cationic ($Na^+$) and water molecules. The alkali-metal ions are engaged in Coulombic interactions with the carboxylic groups, whereas the water molecules form hydrogen bonds among them and with the hydroxyl and carboxyl groups of the NaDC anions. These interactions, together with the hydrogen bonds among NaDC, stabilize the helix.

In recent years, much attention has been paid to the aggregation of mixed systems containing bile salts with

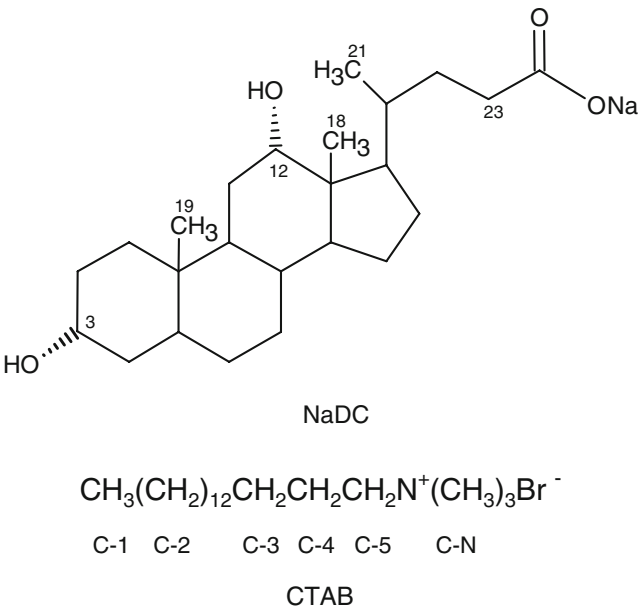


**Fig. 1** The formula and proton numberings of NaDC and CTAB molecules

other surfactants [18–25]. Mixtures of bile salts with anionic [18, 19], cationic [20–23], and nonionic surfactants [24, 25] have been investigated and the micellar characteristics examined for varying mole ratios using different methods. Interestingly, a 1:1 interacted species between cetyltrimethylammonium bromide (CTAB) and bile salt has been found to form in solution at concentration below the critical micelle concentration (cmc) of the components [20]. Fluorescence quenching studies [21] of the mixed micelles of bile salts and cetyltrimethylammonium halides indicated that the micelles tended to grow in size into rod-like micelles for the CTAX/NaDC system.

Nuclear magnetic resonance (NMR) spectroscopy, especially 2D NMR technique, has been proved to be a very powerful method for studying the detailed structure of the micelles and for posing and answering many questions regarding the microenvironments encountered. Recently, two-dimensional phase-sensitive rotating frame nuclear Overhauser enhancement spectroscopy (ROESY) [26, 27] has been used to investigate the structure of micelles of bile salts by quantitatively calculating the distance of interested proton pairs. In this work, we are interested in gaining a deep knowledge of the aggregation of NaDC and the detailed structure of its micelle in its pure aqueous solution and how it interacts with the cationic surfactant CTAB by detecting the variation of its chemical shift, relaxation times, two-dimensional nuclear Overhauser enhancement (2D NOESY) and ROESY at different conditions. A possible mechanism for such interaction is proposed.

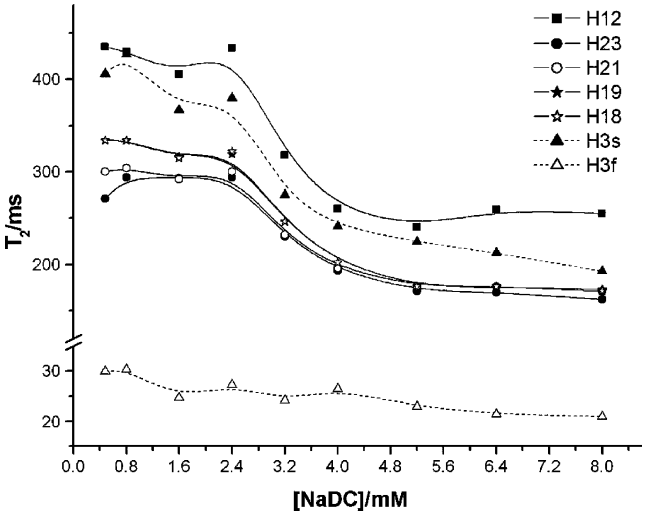


**Fig. 2** The transverse relaxation time ($T_2$, ms) of protons in pure NaDC aqueous solution as a function of the NaDC concentrations

