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Secondary assembly of bile salts mediated by β-cyclodextrin–terbium(III) complex

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Abstract—A fluorescent cyclodextrin–Tb(III) complex is successfully synthesized and can include bile salts in its hydrophobic cavities. Therefore, it can efficiently induce the secondary assembly of small bile salt primary micelles to large micelle aggregates, and the aggregation process can be easily observed by transmission electron microscopy (TEM) and fluorescence microscopy.

1. Introduction

Bile salts are important surfactant-like biological amphipathic compounds composed of a steroid skeleton and a hydrophilic tail, and are well known to possess many exciting biological properties.[1] Because their size well matches that of the cavity of β-cyclodextrin (β-CD), a cyclic oligosaccharide with seven D-glucose units linked by α-1,4-glucose bonds, the molecular recognition of bile salts by β-CDs and modified β-CDs has been extensively studied,[2] and some typical inclusion models of β-CDs or modified β-CDs with bile salt molecules have been clearly presented.[3] However, these studies generally restrict bile salt concentration below the critical micelle concentration (CMC_1). In fact, bile salts usually exist in biological environment with a concentration higher than CMC_1, and convert lipid-soluble material into water-soluble excreta in the form of micelles and micellar aggregates.[4] Moreover, β-CDs can encapsulate drug molecules in their hydrophobic cavities to afford host–guest complex or supramolecular species in aqueous solution and thus be utilized as drug carriers.[5] In the gastrointestinal tract, bile salt micelles promote the dissociation of β-CD/drug complexes and release drug molecules through a competitive exchange with the guest drug molecule.[6] Besides, the combinations of β-CDs with bile salt micelles have been used successfully for chiral drug separation.[7] Camilleri and co-workers reported a possible interaction mechanism of bile salt micelles with β-CDs.[8] Generally, at high surfactant concentrations, there will be an equilibrium between bile salt micelles and free bile salt molecules. β-CDs bind to the free bile salt molecules and shift the equilibrium away from the micellar phase. Then, β-CDs combined with bile salt molecules form a 'pseudo-stationary' phase. Herein, we prepare a pyridine-2,6-dicarboxylic acid-modified β-CD **1**, which can form a stoichiometric 3:1 complex (**2**) with Tb(III) cation. (Scheme 1) The pyridine groups in **2** can sensitize the luminescence of terbium(III) cation to give strong green fluorescence. Another interesting discovery is that **2** can induce the secondary assembly of a typical bile salt, that is, sodium deoxycholate (NaDC). Generally, NaDC exhibits two critical micelle concentrations at ca. 7 mM (CMC_1) and ca. 60 mM (CMC_2). Above CMC_1, NaDC forms small back-to-back micelles (composed of ca. 10 bile salt molecules) with the hydrophilic groups pointing outward, and these micelles convert to large rod-like micelles (ca. 4 nm) with a central hydrophilic core filled with water and ions above CMC_2.[9] Significantly, in the presence of $Tb{\cdot}\mathbf{1}_3$ complex, the small micelles of bile salt can aggregate to large micelle aggregates at a concentration above CMC_1, and the formation of the aggregates can be easily observed by TEM measurement and fluorescence microscopy. These results may provide an efficient and convenient method to label and capture bile salt micelles, which will have the potential application in diagnostics and separation sciences.

