

Micellar aggregates and hydrogels from phosphonobile salts†‡

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The aggregation properties of novel bile acid analogs—phosphonobile salts (PBS)—have been studied. The critical micellar concentration of 23 and 24-phosphonobile salts were measured using fluorescence and ³¹P NMR methods. All the ten synthesized phosphonobile salts formed gels at different pH ranges in water. The pH range at which individual PBSs could gelate water was narrow and influenced by the number and conformation of hydroxyl groups. A reversible thermochromic system has been developed (with 23-phosphonodeoxycholate at pH 3.3), which changes color upon gelation. The investigation of the first hydrogels derived from *trihydroxy* bile acid analogs **1** and **6** was made using fluorescence, ³¹P NMR, X-ray crystallography, circular dichroism and SEM. The present studies reveal that the gel network consists of a chiral, fibrous structure possessing hydrophobic interiors.

Introduction

Bile salts are naturally occurring amphipathic 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.¹ 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.² Bile salt micelles have been studied under different conditions (pH, ionic strength and temperature) by over fifty physical techniques.³ Apart from the formation of micelles, bile salts also form hydrogels close to neutral pH through self-assembled fibrillar network (SAFIN) formation.⁴ Sodium deoxycholate has been shown to form helical aggregates in the gel network.⁵ Hydrogel derived from sodium deoxycholate has also been studied for drug delivery applications.⁶

Polymer based hydrogels are an important class of materials successfully employed in drug delivery,⁷ tissue engineering,⁸ cosmetics and food materials. Organo⁹ and hydrogels^{10,11} derived from low molecular mass species have received considerable attention during the past decade because of the unique properties¹² displayed by these solid-like liquid materials.

We recently reported the synthesis of phosphonobile acids from natural bile acids.^{13,14} In this paper, we report studies on 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 thermochromic gel has also been developed.

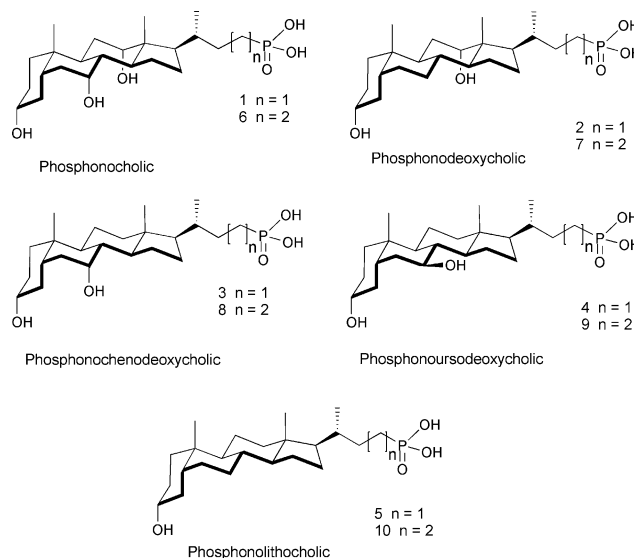


Chart 1 Structures of 23 and 24-phosphonobile acids.

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_3/I_1 as a function of PBS concentration to obtain the CMC.¹⁵ In the second method, the aggregation-sensitive chemical shift of the ³¹P nuclei¹⁶ 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 values¹⁷ (below CMC) of **1** (and **6**) were found to be ~2.4 (pK₁) and ~8.1 (pK₂), which are similar to the pK_a values reported for alkylphosphonic acids.¹⁸

(a) CMC measurement using pyrene fluorescence method. Typical plots of change in pyrene I_3/I_1 as a function of added 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>

‡ List of abbreviations: ANS—1-anilino-naphthalene-8-sulfonic acid, BS—bile salts, CGC—critical gelation concentration, CMC—critical micellar concentration, CR—congo red, ICD—induced circular dichroism, PBS—phosphonobile salts, PXRD—powder X-ray diffraction and SAFIN—self-assembled fibrillar network.