## Materials and methods

NaDC (>98%, Fluka), sodium cholate (NaC, >99% Sigma), sodium taurocholate (NaTC, >95%, Sigma), sodium chenodeoxycholate (NaCDC, >98%, Sigma), sodium ursodeoxycholate (NaUDC, >99%, Fluka), and CTAB (AR, Jining Institute of Chemical Engineering, China) were all used as received. $D_2O$ (99.8%) is the product of the Beijing Chemical Plant (China). NMR experiments were performed at 298 K on a Varian INOVA-500 NMR spectrometer with the $^1H$ frequency of 500.13 MHz. Carr–Purcell–Meiboom–

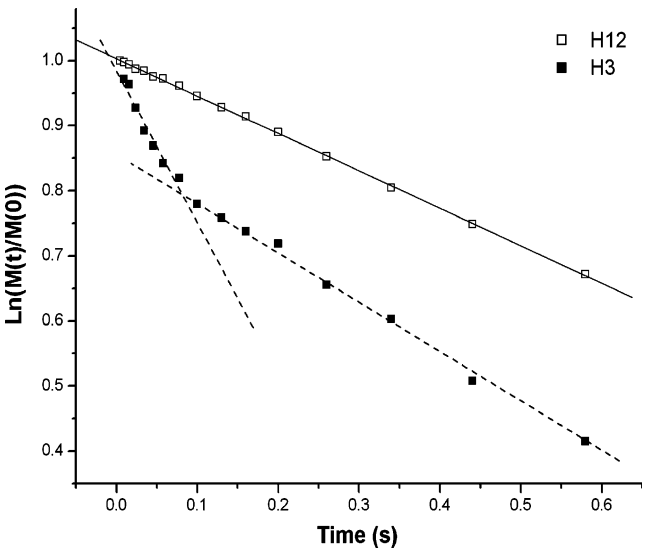


**Fig. 3** Typical bi-exponential and single-exponential transverse relaxation for H3 and H12 at a concentration of 0.8 mM NaDC in aqueous solution. *Square symbols* (*filled and open*) represent experimental results

**Table 1** Transverse relaxation parameters for H3 of NaDC in aqueous solution at various concentrations within experimental and simulation errors (<10%)

| Concentration (mM) | H3 | | | |
|---|---|---|---|---|
| | $T_{2f}$ (ms) | $W_f$ | $T_{2s}$ (ms) | $W_s$ |
| 0.48 | 30 | 0.39 | 406 | 0.61 |
| 0.8 | 30 | 0.40 | 427 | 0.60 |
| 1.6 | 25 | 0.36 | 366 | 0.64 |
| 2.4 | 27 | 0.44 | 380 | 0.56 |
| 3.2 | 24 | 0.36 | 275 | 0.64 |
| 4.0 | 26 | 0.38 | 241 | 0.62 |
| 5.2 | 23 | 0.41 | 224 | 0.59 |
| 6.4 | 21 | 0.41 | 212 | 0.59 |
| 8.0 | 21 | 0.40 | 192 | 0.60 |

Gill pulse sequences were used for transverse relaxation time ($T_2$) measurements. Self-diffusion coefficients were measured by the longitudinal eddy-current delay with bipolar pulse pair pulse sequence [28]. $D_2O$ was used as solvent instead of water in order to weaken the water signal. Meanwhile, the presaturation method was used to further suppress the proton signal of solvent. 2D NOESY experiments were performed with standard three-pulse sequence [29] with a mixing time of 100 ms. 2D ROESY were also performed to avoid spin diffusion in these systems of slow motion.

## Results and discussion

### Pure NaDC aqueous solution

The molecular structure and proton numbering of NaDC and CTAB are shown in Fig. 1. Assignments of NMR signals are made according to [30, 31]. It has been reported [31] that $^1$H chemical shifts of NaDC show slight concentration dependence that β-protons are shifted upfield when the concentration of NaDC increases from 2.4 to 240 mM, whereas α-protons exhibit downfield shift. In our experiments with the range of concentration from 0.48 to 8.0 mM, the chemical shifts exhibit nearly invariable. It indicates that at low concentrations, the chemical shifts are not sensitive to the change in concentration.