Keywords: Cyclodextrins; Bile salts; Micelles; Lanthanide; Fluorescence.
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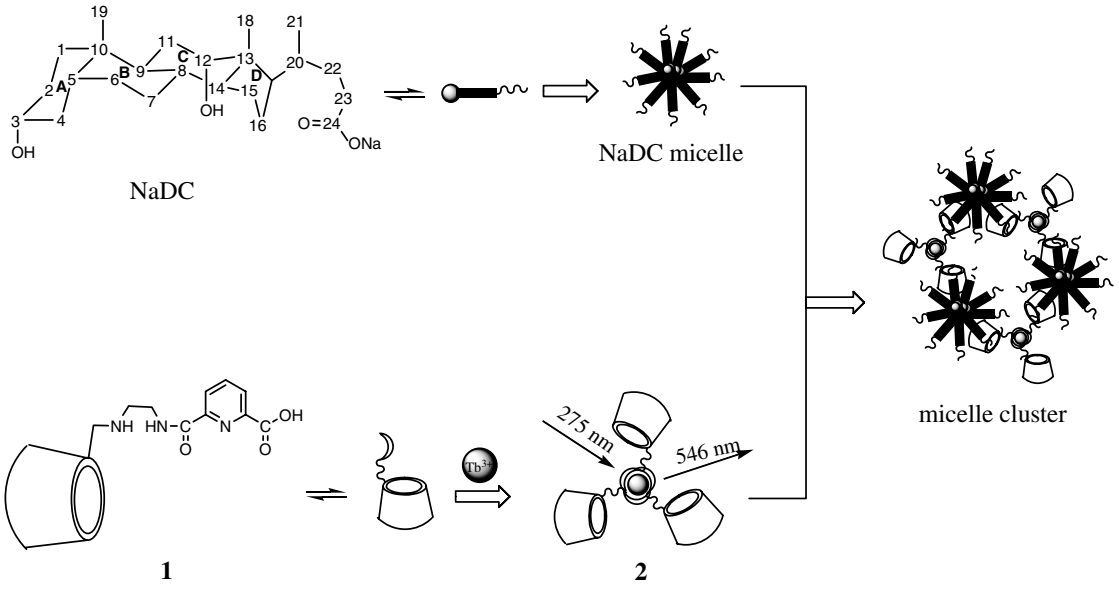

Scheme 1.

2. Results and discussion

2.1. Fluorescence behavior of 2

It is reported that pyridine-2,6-dicarboxylic acid form 3:1 fluorescent complex with Tb^{3+}, and the three-degree stability constants ($\log K$) of this complex are 8.68, 16.11, and 22.03, respectively.[10] Herein, the coordination stoichiometry of **1** with Tb^{3+} is determined by the continuous variation method using fluorescence spectrometry. As can be seen in Figure 1, the fluorescence intensity of the **1**/Tb^{3+} system shows a maximum at a molar ratio of Tb^{3+} of 0.25, which corresponds to a 3:1 **1**/Tb^{3+} stoichiometry. The possible interaction mode of **1** with Tb^{3+} is as follows. In each unit of **1**, the nitrogen atom of pyridine group, one free carboxylate group, and one oxygen atom of the amido group cooperatively complex Tb^{3+}, and three units of **1** complete the 9-coordination of Tb, giving the stable **1**/Tb complex. Moreover, the apparent stability constant ($\log K$) of **1** with Tb^{3+} is also measured to be 6.42 by fluorescence titration. Through an approximate calculation based on the concentrations of **1** and Tb^{3+} employed in our experiments as well as the apparent stability constants between **1** and Tb^{3+}, we deduce that most of **1** is converted to **2** in a 3:1 **1**/Tb^{3+} mixture. Therefore, **2** can be prepared in situ by mixing **1** and $Tb(NO_3)_3$ with a molar ratio of 3:1 in our experiments.

Benefiting from the fascinating photophysical property of Tb^{3+}, **2** displays a satisfactory luminescent behavior. When excited at 275 nm (the absorption maximum of **1**), **2** shows four emission peaks at 492 nm ($^5D_4 \rightarrow {}^7F_6$), 546 nm ($^5D_4 \rightarrow {}^7F_5$), 586 nm ($^5D_4 \rightarrow {}^7F_4$), and 622 nm ($^5D_4 \rightarrow {}^7F_3$). (Fig. 2) The intensities of these emissions show no appreciable changes in a pH range of 6–11 or even in the presence of 100 equiv of NaDC, which indi-

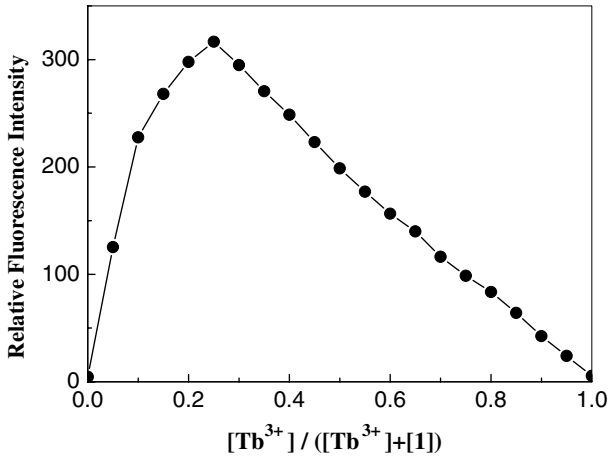

Figure 1. Job's plot of **1**/Tb^{3+} system in aqueous buffer solution at 25 °C and pH 9.18 ($[\mathbf{1}] + [Tb^{3+}] = 1.0 \times 10^{-5}$ M) produced with data taken from fluorescence spectra (λ_{ex} = 275 nm, λ_{em} = 492 nm).

