Micellar aggregates and hydrogels from phosphonobile salts†‡

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Introduction

Bile salts are naturally occurring amphiphilic molecules, possessing hydrophobic and hydrophilic surfaces. They have a remarkable capacity to solubilize cholesterol and emulsify fat in the intestinal tract by forming mixed micelles with lipids. The classical surfactants with flexible alkyl chains form spherical or ellipsoidal micelles, whereas micelles derived from bile salts are flattened and ellipsoidal in shape. In the most accepted model, the hydrophobic faces of bile salts interact with each other and the hydrophilic regions face water.‡ Bile salt micelles have been studied under different conditions (pH, ionic strength and temperature) by over fifty physical techniques. Apart from the formation of micelles, bile salts also form hydrogels close to neutral pH through self-assembled fibrillar network (SAFIN) formation.‡ Sodium deoxycholate has been shown to form helical aggregates in the gel network. Hydrogel derived from sodium deoxycholate has also been studied for drug delivery applications.‡

Polymer based hydrogels are an important class of materials successfully employed in drug delivery, tissue engineering, cosmetics and food materials. Organo† and hydrogels‡‡ derived from low molecular mass species have received considerable attention during the past decade because of the unique properties‡‡ displayed by these solid-like liquid materials.

We recently reported the synthesis of phosphonobile acids from natural bile acids. The aggregation properties of 23 and 24-phosphonobile acids (Chart 1) using fluorescence and 31P NMR methods. The first examples of pH dependent hydrogel formation by cholic acid analogs 1 and 6 have been studied using a variety of techniques like ANS fluorescence, 31P NMR, induced circular dichroism and SEM. A novel thermochoic gel has also been developed.

Results and discussion

Measurement of critical micellar concentration

The critical micellar concentrations of various phosphonobile salts were measured by two distinct techniques. One, using the hydrophobic dye pyrene (invasive), involved plotting the ratio of pyrene vibronic emission bands I$_{1}$/I$_{3}$, as a function of PBS concentration to obtain the CMC.‡‡ In the second method, the aggregation-sensitive chemical shift of the 31P nuclei present in the PBSs was exploited for the CMC measurement (non-invasive). All the CMC measurements were done under small intestinal conditions (pH 6.4 maintained by 50 mM P$i^{-}$, 0.15 M Na$^+$). At this pH the phosphonobile salts exist predominantly as monoionic species. The measured apparent dissociation constant values were found to be ∼2.4 (pK$_{a}$) and ∼8.1 (pK$_{b}$), which are similar to the pK$_{a}$ values reported for alkylphosphonic acids. The critical micellar concentrations of various phosphonobile salts are shown in Fig. 1, and the CMC values estimated by this method are presented in Table 1. The CMC

‡ Electronic supplementary information (ESI) available: plots of CGC measurement of 6, packing diagrams of 6, PXRD pattern of disodium salt of 6 (simulated) and xerogel of NaDC gel and crystallographic information file (CIF). See http://dx.doi.org/10.1039/b504656d

| Chart 1 Structures of 23 and 24-phosphonobile acids. |
The equilibrium between the monomer and micellar aggregates. The line width, however, remained constant, suggesting fast formation is large and varied (0.1–0.4 ppm) for different PBSs. The change in chemical shift upon micelle concentration of PBS showed a break point, which was taken as the CMC (Fig. 2). The change in chemical shift difference ($\Delta$) as a function of the reciprocal concentration of PBS showed a break point, which was taken as the CMC (Fig. 2). The change in chemical shift upon micelle formation is large and varied (0.1–0.4 ppm) for different PBSs. The line width, however, remained constant, suggesting fast equilibrium between the monomer and micellar aggregates. The values of 23-PBSs vary from 2 to 9 mM depending upon the structure and number of hydroxyl groups present in the steroid backbone. The chenodeoxy (3) and the deoxy (2) derivatives showed the lowest CMC values followed by cholic (1) and ursodeoxycholic acid (4) analogs. A similar trend was followed for 24-PBSs, except that the CMC values were slightly (about 1 mM) lower than the corresponding 23-PBSs. This marginal reduction in the CMC value of 24-PBSs is expected because of the homologation. In general, the CMC values of PBSs were found to be lower compared to unconjugated bile salts (at pH 10.0) and comparable to those of conjugated bile salts. An increase in the bulk pH increased the CMC and the addition of NaCl decreased the CMC of the PBSs.