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

Backbone structure	CMC/mM of PBSs (at pH 6.4, 0.15 M Na ⁺) using I ₃ /I ₁ of pyrene (³¹ P NMR method)		CMC of unconjugated (glycoconjugated) bile salts ^b
	23-PBS	24-PBS	
Cholate	1: 5–6 (5–6)	6: 4–5 (4)	11 (10)
Deoxycholate	2: 2–2.5 (2–2.5)	7: 1–1.5 (1.5)	3 (2)
Chenodeoxy	3: 2–2.3 (1.5–2)	8: 1.5–1.75 ^a	4 (1.8)
Ursodeoxy	4: 8–9 (8)	9: 6–7 (6)	7 (4)

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

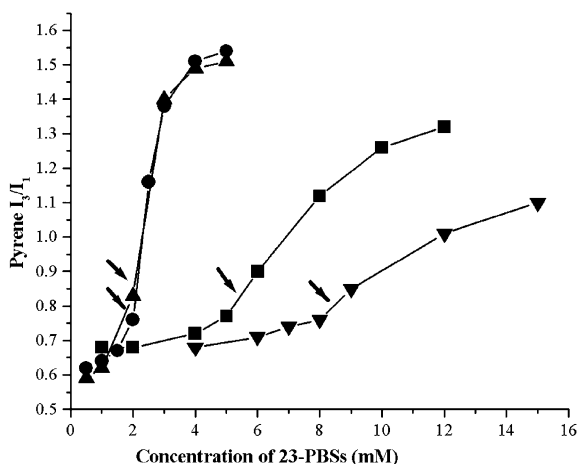


Fig. 1 Plots of pyrene I₃/I₁ vs. concentration of **1** (■), **2** (●), **3** (▲) and **4** (▼) in pH 6.4 phosphate buffer. The arrows indicate the CMC values.

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.¹⁹ 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 ³¹P NMR¹⁶. The plot of ³¹P chemical shift difference ($\Delta\delta$) 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

CMC values estimated by this method (Table 1) for 23- and 24-PBSs are in good agreement with those determined by the fluorescence method.

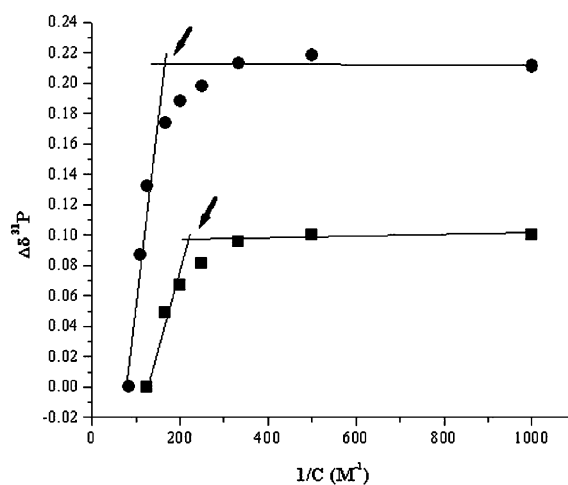


Fig. 2 Plot of ³¹P $\Delta\delta$ as a function of the inverse of the concentration of salts of **1** (■) and **6** (●) at pH 6.4. The arrows indicate the CMC values.

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 hydrogel formation by **1** and **6**. The phosphonolithocholate derivatives **5** and **10** formed gels at pH 7–7.5. The other dihydroxy 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 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

Backbone structure	Conditions for hydrogel formation		Natural BS	Remarks ^a
	23-PBS	24-PBS		
Cholate	1: >4 (1.7–2.4)	6: >3 (1.7–2.5)	No gelation ^b	TG from both
Deoxy	2: >2 (3.1–3.4)	7: >2 (6.3)	>20 (7.4–7.2)	7 forms TG after 6 h
Chenodeoxy	3: >15 (6–6.5)	8: >15 (6.3 + 0.15 M NaCl)	>30	TG from both
Ursodeoxy	4: >20 (~6)	9: >20 (~6)	>40 + 0.15 M NaCl	Turbid gel from both
Litho	5: >15 (7–7.5)	10: >15 (7–7.5)	(~8.5)	TG from both

