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Bile salt–phospholipid aggregation at submicellar concentrations

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Abstract

The aggregation behavior of the bile salts taurodeoxycholate (NaTDC) and sodium cholate (NaC), are followed at concentrations below critical micelle concentrations (CMCs) using the environment sensitive, fluorescent-labeled phospholipid, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD-C_6-HPC). A buffer solution containing NBD-C_6-HPC is titrated with increasing NaC or NaTDC and the fluorescence changes followed. Both bile salts induced fluorescence changes below their critical micelle concentration indicating the presence of a bile salt–phospholipid aggregate. A critical control experiment using 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoic acid (NBD-X) shows that the bile salts are interacting with the longer, C16 hydrocarbon tail, not the NBD probe. The fluorescence curves were fitted to the Hill equation as a model for cooperative aggregation. The cooperativity model provides a minimum estimate for the number of bile salts to give maximal fluorescence. This number was calculated for NaC and NaTDC to have a minimum value of ∼2. A small aggregation number supports the existence of primary micellar aggregates at submicellar concentrations for bile salt–phospholipid aqueous solutions.

Keywords: Critical micelle concentration; Sodium cholate; Taurodeoxycholate; Fluorescent phospholipid; Aggregation number

1. Introduction

Bile salts are amphiphilic molecules that assist in the absorption of dietary lipids at the intestinal wall [1]. These molecules act as biosurfactants to solubilize otherwise insoluble lipids in the body by forming mixed micelles [2]. Micelle formation of bile salts occurs differently than for phospholipids or a conventional aliphatic surfactant like SDS due to differences in molecular structure (steroid ring vs. aliphatic carbon chain). Because of their amphipathic nature and unique shape, bile salts aggregate at concentrations below their critical micelle concentration (CMC). In addition, in mixed bile salt–phospholipid systems, lysophospholipid monomers have been shown to act as seeds in bile salt aggregation [3,4].

A number of models have been proposed to describe the structure of bile salt aggregates in solution including the stepwise model of Small and Carey [2] and Small [5], the disk-like model of Kawamura et al. [6], and the helical, hydrogen bonded structure of Giglio et al. [7]. The size and shape of bile salt aggregates and the mechanism of aggregation have also been shown to depend on such factors as the actual structure of the bile salt (dihydroxy vs. trihydroxy, conjugated vs. unconjugated), the ionic strength of the solution, and even the experimental method used to investigate aggregation [for a review see 8]. Aggregation formation in this class of molecules is clearly not fully understood. The present work adds to the body of evidence supporting the step-wise model first proposed by Small by identifying small, primary aggregates in submicellar bile salt solutions. These solutions have been seeded with a fluorescent phospholipid that has not previously been used at submicellar concentrations to study bile salt aggregation.

The aggregation behavior of two different bile salts, sodium cholate (NaC) a trihydroxy bile salt, and sodium taurodeoxycholate (NaTDC) a conjugated dihydroxy bile salt (Fig. 1A and B) are monitored with a commercially available fluorescent phospholipid. The steady-state fluorescence of the environment sensitive fluorescent phospholipid 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl- *sn*-glycero-3-phosphocholine (NBD-C_6-HPC) (Fig. 1C) was monitored in solutions containing one of the two bile salts. In order to study the formation of aggregates *below* the CMC, the CMC values for both the phospholipid and bile salts have been established under