In NMR experiments, the variation in line width is associated with the transverse relaxation times ($T_2$) of concerned protons. Knowledge of $T_2$ values of different segments will help to construct a more detailed picture for the molecular aggregation. Figure 2 shows the dependence of the $T_2$ values of the protons, directly calculated by single- or bi-exponential decay, on the concentration of NaDC. It is surprising to note that the spin–spin relaxation of H3 proton obeys bi-exponential behavior. Therefore, two separate sets of $T_2$ values for H3 proton (H3s and H3f) are displayed in Fig. 2. While $T_2$ of all the other resolvable protons relax single-exponentially. In spite of the difference in relaxation decay behavior, there are turning points for all the protons, including H3f, at the concentration about 2.4 mM. The $T_2$ values of H3f are remarkably shorter than those of the other component (H3s), which are almost of the same order of those of all the other resolvable protons (H12, H18, H19, H21, and H23). In contrast to H3, although H12 is also a methine proton of a hydroxyl carbon, its spin–spin relaxation obeys single-exponential decay. Consequently, it is difficult to consider that the presence of the two components for H3 is due to the intrinsic property of a NaDC monomer.

Figure 3 shows the typical results of the single-exponential decay for H12 and the bi-exponential decay for H3 at 0.8 mM of NaDC. The $T_2$ values of the two components for H3 were obtained by fitting the data to the following equation:

$$M(t) = M(0)\left[W_f * \exp(-t/T_{2f}) + W_s * \exp(-t/T_{2s})\right] \quad (1)$$

where $W_f + W_s = 1$; they stand for the fractions (weighting factors) of protons with fast and slow relaxation, respec-