(b) CMC measurements using $^{31}$P NMR$^{25}$. The plot of $^{31}$P chemical shift difference ($\Delta$) as a function of the reciprocal concentration of PBS showed a break point, which was taken as the CMC (Fig. 2). The change in chemical shift upon micelle formation is large and varied (0.1–0.4 ppm) for different PBSs. The line width, however, remained constant, suggesting fast equilibrium between the monomer and micellar aggregates. The phosphonocholates (1 and 6) formed gels at acidic pH (1.7–2.5). This reversible hydrogel formation is unprecedented for cholic acid or its analogues. The presence of an additional hydroxyl functionality in the side chain, lacking in cholic acid (Chart 2), may be crucial for the gelation of deoxycholate derivatives 5 and 10 formed gels at pH 7–7.5. The other dihydroxyl derivatives 2, 3, 4, 7, 8 and 9 formed gels between pH 3–6.5. It has been suggested that the gelation of deoxycholate is influenced by two factors: the pH and the concentration of the PBS solution. The pH-dependent hydrogelation of PBSs

During the studies on PBS micelles, we observed that the reduction in the pH of the PBS solutions led to an increase in the viscosity subsequently forming hydrogels at low concentrations. The number and the orientation of hydroxyl groups present in the steroid backbone greatly influenced their gelling pH in water, which varied from 1.7 to 7.5 (Table 2). The phosphonocholates (1 and 6) formed gels at acidic pH (1.7–2.5). This reversible hydrogel formation is unprecedented for cholic acid or its analogues. The presence of an additional hydroxyl functionality in the side chain, lacking in cholic acid (Chart 2), may be crucial for the gelation of deoxycholate derivatives 5 and 10 formed gels at pH 7–7.5. The other dihydroxyl derivatives 2, 3, 4, 7, 8 and 9 formed gels between pH 3–6.5. It has been suggested that the gelation of deoxycholate

Table 1 Comparison of CMC values (in mM) of 23- and 24-PBSs measured using fluorescence (at 25 °C) and $^{31}$P NMR (at 30 °C, in parentheses) with those of bile salts

<table>
<thead>
<tr>
<th>Backbone structure</th>
<th>23-PBS</th>
<th>24-PBS</th>
<th>CMC of unconjugated (glycoconjugated) bile salts$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholate</td>
<td>1: 5–6 (5–6)</td>
<td>6: 4–5 (4)</td>
<td>11 (10)</td>
</tr>
<tr>
<td>Deoxycholate</td>
<td>2: 2–2.5 (2–2.5)</td>
<td>7: 1–1.5 (1.5)</td>
<td>3 (2)</td>
</tr>
<tr>
<td>Chenodeoxy</td>
<td>3: 2–2.3 (1.5–2)</td>
<td>8: 1.5–1.75</td>
<td>4 (1.8)</td>
</tr>
<tr>
<td>Ursodeoxy</td>
<td>4: 8–9 (8)</td>
<td>9: 6–7 (6)</td>
<td>7 (4)</td>
</tr>
</tbody>
</table>

* Compound 8 formed a transparent gel and compounds 5 and 10 were insoluble under identical conditions. $^a$ The CMC values, measured by surface tension, of unconjugated and glycoconjugated (values in parentheses) bile salts have been taken from ref. 19.

Table 2 Conditions for the hydrogel formation by 23- and 24-PBSs and bile salts. The values in parentheses refer to the pH. Concentrations are in mM

<table>
<thead>
<tr>
<th>Backbone structure</th>
<th>23-PBS</th>
<th>24-PBS</th>
<th>Natural BS</th>
<th>Remarks$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholate</td>
<td>1: &gt;4 (1.7–2.4)</td>
<td>6: &gt;3 (1.7–2.5)</td>
<td>No gelation$^a$</td>
<td>TG from both</td>
</tr>
<tr>
<td>Deoxy</td>
<td>2: &gt;2 (3.1–3.4)</td>
<td>7: &gt;2 (6.3)</td>
<td>&gt;20 (7.4–7.2)</td>
<td>7 forms TG after 6 h</td>
</tr>
<tr>
<td>Chenodeoxy</td>
<td>3: &gt;15 (6.6–6.5)</td>
<td>8: &gt;15 (6.5 + 0.15 M NaCl)</td>
<td>&gt;30</td>
<td>TG from both</td>
</tr>
<tr>
<td>Ursodeoxy</td>
<td>4: &gt;20 (~6)</td>
<td>9: &gt;20 (~6)</td>
<td>&gt;40 + 0.15 M NaCl</td>
<td>Turbid gel from both</td>
</tr>
<tr>
<td>Litho</td>
<td>5: &gt;15 (7–7.5)</td>
<td>10: &gt;15 (7–7.5)</td>
<td>(~8.5)</td>
<td>TG from both</td>
</tr>
</tbody>
</table>

$^a$ TG—Transparent gel. $^c$ Calcium cholate has been shown to form an irreversible translucent hydrogel at 50 °C.