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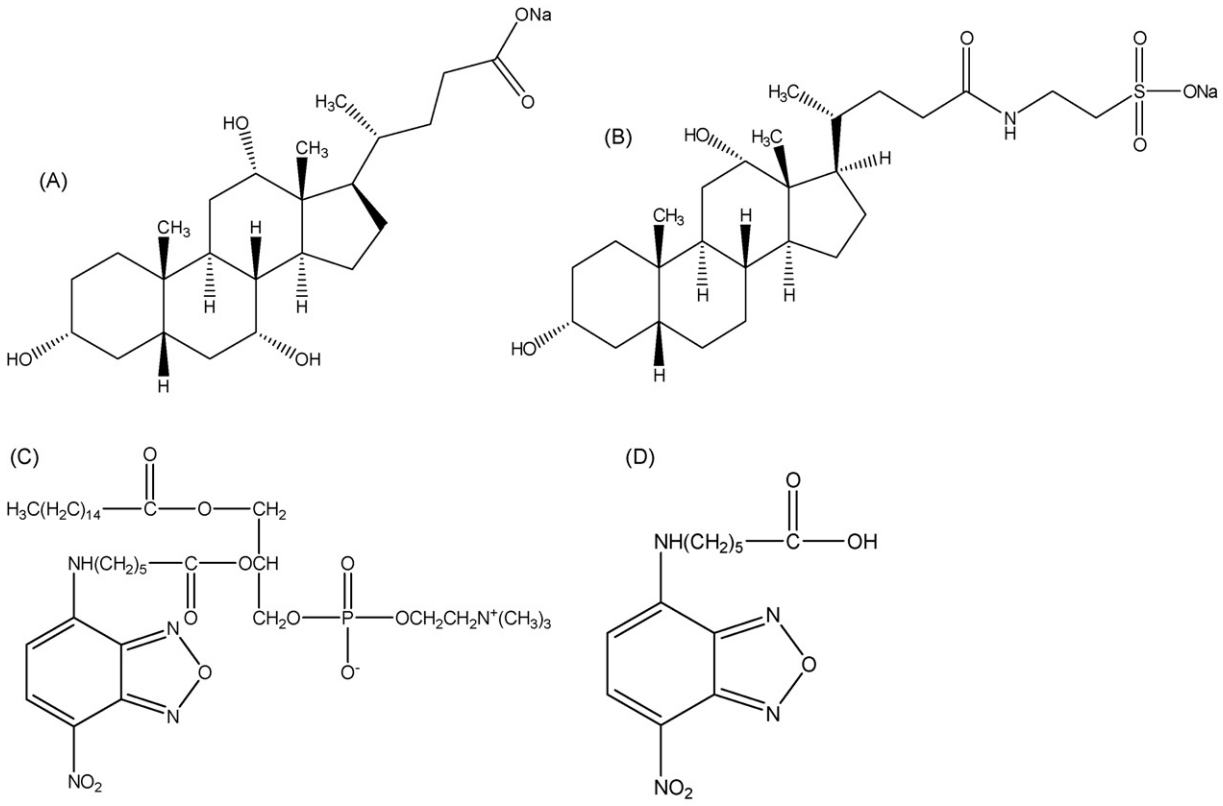

Fig. 1. (A) Structure of sodium cholate (NaC); (B) structure of sodium deoxycholate (NaTDC); (C) structure of 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD-C_6-HPC); and (D) structure of 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid (NBD-X).

our experimental conditions. Literature CMC values for the bile salts vary based on method of analysis and ionic concentration [9,10]. We establish an experimental CMC for NBD-C_6-HPC and use submicellar concentrations of this probe to investigate bile salt–phospholipid aggregation.

2. Experimental

2.1. Materials

NaTDC (Sigma, 97%) and NaC (Alfa Aesar, 99%) were used as received. The fluorescent lipid NBD-C_6-HPC was received as a powder (Molecular Probes) and dissolved into 1 mL of ethanol. From this stock solution, 10-μL aliquots were evaporated under a stream of N_2 and held under vacuum for ∼20 min to remove all solvent. The aliquots were stored at −80 °C until needed. Prior to experiment, an aliquot was reconstituted in buffer to a stock concentration of 100 μM. NBD-X (Fig. 1D) was received as a powder (Molecular Probes) and stored in the dark at room temperature as recommended by the manufacturer. A 0.2 mg/mL solution was prepared from the powder using anhydrous acetonitrile as the solvent. From this stock, 20-μL aliquots were dried down with N_2. Prior to an experiment, an aliquot was reconstituted in buffer to a stock concentration of 100 μM.

NaCl, THAM (molecular biology grade), and sodium azide were purchased from Fisher Scientific. Millipore ultrapure water and deionized ultra-filtered water were used over the course of the experiments with no differences in results. HEPES was purchased from Sigma–Aldrich. Stock solutions of the NaTDC (0.1 M) were prepared in HEPES-buffered saline [HBS; 150 mM NaCl, 10 mM HEPES, 0.04% NaN_3, pH 7.4] and of NaC (1.0 M) in Tris-buffered saline [TBS; 150 mM NaCl, 10 mM Tris, 0.04% NaN_3, pH 8.6] and stored in the refrigerator. The two buffers were used to ensure complete ionization of the bile salts in solution based on their pK_a values of 2.0 for TDC and 5.0 for cholate [5]. The buffers were degassed under vacuum for 15–30 min prior to use in experiments in order to minimize dissolved O_2.