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

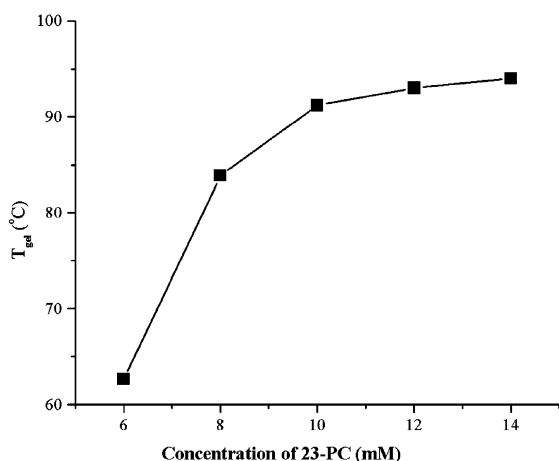


Fig. 5 T_{gel} as a function of concentration of **1** (at pH 2.1, ■).

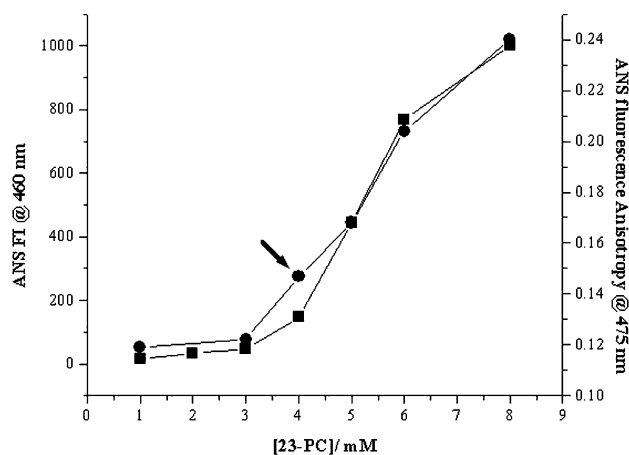


Fig. 6 Plot of ANS fluorescence intensity (at 460 nm, ■) and anisotropy (at 475 nm, ●) vs. concentration of **1** (at pH 2.1). The arrow indicates the visual gelling point.

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).

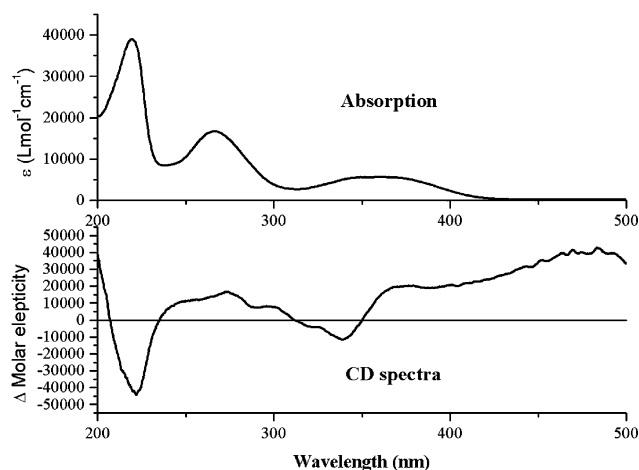


Fig. 7 Absorption (top) and CD (bottom) spectra of ANS (0.425 mM) in the gel of **6** (18 mM).

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

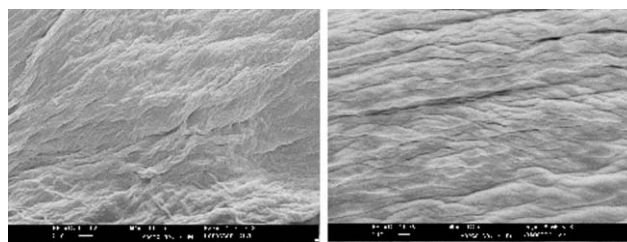


Fig. 8 SEM pictures of xerogels obtained from gel of **1** (left) and **6** (right).