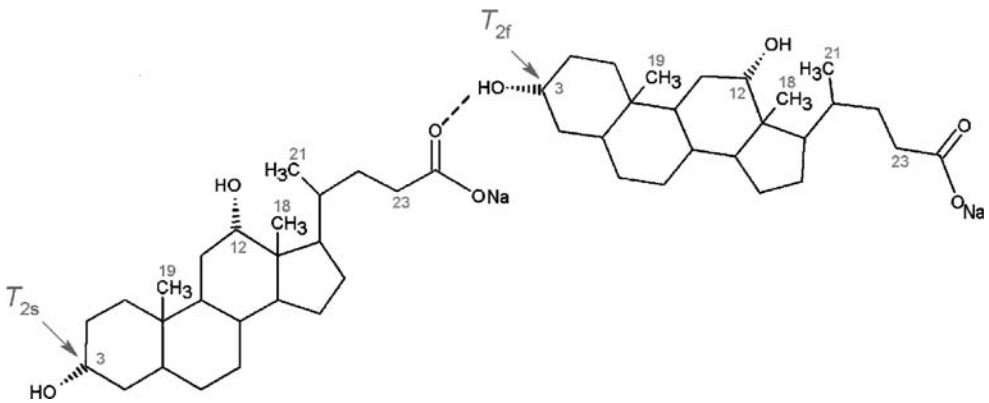


**Fig. 4** Sketch map of the molecular pairs via hydrogen bonds for NaDC molecules

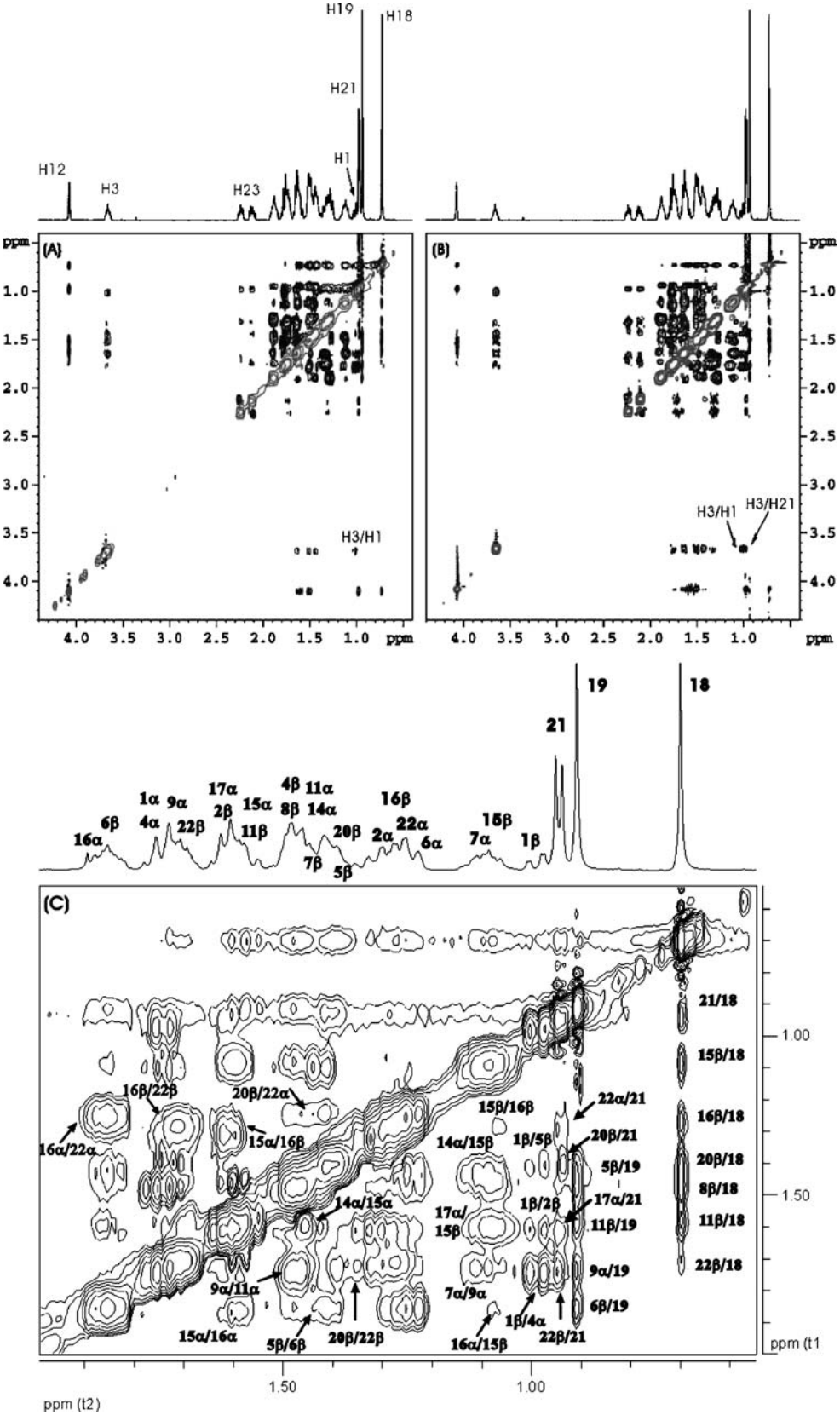


**Fig. 5** **a**–**c** Contour plots of the 2D ROESY spectra for pure NaDC solution with various concentrations with different mixing times: **a** 0.48 mM, $\tau_m$=250 ms; **b** 8.0 mM, $\tau_m$=100 ms; **c** a partial ROESY spectrum ($0.5<\delta<2.0$ ppm) of a 0.48 mM NaDC solution

tively, and $T_{2f}$ and $T_{2s}$ are the corresponding transverse relaxation times. Simulation results are listed in Table 1. The results in Table 1 reveal that the $T_2$ of H3 consists of two components, one of several decades of milliseconds ($T_{2f}$) and the other of hundreds of milliseconds ($T_{2s}$). This implies that H3 exists in two significantly distinguished states in dilute aqueous solutions from 0.48 to 8.0 mM. The motion of the slow relaxation portion ($T_{2s}$, $W_s$) is free, which is the same as those of the other protons, and the other portion ($T_{2f}$, $W_f$) is relatively restricted. It is noticeable that the fractions of the two components ($W_{2f}$ and $W_{2s}$) remain unchanged in the concentration range of this study, within experimental and simulation errors (<10%). The fraction of the proton with restricted motion is remarkable. It may originate from the formation of hydrogen bond between 3-OH of one NaDC molecule and the carboxyl oxygen atom of another NaDC molecule. That is to say, NaDC molecules are probably associated in pairs through hydrogen bonding in the dilute solution as shown in Fig. 4. These pairs begin to form lager aggregates at the concentration of 2.4 mM, as evidenced by the decrease in the $T_{2s}$ values at this concentration (Fig. 2). Thus, the fast relaxation part ($T_{2f}$) of H3 still exists in the larger aggregates, which are formed via this pairs. Obviously, neither back-to-back [7–11] nor face-to-face [12, 13] model can explain the two states for H3. While in the helix [14, 17], the H3 is located outer side toward the solvent; thus, its motion should be free in dilute solution. It is difficult to explain the bi-exponential relaxation process of H3.

In order to make clear whether this kind of aggregates for NaDC is a special case or a common phenomenon, $T_2$ measurements of protons for more bile salts in dilute aqueous solutions are also made. The studied concentrations of each bile salt solution are lower than their corresponding cmc values according to [32–34]. Tri-hydroxyl bile salts (NaC and NaTC) have three hydroxyl groups at 3-, 7- and 12-positons, and di-hydroxyl bile salts (NaCDC and NaUDC) have two hydroxyl groups at 3-, 7-positions. Simulations of transverse relaxation decay for H3, H7, and H12 of these bile salts in dilute aqueous solutions are also made. It is similar to NaDC; all proton signals obey single-exponential decay except for H3. Accordingly, it is speculated that bile salts probably form head-to-tail dimer via hydrogen bonds between 3-OH and carboxyl oxygen atom in dilute aqueous solution.