2.2. Fluorescence and light scattering measurements

Steady-state fluorescence measurements were acquired from a K2 multi-frequency cross-correlation phase and modulation fluorometer operating in the steady-state mode (ISS Instruments). The Xenon Arc lamp operated between 17.5 and 18 amps. All slit widths were chosen to be 16 nm for the fluorescence experiments. The excitation wavelength was set at 475 nm as recommended by the manufacturer. An emission spectrum for NBD-C_6-HPC under our conditions led us to choose an emission wavelength of 550 nm for all fluorescence experiments.

Light scattering was measured using this same instrument following the methodology of Shoemaker and Nichols [3]. The parameters consisted of an excitation wavelength of 400 nm,

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8 nm slit width, and collection of scattered light through a KV500 (Schott Glass) cut-off filter.

For all experiments, the sample cuvette was mechanically stirred for 15–30 s after each change in concentration. Ten single point intensity readings were taken at each concentration for all experiments and these values were averaged after transferring the data to a spreadsheet. Any reading with a standard deviation greater than 3% was discarded and the experiment repeated at a given concentration. Normalized fluorescence was plotted versus concentration for analysis.

Fluorescence output was normalized (F_N) by subtracting the background fluorescence (F_o) from the measured value (F) and dividing by the maximum fluorescence found at the highest bile salt concentration $[(F - F_o)/(F_{max} - F_o)]$.

3. Results and discussion

3.1. Determination of bile salt CMCs

The CMCs for both NaC and NaTDC were examined under the solution conditions and concentration ranges used in the bile salt–phospholipid experiments via light scattering. The common practice of fitting data to bi-phasic linear functions and identifying the CMC at the intersection of the lines was employed [3,5,10]. This established a NaC CMC of 11.5 ± 0.7 mM and a CMC of 1.1 ± 0.4 mM for NaTDC. The values determined in our lab fell within the range of established CMC values for the two bile salts, namely ~10 mM for NaC and 1–2 mM for NaTDC [3,5,10]. The values determined were also considered reasonable CMCs for the bile salt–phospholipid experiments since highly flexible molecules such as a hydrocarbon chain modify bile salt aggregates very little [6,11].

3.2. Determination of NBD-C_6-HPC CMC

Aliquots from a stock solution of NBD-C_6-HPC were titrated into a buffer solution over a concentration range of 50–5000 nM. Normalized fluorescence was plotted versus concentration and experimental CMC values for NBD-C_6-HPC were determined. A representative plot is shown in Fig. 2. With increasing concentration, the NBD-fluorophore self-quenches providing an indicator of the CMC [12]. A bi-phasic rise in fluorescence is observed. Both phases were approximated to linear functions. The intersection of the functions determines the CMC value for the fluorophore [3,10,13]. No significant difference was seen in these values using either of the two buffers. The average CMC value over 12 experimental runs was 414 ± 137 nM. Literature values for NBD-C_6-HPC [13] and a 16C lysophopholipid [3] fall within this same range. A concentration of 200 nM NBD-C_6-HPC was used during the bile salt–phospholipid experiments ensuring that we were working below the experimental standard deviation with submicellar fluorophore.

3.3. Bile salt–phospholipid aggregation

Aliquots from a 1 M stock solution of NaC were titrated into a TBS solution containing NBD-C_6-HPC over a concentration range of 0.25–35 mM. Aliquots from a 0.1 M stock solution of NaTDC were titrated into an HBS solution containing the NBD-C_6-HPC over a concentration range of 0.05–5 mM. Normalized fluorescence was plotted against concentration for analysis (Fig. 3). Similar curves have been observed previously using lysophospholipids [3] or by equilibrating a set of bile salt–phospholipid solutions before acquiring measurements [13].