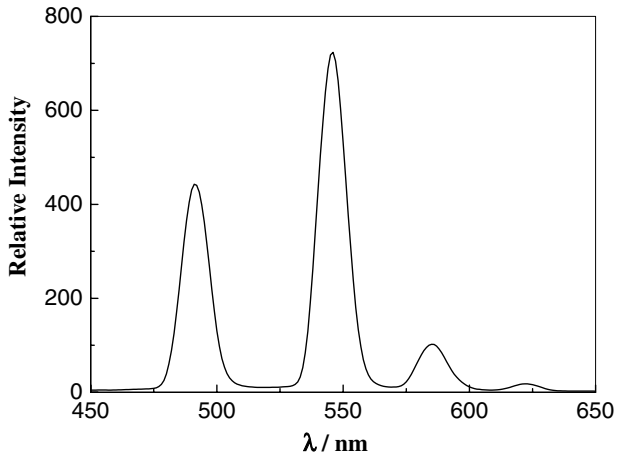

Figure 2. Fluorescence spectrum of Tb·$\mathbf{1}_3$ (0.1 mM) in buffer solution (pH 9.18) at 25 °C (λ_{ex} = 275 nm).

cates a satisfactory stability of **2**. Furthermore, the strong green fluorescence of **2** can be readily distinguished by eye even at a low concentration (0.1 mM). These phenomena indicate an efficient energy transfer from the pyridine groups in **1** to Tb^{3+}.

2.2. 2D NMR spectroscopy

Possessing three hydrophobic β-CD cavities, **2** has a capability of including various organic/inorganic/biological substrates. Herein, we try to investigate the association of **2** with NaDC micelle by NMR spectroscopy. Unfortunately, because terbium is a strong NMR shift reagent, the NMR signals of bile salt change to a broad band in the 1D NMR spectra, and the NOE signals in the 2D NMR spectra rapidly decay in the presence of **2**. Therefore, we select the modified β-CD **1**, the ligand unit of **2**, as host molecules to investigate its inclusion complexation behaviors with bile salt micelle. Figure 3 shows a ROESY spectrum of **1**/NaDC system at a concentration of NaDC below CMC_1. The complete assignment of NaDC protons is made following the reports by Campredon et al., and Barnes and Geckle.[11] The notation used is H-*n* for β-CD protons and P-*n* for bile salt protons. It is well known that only cross-peak interactions with the H-3, H-5, and H-6 protons of β-CDs are considered to analyze the results, because the H-2 and H-4 protons are not facing the inner cavity and the H-1 protons are affected by D_2O. As illustrated in Figure 3, the cross-peaks B correspond to the NOE correlations between the P-18 protons of NaDC and the H-3 protons of β-CD. The cross-peaks A correspond to the NOE correlations between the P-21 protons of NaDC and the H-3/H-5/H-6 protons of β-CD. The cross-peaks C correspond to the NOE correlations between the P-19 protons of NaDC and the H-3/H-5/H-6 protons of β-CD. The cross-peaks D correspond to the NOE correlations between the P-16 protons of NaDC and the H-3 protons of β-CD. The cross-peaks E correspond to the NOE correlations between the P-15/P-16/P-23 protons of the hydrophilic tail of NaDC and the H-3/H-5 protons of β-CD. These NOE correlations indicate that the C ring, D ring, and hydrophilic tail of NaDC may penetrate into one β-CD cavity, and the A ring of NaDC is included by another β-CD cavity, which is similar to the reported 1:2 binding mode of native β-CD and mono-modified β-CDs with NaDC below CMC_1.[2d,e] Therefore, we can deduce that at a concentration below CMC_1, NaDC may mainly form 1:2 inclusion complex with **1**, like the cases reported by Tato et al., although the formation of the 1:1 complexes is not rigorously ruled out.