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,²⁴ 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.²⁵ 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.²⁵ The crystal packing shows a bilayer arrangement, in which the β -surfaces of **6** are facing each other forming a hydrophobic layer, and the α -hydroxyl groups, sodium ions and water molecules form a hydrophilic channel (see ESI† for packing diagrams).

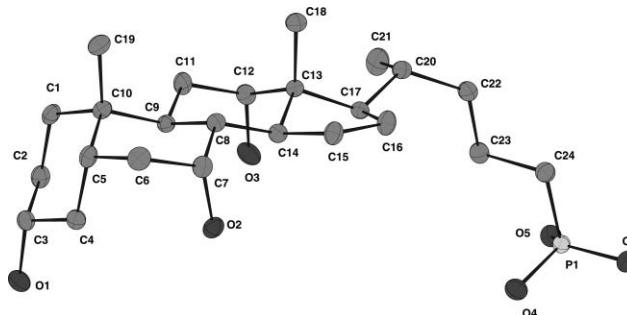


Fig. 9 ORTEP diagram of disodium salt of **6** (hydrogen atoms are removed for clarity).

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 native 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.^{5b} This comparison suggests that a similar molecular organization might be present in the phosphonocholate gels.

§ Crystal data: chemical formula $\text{C}_{24}\text{H}_{38}\text{O}_{14}\text{Na}_2\text{P}_1$, formula weight 627.5, orthorhombic $P2_12_12_1$, $a = 9.408(9)$, $b = 11.050(10)$, $c = 31.834(30)$ Å, $V = 3309.4(53)$ Å³, $Z = 4$, $\rho(\text{calc}) = 1.26$ g cm⁻³, $T = 290$ K, $\mu = 0.169$ mm⁻¹, reflections measured = 23220, unique reflections = 5613, reflections observed [$I > 2\sigma(I)$] = 5313, $R(\text{int}) = 0.0269$, $R1_{\text{obs}} = 0.054$, $wR2_{\text{obs}} = 0.153$, $F(000) = 1323.8$, 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.

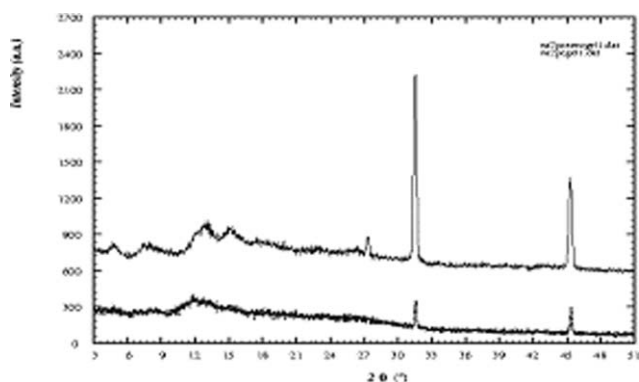


Fig. 10 Powder XRD patterns of xerogel (top) and native gel (bottom) of **6**. Y-Axis is not scaled to absolute intensity.

Conclusions

Micellar aggregation of 23- and 24-phosphonobile salts has been examined by fluorescence and ^{31}P NMR methods, which were in good agreement with each other. Phosphonobile acids **2**, **3**, **7** and **8** formed micelles at lower concentrations compared to compounds **1**, **6** and **4**, **9**. All the phosphonobile salts formed hydrogels at different pH values ranging from 1.7 to 7.5. The pH range at which hydrogels were formed by the *individual* phosphonobile salts 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 phosphonobile acids 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 phosphonobile acids 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 ^{31}P NMR were done with buffer in 20% D_2O 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_{gel} values were measured by carefully heating 0.5 mL of the gel in a sealed, inverted pyrex test tube (5 cm \times 0.5 cm id) in an oil bath. The temperature at which the gel started to flow downwards was noted as the T_{gel} .