Fig. 1 Plots of pyrene I/I vs. concentration of 1 (■), 2 (○), 3 (▲) and 4 (△) in pH 6.4 phosphate buffer. The arrows indicate the CMC values.

Fig. 2 Plot of $^3$P $\Delta$ as a function of the inverse of the concentration of salts of 1 (■) and 6 (○) at pH 6.4. The arrows indicate the CMC values.
Chart 2 Comparison of the structure of cholic acid (11) and its analogues (1, 6 and 12).

requires partial protonation of the anion to generate a mixture of the detergent and the free acid. We believe that a similar phenomenon is responsible for the pH dependent gelation of the PBSs as observed by us.

Gelation induced colour change by phosphono deoxycholate (2)

Luminescent or thermochromic gels have the potential of being useful as novel materials. Compound 2 (23-phosphono deoxycholate) was found to gel at pH 3.1–3.4. To develop a thermochromic gel, congo red (CR) was chosen to dope this gel, since it has a pK<sub>a</sub> within this range. CR shows a violet colour at pH 3.0 and orange–red colour at pH >5.2. The violet color of CR incorporated in the gel of 2 (pH 3.3) changed to magenta–red upon heating the gel (Fig. 3). Thus the gel to sol transition or the gel formation was visibly observed. This strategy can be explored further to develop inexpensive thermal sensors.

Fig. 3 Photograph of a gel (violet colour) and sol (magenta–red colour) of 2 doped with congo red (in pH 3.3 buffer) in an inverted cuvette (1 mm). The top half was heated with a heat gun and photographed immediately.

To understand the mechanism of colour change the absorption spectrum of CR was recorded under different conditions. The absorption maxima of CR at pH 3.3 and 6.6 are at ~565 and ~495 nm, respectively. CR (0.167 mM) in the gel of 2 at pH 3.3 showed a violet colour with λ<sub>max</sub> ~ 540 nm (shoulder ~460 nm). When the gel was heated to 70 °C the colour changed to magenta–red with the λ<sub>max</sub> blue-shifting to 510 nm in the sol (Fig. 4).

Fig. 4 Absorption spectra of congo red at pH 6.6 (magenta–red, dotted line) and pH 3.3 buffer (violet, solid line) and in the gel of 2 (violet, dotted line) and sol (magenta–red, solid line).

CR showed a violet colour both in pH 3.3 buffer and in the gel of 2, suggesting the absence of any interaction between the dye and the gel fibers. In the sol (at a higher temperature), the colour change may occur due to either (i) bulk pH change upon heating the gel to sol or (ii) preferential solubilization of the purple form of the dye in the aggregates present in the sol. The former can be ruled out as there was no colour change observed when CR dissolved either in pH 3.5 buffer or in the presence of non-gelling concentration of 2 was heated to 70 °C. Thus, it is very likely that micelle like aggregates in the sol of 2 are responsible for the colour change by altering the equilibrium position between the two chromophoric forms of CR.

The pH responsive hydrogelation by phosphonocholates

As mentioned earlier, hydrogel formation by cholic acid or its analogs (11 and 12) have not been reported. Avicholic acid, another trihydroxy bile acid, also failed to gel water. Thus, a detailed study of this unusual hydrogelation by phosphonocholates was undertaken.

Gels of phosphonocholates showed high gel to sol transition temperatures (T<sub>gel</sub>) and were thermally reversible. Interestingly, some of the T<sub>gel</sub> values were higher than the boiling point of water when measured in sealed tubes. The thermal stability of the gels increased with an increase in the gelator concentration and decrease in the pH (Fig. 5). Critical gelator concentrations (CGC) were measured by both fluorescence and 31PN M R . The change in the steady state fluorescence intensity and anisotropy of ANS were utilized for the determination of CGC. ANS, which has been used to characterize the partially folded/native state of proteins, showed a significant increase in the fluorescence intensity upon gelation, with a blue-shift in the emission maxima. The increase in the ANS fluorescence intensity, the fluorescence anisotropy and emission λ<sub>max</sub> shift in the gel state are possibly due to the binding of ANS molecules in the hydrophobic pockets of gel aggregates formed by 1 and 6 (Fig. 6).