Controls were performed with both NaC and NaTDC in their respective buffers. These experiments contained 200 nM

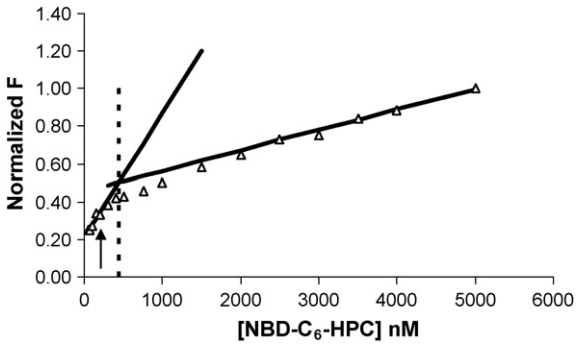

Fig. 2. Critical micelle measurement for NBD-C_6-HPC (open triangles). NBD-C_6-HPC was reconstituted in buffer to provide a 100 mM stock solution. Aliquots were titrated into buffer over the range of 50–5000 nM. A concentration of 200 nM was selected for use in the experiments (arrow) where micelle formation would be negligible. The dashed line goes through the intercept, which represents the experimental CMC value. The average value CMC value determined from 12 data sets is 414 ± 137 nM.

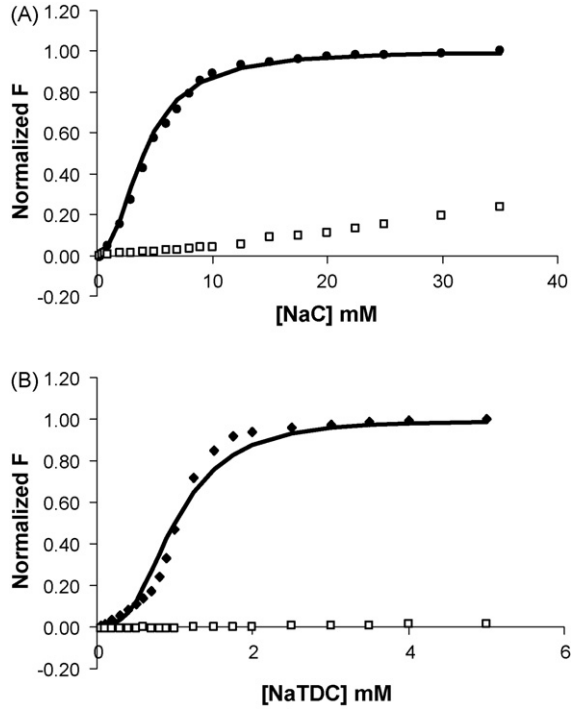

Fig. 3. (A) Titration experiment for NaC in the presence of 200 nM NBD-C_6-HPC (circles) fit to the Hill equation and compared with the control compound 200 nM NBD-X (open squares). (B) Titration experiment for NaTDC in the presence of 200 nM NBD-C_6-HPC (diamonds) fit to the Hill equation and compared with the control compound 200 nM NBD-X (open squares).

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NBD-X. NBD-X was used at this concentration to compare its fluorescence changes with those of NBD-C_6-HPC. NBD-X is more polar than most naturally occurring fatty acids [14] and since the hydrocarbon chain portion of NBD-X is only six carbons in length, this molecule does not behave as a surfactant in water and does not aggregate in aqueous solution [15]. In both control experiments, the bile salts were titrated over the same concentration ranges as in the NBD-C_6-HPC experiments. The normalized fluorescence of the controls versus concentration is overlaid with the aggregation experiment for comparison in Fig. 3. The relatively constant fluorescence of the control with increasing bile salt concentration indicates that the effect observed with the NBD-C_6-HPC is not reproducible with a small six carbon aliphatic chain NBD.

3.4. Hill analysis of aggregation

To quantitate the interaction between phospholipid monomer and bile salt monomer the bile salt–phospholipid data was fitted to the Hill equation [16] modified as

$$F_N = \frac{[BS]^n}{K' + [BS]^n}$$

where F_N is the normalized fluorescence; [BS] the bile salt concentration in millimolar; and n is the minimum number of bile salts that bind (aggregate) to the fluorophore. As described in Table 1, K' is a constant that contains the dissociation constant, K_{BS}, as well as other interaction factors that serve to describe the cooperative behavior of the system. A third constant, K^*, can be derived from the first two constants as the nth root of K' and is equivalent to half-maximal fluorescence of the probe. In this fluorescence analogy to the Hill equation, each bile salt that binds is assumed to increase the fluorescence yield of the NBD-C_6-HPC by $1/n$ of the maximum fluorescence. In this system, n is replaced with n_{app} noting that n_{app} determined experimentally will likely not be a whole number [16]. n_{app} represents a *minimum* number of bile salt monomers that associate with a single phospholipid. The relationship between K^* and K' becomes $K^* = K'^{1/n_{app}}$. This method has been shown previously [3] to be a viable method for approximating an aggregation number below the CMC.