Direct evidence about the space relationship among the molecules will help to confirm the microstructure of aggregates. This can be achieved by the 2D NOESY experiment. Whereas 2D ROESY has more advantages over NOESY, that spin diffusion and chemical exchange peaks are negative to cross-peaks in 2D ROESY. The ROESY spectra of 0.48 and 8.0 mM NaDC solutions were recorded as shown in Fig. 5. The mixing times are 250 and 100 ms, respectively. The diagonal peaks are negative to the cross-peaks, which occur for a pair of two signals of protons spatially close to each other. The cross-peaks in ROESY spectrum of 0.48 mM NaDC solution will be ascribed to the proximity between the protons in a NaDC monomer. In order to obtain a detailed view, ROESY spectrum of 0.48 mM NaDC is expanded between 0.5 and 2.0 ppm. The cross-peaks are signed as shown in Fig. 5c. The cross-peaks in 2D ROESY spectra are assigned as listed in Table 2. The additional cross-peak for proton pair H21–H3 is observed in ROESY spectrum of 8.0 mM NaDC. H3 and H21 are both directed to the hydrophobic convex backside, but locate two ends of the molecule, respectively. The cross-peaks between them occur only when the molecules form anti-parallel back-to-back aggregates. Combining the results of transverse relaxation time ($T_2$), we speculate that the NaDC molecules form anti-parallel back-to-back aggregates via head-to-tail dimer when the concentration of NaDC exceeds its cmc value. It has been reported that bile salt molecules tend to form small aggregates in dilute solutions. Thus, the anti-parallel back-to-back aggregates via head-to-tail dimmer are likely to be tetramers [7–11, 26]. The counter sodium ions are located outward toward the aqueous medium.

**Table 2** The cross-peaks of protons in 2D ROESY spectra for pure NaDC solution with various concentrations

| Proton pairs | 0.48 mM | 8.0 mM | Proton pairs | 0.48 mM | 8.0 mM |
|---|---|---|---|---|---|
| 1β–2β | * | * | 12β–21 | * | * |
| 1β–3β | * | * | 14α–15α | * | * |
| 1β–4α | * | * | 14α–15β | * | * |
| 1β–5β | * | * | 15α–16α | * | * |
| 2α–3β | * | * | 15α–16β | * | * |
| 2β–3β | * | * | 15β–16α | * | * |
| 3β–4α | * | * | 15β–16β | * | * |
| 3β–4β | * | * | 15β–17α | * | * |
| 3β–5β | * | * | 15β–18 | * | * |
| 3β–21 | | * | 16α–22α | * | * |
| 5β–6β | * | * | 16β–18 | * | * |
| 5β–19 | * | * | 16β–22β | * | * |
| 6β–19 | * | * | 17α–21 | * | * |
| 7α–9α | * | * | 18–20β | * | * |
| 8β–18 | * | * | 18–21 | * | * |
| 9α–11α | * | * | 18–22β | * | * |
| 9α–19 | * | * | 20β–21 | * | * |
| 11α–12β | * | * | 20β–22α | * | * |
| 11β–12β | * | * | 20β–22β | * | * |
| 11β–18 | * | * | 21–22β | * | * |
| 11β–19 | * | * | 21–23 | * | * |
| 12β–18 | * | * | 22–23 | * | * |

CTAB/NaDC binary system

The CTAB/NaDC binary solution contains a constant concentration of 1.6 mM of NaDC, which is lower than its cmc value, and various concentrations of CTAB ranging from 0.8 to 6.4 mM, which are corresponding to various molar ratios of CTAB/NaDC from 0.5:1 to 4:1. The chemical shift changes of concerned protons of NaDC induced by the presence of different additions of CTAB in the mixed solutions are shown in Fig. 6. Different from pure NaDC solution, the variations in chemical shift of NaDC protons are quite large in mixed CTAB/NaDC binary solution. Chemical shift changes of the protons reach maximum at a concentration of 1.6 mM CTAB and then keep relatively invariable. This turning point of CTAB concentration corresponds to a molar ratio of 1:1 for CTAB/NaDC in the mixed solution. Many studies on mixed micelle of cationic and anionic have been published, and the strong synergism due to electrostatic interaction at the 1:1 ratio has been confirmed [20–23, 35, 36]. Consequently, the turning point in Fig. 6 indicates that strong interaction must occur between NaDC and CTAB, especially for equal-molar conditions. Besides, variations in chemical shift for H3 and H21 are much lager than the other protons, but negative and positive, respectively. Consequently, it can be speculated that the points of interaction between NaDC and CTAB are mostly located near the 3- and 21-position of NaDC molecule.