In the NMR method, the change in the local environment around the phosphorus atom during gelation caused an up-field shift. Furthermore, upon gelation the line-width at half height of the 31P peak increased (figure not shown), possibly due to the restricted motion of the molecules in the SAFINs. The estimated CGC of gelators 1 and 6 in pH 2.1 buffer by both the methods are 4 and 3 mM, respectively.

Fig. 5 \( T_{gel} \) as a function of concentration of 1 (at pH 2.1, ■).

Fig. 6 Plot of ANS fluorescence intensity (at 460 nm, ■) and anisotropy (at 475 nm, ◦) vs. concentration of 1 (at pH 2.1). The arrow indicates the visual gelation point.

The chiral nature of gel aggregates was studied by induced circular dichroism spectroscopy. ANS (0.425 mM) was used as an achiral chromophore in the gel of 6. Indeed, ANS showed a chiral signature in the gel of 6, suggesting its inclusion within a chiral environment in the gel network (Fig. 7).

Fig. 7 Absorption (top) and CD (bottom) spectra of ANS (0.425 mM) in the gel of 6 (18 mM).

Morphological studies

In order to see the nature of the aggregates formed, gels samples (without buffer) of 1 and 6 were examined by SEM, which showed a collapsed fibrous network structure (Fig. 8). The apparent absence of macroscopic chirality in the gel fibers does not, however, rule out the presence of chiral supramolecular structures.

Bile acids/salts are known to organize in a specific manner in the crystal lattice, and thus it was of interest to us to examine a phosphonobile salt by single crystal X-ray analysis.§ It is of course known from several studies that the packing modes of gelator molecules in the crystal and in the gel form are not similar. We found that in the structure of the disodium salt of 6 there are no abnormal bond lengths/angles, but the side chain conformation is different from bile salts. The dihedral angle of C17–C20–C22–C23 in 6 is 64.03° while that of sodium cholate is 170.51° (Fig. 9). The observed short torsion angle is due to a rather unusual conformation of the side chain (gauche), which is not observed in the crystal structure of any of the reported bile acids/salts and their derivatives. The crystal packing shows a bilayer arrangement, in which the \( \beta \)-surfaces of 6 are facing each other forming a hydrophobic layer, and the \( \alpha \)-hydroxyl groups, sodium ions and water molecules form a hydrophilic channel (see ESI† for packing diagrams).

Fig. 8 SEM pictures of xerogels obtained from gel of 1 (left) and 6 (right).

Fig. 9 ORTEP diagram of disodium salt of 6 (hydrogen atoms are removed for clarity).

Although XRD shows a layered structure for the salt of 6 in the crystalline state, the molecular packing in the gel phase (at pH 2.1) need not be the same. So, the powder XRD pattern of the xerogel and the gel were examined. In the case of the xerogel and the native gel (Fig. 10) the two most prominent peaks are observed in the PXRD pattern at 31.3° and 45.4°. The lack of lower angle peaks in the PXRD of gel and xerogel imply that the molecular packing in the gel is not a layered structure. A comparison with the PXRD pattern of the xerogel of sodium deoxycholate gel (NaDC) showed the same pattern and peaks positions. NaDC gel has been shown to form helical aggregates in the gel by fiber XRD studies. This comparison suggests that a similar molecular organization might be present in the phosphonocholate gels.