The ^{1}H NMR and ROESY spectra of **1**/NaDC system at a concentration of NaDC above CMC_1 are also performed under the same condition. (Figs. 4 and 5) Figure 4 shows the ^{1}H NMR spectra of NaDC above CMC_1 in

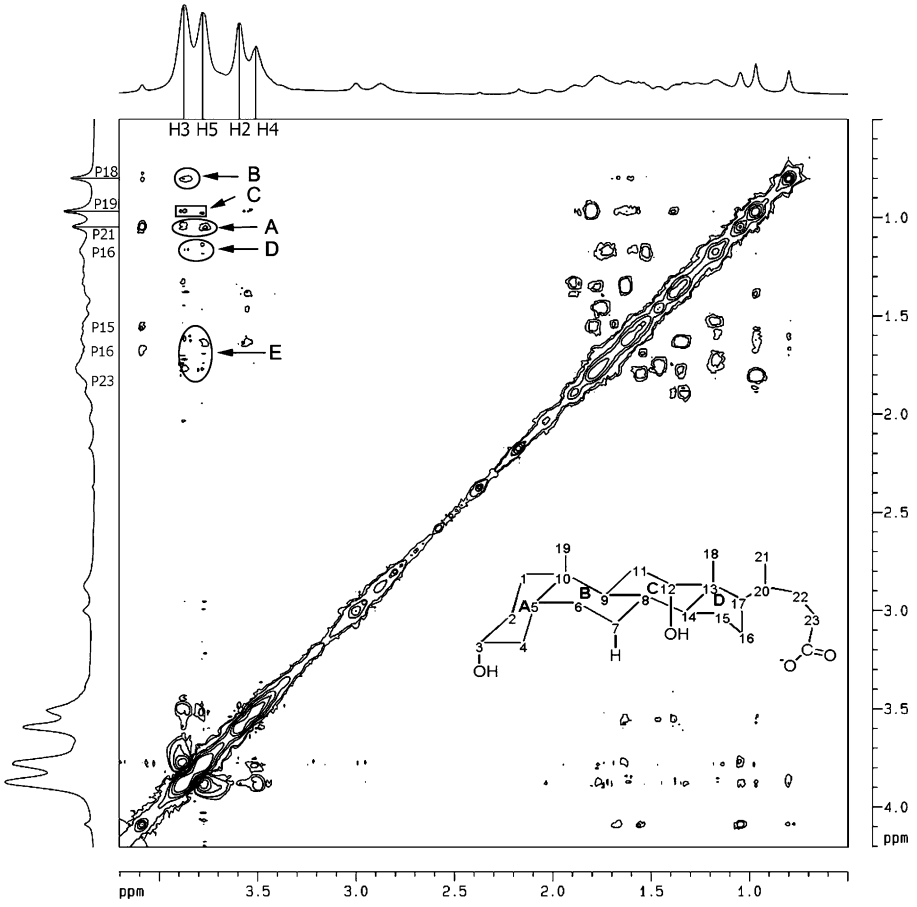

Figure 3. ROESY spectrum of NaDC (3 mM) in the presence of **1** (3 mM) in a pD 9.18 buffer with a mixing time of 600 ms.

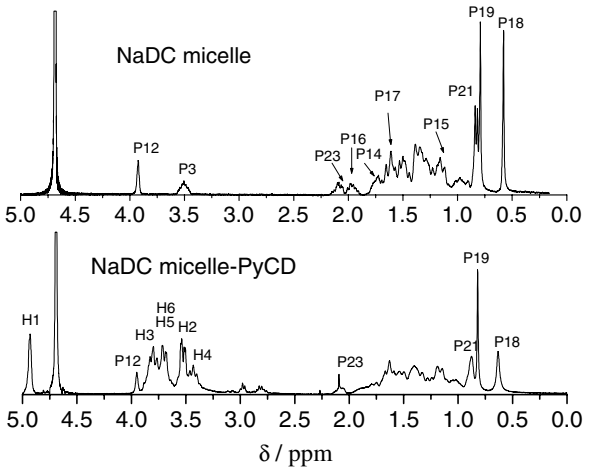

Figure 4. ^{1}H NMR spectra of NaDC (10 mM) in the absence (top) and presence (bottom) of **1** (3 mM) at 298 K in a pD 9.18 buffer.

the absence and the presence of **1**. As can be seen in Figure 4, the P-19 protons of NaDC show appreciable downfield shifts ($\Delta\delta$ 0.029) in the presence of **1**. Because the formation of bile salt micelles will result in the downfild shifts of P-19 protons of bile salt,[1a] these results indicate that the introduction of **1** favors the formation of micelles, which is similar to Ghorab's reports that cyclodextrin at a low concentration will promote micelle formation.[6a]