Figure 7 shows the self-diffusion coefficients of NaDC and CTAB in the mixed solution, with the concentration of CTAB ranging from 0.8 to 6.4 mM. In the absence of CTAB, the self-diffusion coefficient of NaDC is about $3.3 \times 10^{-10}$ m$^2$ S$^{-1}$. The addition of CTAB causes the self-diffusion coefficient of NaDC decrease rapidly at first and then remains almost unchanged. This variation trend can be divided into different concentration ranges:

1. When $C_{CTAB} < C_{NaDC}$, $D_{NaDC}$ decreases with the continuously addition of CTAB. This implies that more and more mixed species having diffusion coefficient smaller than that of NaDC are formed because the observed diffusion coefficient is the weighted value of those of NaDC and the mixed species.

2. When $C_{CTAB} = C_{NaDC}$, the self-diffusion coefficients of two components in the mixed solution are approximately the same, $D_{mix} \approx D_{NaDC} \approx D_{CTAB} \approx 0.5 \pm 0.1 \times 10^{-10}$m$^2$ S$^{-1}$. It indicates that a 1:1 mixed species is formed between CTAB and the bile salt in the mixed solution. Comparing with the pure NaDC solution, the size of the mixed aggregates seems to be much lager.

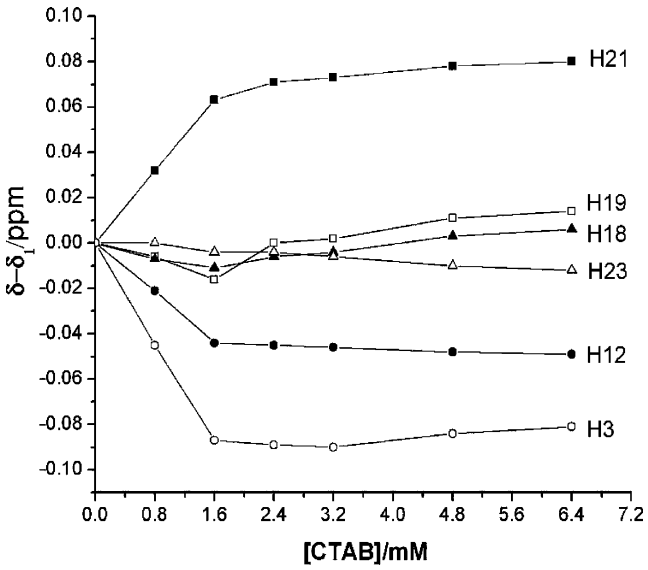


**Fig. 6** Chemical shift changes ($\delta$–$\delta_1$) of NaDC protons in mixed CTAB/NaDC solution at a constant concentration of NaDC of 1.6 mM with various concentrations of CTAB