§ Crystal data: chemical formula C24H38O14Na1P1, formula weight 627.5, orthorhombic \( P2_1 2_1 2_1 \), \( a = 9.408(9), b = 11.050(10), c = 31.834(30) \text{ Å}, V = 3309.4(53) \text{ Å}^3 \), \( Z = 4, \rho(\text{calc}) = 1.26 \text{ g cm}^{-3}, T = 290 \text{ K, } \mu = 0.169 \text{ mm}^{-1}, \) reflections measured = 23220, unique reflections = 5613, reflections observed \( |I > 2\sigma(I)| = 5313, R(\text{int}) = 0.0269, R_l_\text{obs} = 0.054, wR^2_\text{obs} = 0.153, F(000) = 1323.8, \text{ G.o.f} = 1.091. \) CCDC reference number 240096. See http://dx.doi.org/10.1039/b504656d for crystallographic data in CIF or other electronic format.
Micellar aggregation of 23- and 24-phosphonobisalts has been examined by fluorescence and $^3$P NMR methods, which were in good agreement with each other. Phosphonobis acids 2, 3, 7 and 8 formed micelles at lower concentrations compared to compounds 1, 6 and 9. All the phosphonobisalts formed hydrogels at different pH values ranging from 1.7 to 7.5. The pH range at which hydrogels were formed by the individual phosphonobisalt was rather narrow. This property might enable these hydrogels to be useful for drug delivery and other pH-responsive systems with further developmental work. A thermochromic system that changes color from violet to red upon hydrogel formation was designed and demonstrated. Furthermore, hydrogel formation by compounds 1 and 6 has been studied in detail using a variety of techniques. These present physico-chemical studies on phosphonobisalts will be useful for the biological evaluation of these novel analogs as bile acid metabolism modifiers in vivo, and towards the design of newer hydrogelators.

Experimental

CMC measurements

All the reported CMC values were measured using the disodium salts of phosphonobisalts dissolved in 50 mM pH 6.4 phosphate buffer at 25 °C. A solution of PBS together with pyrene (1 µM) was equilibrated for 12–24 h before recording the emission spectra. All the fluorescence experiments were carried out in a fluorescence micro cell (0.5 cm) on a Perkin-Elmer LS 50B spectrophotometer with a flow-type temperature controller. All the CMC and CGC measurements by $^3$P NMR were done with buffer in 20% D$_2$O on a Bruker AMX 400 MHz spectrometer at 30 °C. All the CGC measurements were done using phosphate buffer (50 mM) and the values reported here are the average of two experiments. The CD and absorption spectrum of the gel of 6 (prepared by neutralizing a solution of disodium salt of 6 containing ANS with dil. HCl) was recorded using a 0.1 cm cell. All the $T_{mp}$ values were measured by carefully heating 0.5 mL of the gel in a sealed, inverted pyrex text tube (5 cm × 0.5 cm id) in an oil bath. The temperature at which the gel started to flow downwards was noted as the $T_{mp}$.

X-Ray diffraction studies

X-Ray quality single crystals of disodium salt of 6 were obtained by slow evaporation of a methanolic solution. The single crystal data were collected at room temperature on a Bruker AXS SMART APEX CCD diffractometer. The X-ray generator was operated at 50 kV and 35 mA using MoK$_\alpha$ radiation. Data were collected with $\omega$ scan width of 0.3 A. A total of 606 frames were collected in three different settings of $\omega$ (0°, 90°, 180°) keeping the sample to detector distance fixed at 6.03 cm and the 2θ value fixed at −25°. The data were reduced using SAINTPLUS and an empirical absorption correction was applied using the package SADAB. The crystal structure was solved by direct methods using SIR92 and refined by full matrix least squares method using SHELXL97 present in the program suite WinGX (Version 1.63.046). The hydrogen atoms were located and refined isotropically. ORTEP plot and packing diagram were generated using CAMERON. Geometrical calculations were done using PARST97. Powder diffraction data was collected on a Philips X’Pert diffractometer operating at 40 kV and 30 mA using WKr radiation and a curved graphite crystal as a monochromator.

Fig. 10 Powder XRD patterns of xerogel (top) and native gel (bottom) of 6. Y-Axis is not scaled to absolute intensity.

Conclusions

Scanning electron micrographs

To observe the morphology of the xerogels 200 Å thick films were deposited by dc sputtering, and were examined by using a Leica 440i scanning electron microscope with a LaB$_6$ emitter.

Acknowledgements

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References

12 Response to external stimuli, thermal reversibility, low melt viscosity, potential biocompatibility, etc.
The pK\textsubscript{a} values of 1 and 6 were measured using change in 31P chemical shift as a function of pH in the buffer solutions. A nonlinear regression curve fitting was done using the following equation: \[ \text{pH} = \text{pK}_\text{a} + \log \frac{(d - d_a)}{(d - d_b)} \]; pK\textsubscript{a}, δ\textsubscript{a}, and δ\textsubscript{b} are variable parameters, δ\textsubscript{a} is limiting δ in the acidic medium and δ\textsubscript{b} is limiting δ in the basic medium.