Fig. 3A and B show data points for NaC and NaTDC, respectively, plotted with curves fit to the Hill equation. Table 1 shows the average calculated constants derived from the fits. The data used to generate these fits corresponds to the submicellar regions of the plots (NaC data from 2–6 mM and NaTDC data from 0.5–1 mM). The average calculated n_{app} values are 2.06 for NaC and 2.42 for NaTDC. These represent *minimum* aggregation numbers. The large differences in K' and K^* for the two bile salts is not surprising since the bile salts have different CMC values producing very different concentrations at half-maximal fluorescence for the two bile salts. For comparison, these values are in the same range albeit slightly higher than values reported by Shoemaker and Nichols who used a slightly different phospholipid probe whose NBD-label was covalently bonded to an ethanolamine head group and contained a single 16C chain [3].

Table 1
Average[a] values for constants[b] determined from fluorescence data fit to Hill equation

Bile salt	n_{app}	K'	K^*
NaC	2.06	103.0	5.37
NaTDC	2.42	1.12	1.05

[a] The average values were determined from fits to ten data sets for NaC and eight data sets for NaTDC over two 4 month periods.

[b] n_{app} = estimate of the minimum number of binding areas (represents the aggregation number) per phopholipid molecule producing maximum fluorescence assuming a high degree of cooperativity of the system; K' = a constant that includes the dissociation constant, K_{BS} and other interaction factors; $K^* = K'^{1/n_{app}}$, corresponding to the concentration of bile salt at half-maximal fluorescence.

Keeping in mind that n_{app} estimates a minimum number for aggregation, low aggregation numbers of ~3 have previously been reported for NaC under similar electrolyte conditions (0.100–0.150 M NaCl) by other groups using different methods [17,18]. Due to the increased hydrophobicity in dihydroxy bile salts like NaTDC, higher aggregation numbers have been calculated than for trihydroxy bile salts. NaTDC values in the literature range from around 5 [9] to 22 [5, for a review see 9] which are much higher than our determined value. However, Li and McGown have shown that bile salt solutions are polydisperse using dynamic fluorescence anisotropy measurements and suggest that NaTDC and NaTC dimers [19,20] are present. In addition, Kawamura et al. [6] and more recently Funasaki et al. [21] have reported the co-existence of more than one size and type of micelle present in NaTDC solutions. Our work provides more evidence for the existence of smaller, primary bile salt aggregates in submicellar bile salt–phospholipid mixtures.

3.5. Primary aggregation

The primary–secondary model for aggregate formation proposed by Small and Carey [2] and Small [5] attributes the low aggregation numbers seen at submicellar concentrations observed here to primary micelles. Other groups have also observed primary aggregation. Matsuoka et al. have performed steady-state fluorescence experiments with several different bile salts (including NaC and NaTDC) using pyrene and identify concentration ranges of primary and secondary aggregate formation [22,23] which they term first and second CMC ranges. These ranges are based on discontinuities (abrupt changes in slope) seen over two concentration regions in their fluorescence titrations. Our data sets do not display such discontinuities and provide a more gradual increase in fluorescence over the same range indicating a more cooperative behavior for aggregate formation. Despite this difference, Matsuoka et al. measure a first CMC for NaTDC in 0.15 M NaCl as 1.1 mM and a first CMC for aqueous NaC of 6.3 mM which actually are within the submicellar ranges studied here implying that we are measuring the same phenomenon with different probes. We attribute our differences in fluorescence trace to differences in bile salt aggregation with pyrene versus a phospholipid. Other groups also identify primary bile salt aggregates. More recently, Megyesi and Biczók [24] suggest the existence of a primary NaC aggregate at concentrations of 2–10 mM based on fluorescence lifetime

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measurements using a cationic fluorophore. This complements the evidence of small, primary aggregates by others using fluorescence lifetime analysis [19,20] and ESR [6]. The use of an actual phospholipid molecule as the probe in this study ensures a more realistic view of phospholipid solubilization by bile salts in the intestine. In conclusion, this paper supports the existence of primary aggregates of at least two monomers in aqueous solution (0.15 M NaCl) for both NaTDC, a dihydroxy bile salt and NaC, a trihydroxy bile salt using a phospholipid probe.

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