As illustrated in Figure 5, the cross-peaks A correspond to the NOE correlations between the P-18 protons of NaDC and the H-3 protons of β-CD. The cross-peaks B correspond to the NOE correlations between the P-21 protons of NaDC and the H-3/H-5/H-6 protons of β-CD. The cross-peaks C correspond to the NOE correlations between the P-15/P-16 protons of NaDC and the H-3 protons of β-CD. The cross-peaks D correspond to the NOE correlations between the P-12 protons of NaDC and the H-3 protons of β-CD. However, no NOE correlations between the P-19 protons of NaDC and the H-3/H-5/H-6 protons of β-CD can be observed. Since the H-3 protons are located at the wide opening of the β-CD cavity, while H-5/H-6 protons near the narrow opening, these NOE correlations indicate that the C ring and D ring of NaDC may be included in the β-CD cavity from the wide opening. Based on the above comparison, we deduce that NaDC forms the stable back-to-back aggregates at a concentration exceeding CMC_1, which cannot be disrupted by the inclusion complexation of **2**. Therefore, the β-CD cavities of **2** can only insert into the surface of NaDC micelle by including the hydrophilic side (C ring, D ring, and hydrophilic tail) of NaDC, where the β-CD cavity mainly includes the C ring and D ring of NaDC, and the carboxylate group of NaDC is located close to the Tb-coordinated linker. Under our experimental conditions, the carboxylate group of NaDC is not protonated and should exist as a carboxylate anion. Therefore, the electrostatic interac-

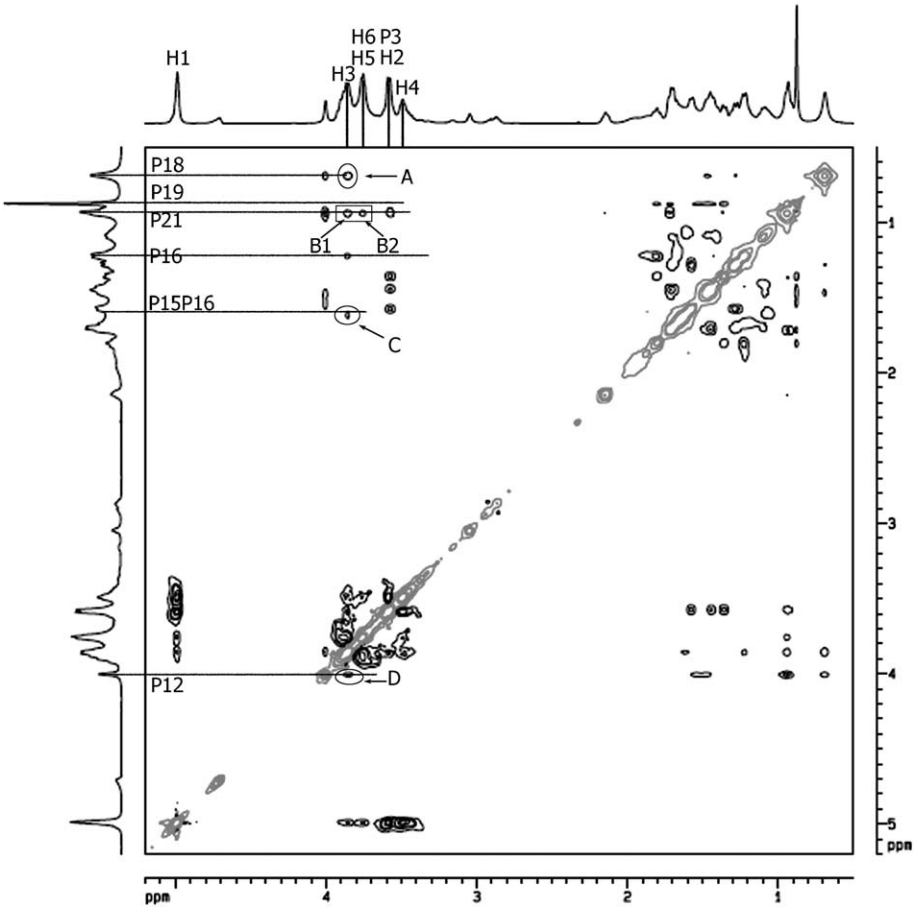

Figure 5. ROESY spectrum of NaDC (10 mM) in the presence of **1** (3 mM) in a pD 9.18 buffer with a mixing time of 600 ms.

tions between the Tb-coordinated linker and the anionic carboxylate tail of NaDC may favor the inclusion complexation to some extent. Consequently, the complexation of **2** with the surface of NaDC micelle links little micelles to form large micelle clusters as shown in Scheme 1.