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 $\text{MoK}\alpha$ radiation. Data were collected with ω scan width of 0.3 Å. A total of 606 frames were collected in three different settings of ρ (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 SAINTPLUS²⁶ and an empirical absorption correction was applied using the package SADABS.²⁷ The crystal structure was solved by direct methods using SIR92²⁷ and refined by full matrix least squares method using SHELXL97²⁸ present in the program suite WinGX (Version 1.63.04a).²⁹ The hydrogen atoms were located and refined isotropically. ORTEP plot and packing diagram were generated using CAMERON.³⁰ Geometrical calculations were done using PARST97.³¹ Powder diffraction data was collected on a Philips X'Pert diffractometer operating at 40 kV and 30 mA using $\text{W}\text{K}\alpha$ radiation and a curved graphite crystal as a monochromator.

Scanning electron micrographs

To observe the morphology of the xerogels 200 Å thick gold 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

- 1 A. F. Hofmann, in *Bile Acids in Hepatobiliary Disease*, ed. T. C. Northfield, H. A. Ahmed, R. P. Jazrawi and P. L. Zentler-Munro, Kluwer Academic Publications, Dordrecht, 1999, p. 303.
- 2 D. M. Small, *Adv. Chem. Ser.*, 1968, **84**, 31–52.
- 3 M. C. Carey, in *Sterols and Bile Acids*, ed. H. Danielsson and J. Sjövall, Elsevier, Amsterdam, 1985, p. 345.
- 4 H. Sobotka and N. Czechowiczka, *J. Colloid Sci.*, 1958, **13**, 188–191.
- 5 (a) A. Rich and D. M. Blow, *Nature*, 1958, **182**, 423–426; (b) A. Rich and D. M. Blow, *J. Am. Chem. Soc.*, 1960, **82**, 3566–3571.
- 6 C. Valenta, E. Nowack and A. Bernkop-Schnürch, *Int. J. Pharm.*, 1999, **185**, 103–111.
- 7 T. Miyata, T. Urugami and K. Nakame, *Adv. Drug Delivery Rev.*, 2002, **54**, 79–98.
- 8 K. Y. Lee and D. J. Mooney, *Chem. Rev.*, 2001, **101**, 1869–1879.
- 9 (a) P. Terech and R. G. Weiss, *Chem. Rev.*, 1997, **97**, 3133–3159; (b) D. J. Abdallah and R. G. Weiss, *Adv. Mater.*, 2000, **12**, 1237–1247; (c) J. H. van Esch and B. L. Feringa, *Angew. Chem., Int. Ed.*, 2000, **39**, 2263; (d) O. Gronwald, E. Snip and S. Shinkai, *Curr. Opin. Colloid Interface Sci.*, 2002, **7**, 148–156; (e) J. H. Jung, Y. Ono, K. Hanabusa and S. Shinkai, *J. Am. Chem. Soc.*, 2000, **122**, 5008–5009; (f) P. Babu, N. M. Sangeetha, P. Vijaykumar, U. Maitra, K. Rissanen and A. R. Raju, *Chem. Eur. J.*, 2003, **9**, 1922–1932; (g) A. Ajayaghosh, S. T. George and V. K. Praveen, *Angew. Chem., Int. Ed.*, 2003, **42**, 332–335.
- 10 For a recent review see L. A. Estroff and A. D. Hamilton, *Chem. Rev.*, 2004, **104**, 1201–1217.
- 11 (a) F. M. Menger and K. L. Caran, *J. Am. Chem. Soc.*, 2000, **122**, 11679–11691; (b) L. A. Estroff and A. D. Hamilton, *Angew. Chem., Int. Ed.*, 2000, **39**, 3447–3450; (c) U. Maitra, S. Mukhopadhyay, A. Sarkar, P. Rao and S. S. Indi, *Angew. Chem., Int. Ed.*, 2001, **40**, 2281–2283; (d) B. Xing, C.-W. Yu, K.-H. Chow, P.-L. Ho, D. Fu and B. Xu, *J. Am. Chem. Soc.*, 2002, **124**, 14846–14847; (e) H. Kobaashi, A. Friggeri, K. Koumoto, M. Amaike, S. Shinkai and D. N. Reinhoudt, *Org. Lett.*, 2002, **4**, 1423–1426; (f) G. Wang and A. D. Hamilton, *Chem. Commun.*, 2003, 310–311; (g) D. J. Pochan, J. P. Schneider, J. Kretsinger, B. Ozbas, K. Rajagopal and L. Haines, *J. Am. Chem. Soc.*, 2003, **125**, 11802–11803; (h) S. Kiyonaka, S. Shinkai and I. Hamachi, *Chem. Eur. J.*, 2003, **9**, 976–983; (i) K. Köhler, G. Förster, A. Hauser, B. Dobner, U. F. Heiser, F. Ziethe, W. Richter, F. Steiniger, M. Drechsler, H. Stettin and A. Blume, *Angew. Chem., Int. Ed.*, 2004, **43**, 245–247.
- 12 Response to external stimuli, thermal reversibility, low melt viscosity, potential biocompatibility, etc.
- 13 U. Maitra and P. Babu, *Steroids*, 2003, **68**, 459–463.
- 14 P. Babu and U. Maitra, *Steroids*, 2005, **70**, 681–689.
- 15 K. Kalyanasundaram and J. K. Thomas, *J. Am. Chem. Soc.*, 1977, **99**, 2039–2044.
- 16 R. E. Stafford, T. Fanni and E. A. Dennis, *Biochemistry*, 1989, **28**, 5113–5120.