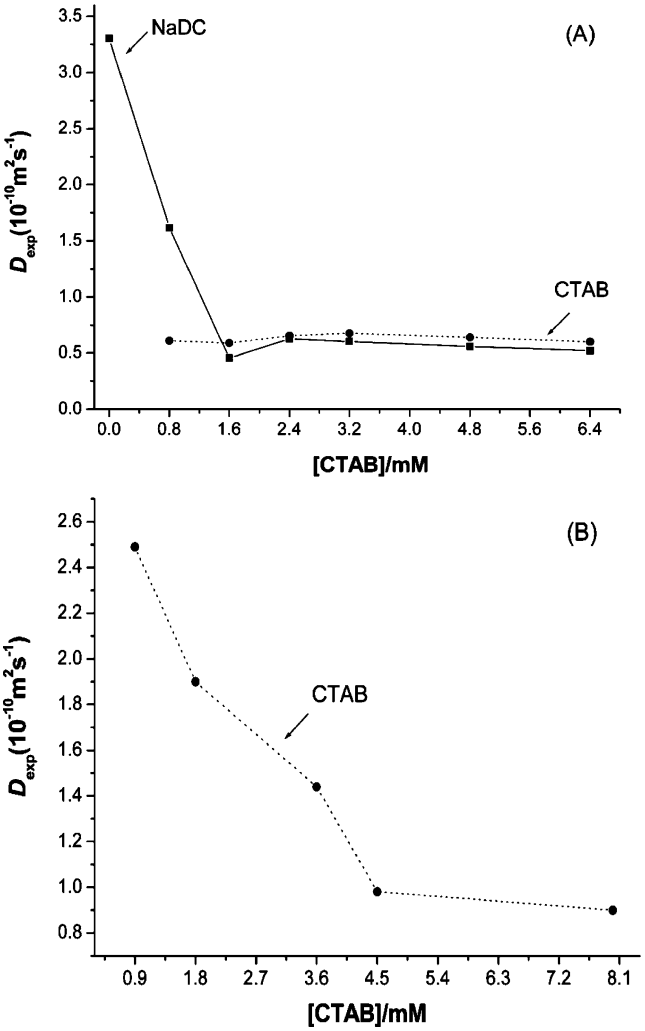


**Fig. 7** **a**–**b** Self-diffusion coefficients **a** at the presence of NaDC, **b** at the absent of NaDC with various concentrations of CTAB

3. When $C_{CTAB} > C_{NaDC}, D_{NaDC} \approx 0.6 \pm 0.1 \times 10^{-10} m^2 S^{-1}$ is approximately equal to $D_{mix}$. It indicates that NaDC molecules are totally involved in mixed aggregates in this concentration region of CTAB, even when the concentration of CTAB increased. The amount of excess CTAB molecules in the mixed solution is likely to form CTAB self-aggregates. Since the self-diffusion coefficient of CTAB micelles is in the same order of $D_{mix}$ of the mixed aggregates (shown in Fig. 7a,b), the observed diffusion coefficient of CTAB ($D_{CTAB}$) in mixed solutions remains almost unchanged in this concentration range.

From the above-mentioned discussion, it can be concluded that the continuous addition of CTAB causes NaDC form more and more mixed aggregates until the concentration of CTAB reaches 1.6 mM, corresponding to a molar ratio of 1:1, after which the excess CTAB molecules form self-associated species.

The simulations of $T_2$ for the concerned protons of NaDC in the mixed solutions fit single-exponential decay. Figure 8 shows the curves of $T_2$ of NaDC protons in these mixed solutions. The $T_2$ values of NaDC protons in these mixed systems are much smaller than those in pure NaDC solution. Furthermore, the curves of $T_2$ exhibit similar trend to the chemical shift results; they also reach extremes at the molar ratio 1:1 for CTAB/NaDC. It confirms that a 1:1 mixed species is formed between CTAB and NaDC in the mixed solution. It is noticeable that the $T_2$ values of H3 exhibit single-exponential decay with the addition of CTAB. The $T_2$ values of H3 in mixed solutions are several decades of milliseconds. It indicates that the motion of the slow relaxation portion ($H_{3s}$) is quite restricted with the addition of CTAB. Whereas the $T_2$ values of H3 in mixed solutions are quantitatively equal to the $T_{2f}$ of H3 with the absent of CTAB, so it is difficult to consider that the original fast relaxation portion ($H_{3f}$) does not exist in mixed solution. Whether the intermolecular hydrogen bond between the hydroxyl group of a NaDC molecule and the carbonyl group of another NaDC molecule are destroyed by CTAB in mixed solution, it is still suspensive.

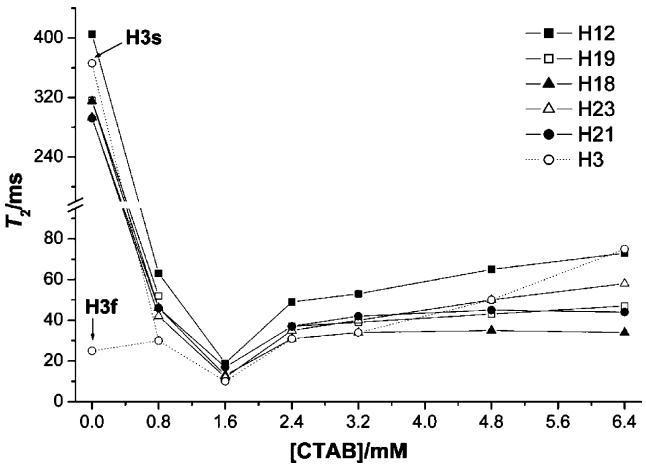


**Fig. 8** The transverse relaxation time ($T_2$, ms) of NaDC protons in mixed CTAB/NaDC solution at a constant concentration of NaDC of 1.6 mM with various concentrations of CTAB