2.3. Morphology of 2-mediated NaDC assembly

According to the reported primary and secondary aggregate model of bile salts by Small[12] NaDC can only form fairly little primary micelles (composed of ca. 10 monomers) at a concentration of 10 mM. However, in the presence of **2**, NaDC forms the far larger assemblies under the same condition. The formation of large assemblies can be monitored easily by examining the turbidity of solution. At a concentration of 10 mM, the NaDC solution (at pH 9.18) is transparent. However, with the gradual addition of **2**, the NaDC solution turns translucent. When the concentration of **2** reaches 0.6 mM, a white turbid solution is formed. This phenomenon indicates the formation of large NaDC assemblies that can scatter the light in solution.

Transmission electron microscopy (TEM) and fluorescence microscopy present the direct information about the morphology of **2**-mediated NaDC assemblies. As shown in Figure 6, the size range of **2**-mediated NaDC assemblies is ca. 30–100 nm, which is even far larger than that of the rod-like secondary aggregates of NaDC (ca. 4 nm length and 0.8 nm radius).

Due to the good luminescent property of **2**, the **2**-mediated large NaDC assemblies can be easily observed by fluorescence microscopy. In the control experiments, NaDC solution (10 mM) or the **2**/NaDC mixture ([NaDC] = 10 mM, [**2**] = 0.3 mM) presents black images. Significantly, the **2**/NaDC mixture ([NaDC] = 10 mM, [**2**] = 0.6 mM) presents a number of large particles with green fluorescence, as illustrated in Figure 7. Based on above experiment results, we can deduce a possible mechanism for the formation of large micelle assemblies as shown in Scheme 1. Through the inclusion complexation of β-CD cavities with NaDC molecules, **2** are immobilized on the surface of small NaDC micelles, and subsequently induce the assembly of the discrete small NaDC micelles to the large secondary assemblies through the intermolecular linkages.

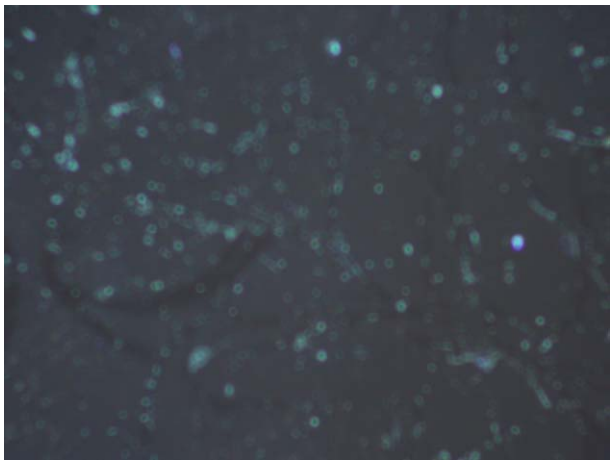

Figure 7. Fluorescence micrograph (magnification 400×) of **2**/NaDC mixture ([NaDC] = 10 mM, [**2**] = 0.6 mM) in buffer solution (pH 9.18).

3. Conclusion

In summary, we successfully prepare fluorescent β-CD–Tb(III) complexes, **2**, as connectors for the secondary assembly of bile salt micelles. Under identical conditions, neither Tb^{3+} nor **1** can induce the assembly of bile salt micelles. From these results, we deduce that the polymeric β-CDs may also have a capability of inducing the secondary assembly of bile salts, which may be important to the application of polymeric β-CDs in the design of the drugs for the liver/gallbladder diseases and the stationary phase for the chiral drug separation.