- 17 The pK_a values of **1** and **6** were measured using change in ^{31}P chemical shift as a function of pH in the buffer solutions. A non-linear regression curve fitting was done using the following equation: $\text{pH} = pK_a + \log [(\delta - \delta_a)/(\delta_b - \delta)]$; pK_a , δ_a and δ_b are variable parameters, δ_a is limiting δ in the acidic medium and δ_b is limiting δ in the basic medium.
- 18 A. J. Kresge and Y. C. Tang, *J. Org. Chem.*, 1977, **42**, 757–759.
- 19 A. Roda, A. F. Hofmann and K. J. Mysels, *J. Biol. Chem.*, 1983, **258**, 6362–6370.
- 20 K. Sugiyasu, N. Fujita, M. Takeuchi, S. Yamada and S. Shinkai, *Org. Biomol. Chem.*, 2003, **1**, 895–899.
- 21 R. Haque and W. U. Malik, *J. Phys. Chem.*, 1963, **67**, 2082–2084.
- 22 S. Mukhopadhyay and U. Maitra, *Org. Lett.*, 2004, **6**, 31–34.
- 23 (a) D. C. Turner and L. Brand, *Biochemistry*, 1968, **7**, 3381–3390; (b) J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Kluwer Academic/Plenum Publishers, New York, 1999.
- 24 K. Kato, M. Sugahara, N. Tohrai, K. Sada and M. Miyata, *Cryst. Growth Des.*, 2004, **4**, 263–272.
- 25 (a) M. George and R. G. Weiss, *J. Am. Chem. Soc.*, 2001, **123**, 10393; (b) A. Ballabh, D. R. Trivedi and P. Dastidar, *Chem. Mater.*, 2003, **15**, 2136–2140; (c) D. K. Kumar, D. A. Jose, P. Dastidar and A. Das, *Chem. Mater.*, 2004, **16**, 2332–2335.
- 26 SMART, SAINT, SADABS, XPREP, SHELXTL. Bruker AXS Inc., Madison, Wisconsin, USA, 1998.
- 27 A. Altomare, G. Casciaro and C. Giacovazzo, A program for crystal structure solution, *J. Appl. Crystallogr.*, 1993, **26**, 343.
- 28 G. M. Sheldrick, SHELXL97, Program for Crystal Structure Refinement, University of Göttingen, Germany, 1997.
- 29 L. J. Farrugia, WINGX, *J. Appl. Crystallogr.*, 1999, **32**, 837.
- 30 D. M. Watkin, L. Pearce, C. K. Prout, CAMERON—A Molecular Graphics Package, Chemical Crystallography Laboratory, University Of Oxford, Oxford, 1993.
- 31 M. Nardelli, *J. Appl. Crystallogr.*, 1995, **28**, 569.