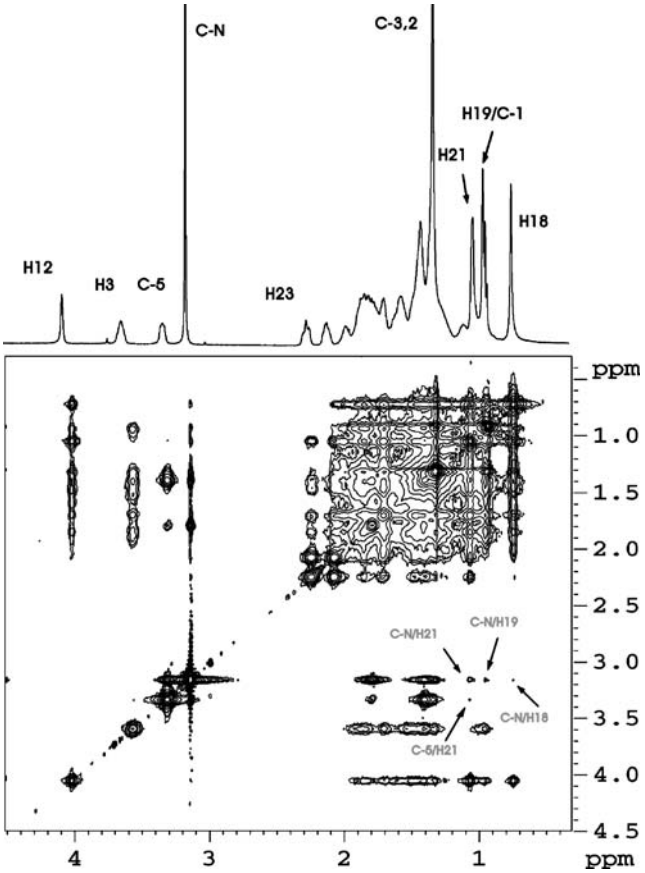


**Fig. 9** Contour plots of the 2D NOESY spectra for mixed 1:1 CTAB/NaDC solution at a concentration of NaDC of 1.6 mM

In order to obtain the details about the arrangement for 1:1 mixed aggregates between NaDC and CTAB, 2D NOESY and ROESY spectra were recorded for the mixed

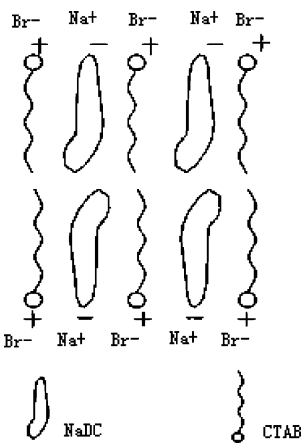


**Fig. 10** The sketch of primary structure for mixed aggregates in mixed NaDC/CTAB solution

solution. Figure 9 show contour plots of the 2D NOESY spectra for 1:1 mixed solutions at a concentration of 1.6 mM NaDC. The cross-peaks of proton pairs between two components (C-N/H21 and C-N/H19) appear in the 2D NOESY spectra. 2D ROESY spectra were also measured in order to avoid the spin diffusion in those systems of slow motion. However, the cross-peaks of proton pairs of C-N/ H21 still appear in the 2D ROESY spectrum except for other proton pairs. As we know, H21 lies in the convex backside of NaDC molecules, so the appearance of cross-peaks of proton pairs H21/C-N indicates that CTAB molecules contact the backside of NaDC, and the polar heads of CTAB are located in the near vicinity of the carboxyl groups in NaDC molecules. It is noticeable that the cross-peaks of H3-H21, which occur in pure NaDC solution at the concentration of 8.0 mM, do not appear. Due to 1:1 interacted species formed between NaDC and CTAB, we propose an alternative arrangement of NaDC/CTAB/NaDC/CTAB for mixed aggregates, as shown in Fig. 10, that either of the CTAB molecules contact the hydrophobic backside of one NaDC molecule and besides the polar heads of CTAB located in the near vicinity of the carboxyl group of NaDC molecule.

## Conclusions

The analysis of $T_2$ values of NaDC protons shows that NaDC molecules are probably associated in pairs through hydrogen bonds in dilute aqueous solution. These pairs begin to form anti-parallel back-to-back aggregates through hydrophobic interactions, when the concentration of NaDC exceeds 2.4 mM. Strong interaction exists between NaDC and CTAB molecules in mixed CTAB/NaDC solution. In addition, it forms 1:1 mixed species between these two components in mixed solution. From 2D NOESY and ROESY spectra we propose a possible primary unit for the mixed aggregates. That is, one CTAB molecule contacts the hydrophobic backside of one NaDC molecule, and the polar heads of CTAB is located in the near vicinity of the carboxyl group of NaDC molecule; these two CTAB molecules approach to each other in the way of anti-parallel through hydrophobic interaction. Larger mixed aggregates are formed by mutual association of different primary units via intermolecular hydrogen bonds between the hydroxyl groups of the NaDC molecules.

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