4. Experimental

4.1. Materials

Sodium deoxycholate (NaDC) was purchased from Sigma and directly used after dried in vacuo at 50 °C for

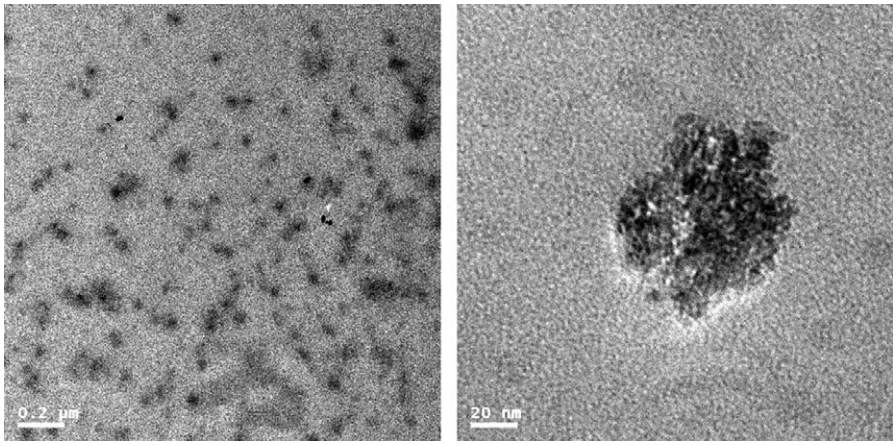

Figure 6. TEM image (left) and high-resolution TEM image (right) of **2**/NaDC mixture ([NaDC] = 10 mM, [**2**] = 0.3 mM).

two days. Terbium nitrate was prepared by dissolving the corresponding oxides of 99.99% purity (Bao-tou Rare Earth Chem. Co.) in 50% aqueous nitric acid. After evaporation, the solid residue was dried in vacuo for several days. The amount of terbium was standardized by EDTA titration with xylenol orange as an indicator. Pyridine-2,6-dicarboxylic acid was purchased from Alfa Aesar. β-Cyclodextrin of reagent grade was recrystallized twice from water and dried in vacuo at 95 °C for 24 h prior to use.

4.2. Measurements

Rotating-frame Overhauser effect spectroscopy (ROESY) experiments were recorded on a Varin Mercury VX600 instrument. Samples were kepy at least 24 h before measurement for equilibration. All 2D NMR experiments were carried out in D_2O buffered with borate to pD 9.18. Fluorescence spectra were measured in a conventional rectangular quartz cell ($10 \times 10 \times 45$ mm) at 25 °C on a JASCO FP750 spectrometer equipped with a constant-temperature water bath, with the excitation and emission slit's width of 5 nm. Transmission electron microscopic (TEM) measurements were performed on Philips Tecnai G2 20 S-TWIN microscope operating at an accelerating voltage of 200 keV. The mixture solution was sonicated for 5 min and then equilibrated for 24 h. Before measurement a drop of the solution was dropped onto a carbon-coated copper plate and dried rapidly in vacuo. Microscopy fluorescence was visualized using a Nikon Eclipse TE2000U high-resolution differential interference contrast microscopy. The images were excited by UV light and recorded with a digital color CCD camera.

4.3. Preparation of pyridine-2,6-dicarboxamide-modified β-CD (1)

To a solution of DMF (50 mL) containing 2.61 g (2 mmol) of mono[6-(2-aminoeythylamino)-6-deoxy]-β-CD and 1.24 g of dicyclohexylcarbodiimide (DCC) was added 0.40 g (2.4 mmol) of pyridine-2,6-dicarboxylic acid in the presence of a small amount of 4 Å molecular sieves. The reaction mixture was stirred for 1 d in an ice bath and another 2 d at room temperature. The precipitate was removed by filtration, and the filtrate was poured into 300 mL acetone. The white precipitate was collected and subsequently purified on a Sephadex G-25 column with deionized water as eluent. After drying in vacuo, a pure sample was obtained in 8% yield. MS (ESI): *m/z* 1344.68 ($M+NH_4^+$); ^1H NMR (D_2O, TMS, ppm): δ 2.6–3.1 (m, 4H), 3.2–4.0 (m, 42H), 4.90 (s, 7H), 7.88 (s, 3H). Anal. Calcd for ($C_{51}H_{79}O_{37}N_3 \cdot 3H_2O$) C, 44.38; H, 6.21; N, 3.05. Found: C, 44.50; H, 6.41; N, 3.18. FT-IR (KBr) ν/cm^{-1} 438, 532, 579,608, 651, 707, 757, 847, 944, 1031, 1079, 1155, 1242, 1369, 1434, 1572, 1615, 2152, 2930, 3337.

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