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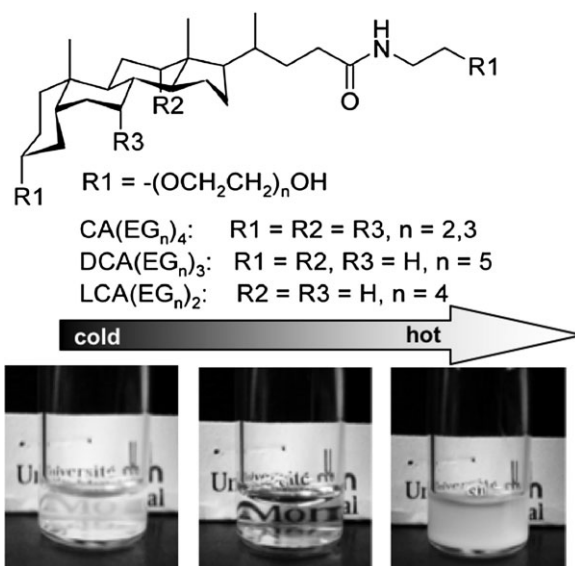
Thermosensitivity of Bile Acid-Based Oligo(ethylene glycol) Stars in Aqueous Solutions^a

Satu Strandman,* Frantz Le Dévédec, X. X. Zhu*

Amphiphilic star-shaped oligo(ethylene glycol)s with a hydrophobic bile acid core and varying number of hydrophilic arms have been made. Their thermal behavior in aqueous solutions depends on the number rather than the length of the arms. The two-armed lithocholate derivative showed the strongest tendency for association and exhibited the lowest cloud point (79 °C) of the oligomers made, as well as another phase separation at a lower temperature (31 °C). The “double thermosensitivity” arising both from the salt-dependent LCST of the oligo(ethylene glycol) segments and the temperature-responsive self-assembly of amphiphilic bile acid derivative provides an interesting path in the design of bile acid-based smart materials.

$R1 = -(OCH_2CH_2)_nOH$
 CA(EG_n)₄: $R1 = R2 = R3, n = 2, 3$
 DCA(EG_n)₃: $R1 = R2, R3 = H, n = 5$
 LCA(EG_n)₂: $R2 = R3 = H, n = 4$

cold hot



Introduction

Owing to its high flexibility and thus excellent steric shielding properties, poly(ethylene glycol) (PEG) is the most commonly employed non-ionic hydrophilic polymer in bioapplications, particularly in polymeric drug carriers and drug-polymer conjugates.^[1,2] Perturbation of the interactions with water by heating will lead to a phase separation (clouding), represented by a lower critical solution temperature (LCST).^[3] In addition, PEG exhibits molar mass- and end group-dependent closed loop-type liquid-liquid immiscibility phase behavior at ambient pressure, i.e., a

two-phase region bounded by an upper and a LCST envelopes, where the upper critical solution temperature (UCST) is above the LCST.^[4] This means that the polymer becomes miscible at a certain temperature and concentration regime above the LCST.

Many amphiphilic oligo(ethylene glycol) derivatives show phase separation upon heating. These include non-ionic surfactants,^[5] oligo(ethylene glycol) methacrylate polymers,^[6,7] as well as more complex architectures such as amphiphilic dendrons,^[8] dendrimers,^[9] and branched amphiphiles bearing oligo(ethylene glycol) chains.^[10] Many of these structures are more hydrophobic than PEG, resulting in lower cloud points than that of PEGs. The solubility of non-ionic surfactants can drastically decrease with decreasing temperature, often leading to phase separation and the formation of hydrated crystals.^[11] To our knowledge, none of the branched architectures above

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has shown similar mixing/demixing behavior in water despite their high hydrophobicity.

In this work, we have studied the temperature-dependent solution behavior of amphiphilic star-shaped oligo(ethylene glycol)s based on bile acids with a bent steroid core bearing 2–4 functional groups allowing PEG attachments. The association of bile acids in aqueous solutions is more complex than that of cholesterol-based or conventional surfactants due to factors such as facial amphiphilicity and incomplete separation of hydrophilic and hydrophobic domains.^[12] Based on our earlier study, PEGylation increases the solubility of bile acids in water and also changes their micellization characteristics.^[13] Since the differences between the amphiphiles are larger when the PEG arms are shorter, our study focuses on short arms of 2–5 units of oxyethylene. Interesting “double thermosensitivity” of the two-armed lithocholate derivative has been observed and studied. The results indicate that steric stabilization may play an important role in the solubility of bile acid derivatives.

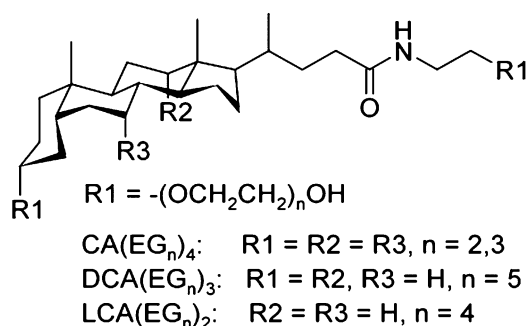
Experimental Section

Materials

Star-like oligomers were synthesized according to a previously reported procedure^[13,14] by anionic polymerization of ethylene oxide. The structures of oligomers are shown in Scheme 1. The molar masses of stars were determined by ¹H NMR spectroscopy, size exclusion chromatography (SEC), and MALDI-TOF mass spectrometry. The values of molar masses and the polydispersities obtained by SEC are listed in Table 1. The numbers of arms were determined by the end group analysis by ¹H NMR using a previously described method.^[13]

Instrumentation and Sample Preparation

Turbidimetric measurements of the aqueous solutions were done on a Varian Cary 300 Bio UV–Vis spectrophotometer. The heating



Scheme 1. The chemical structures of bile acid-based star oligo(ethylene glycol)s. CA, DCA, and LCA refer to cholic acid, deoxycholic acid, and lithocholic acid, respectively.

Table 1. Properties of bile acid-based oligomers.

Oligomer ^{a)}	\bar{M}_n [g · mol ⁻¹]			T_{cp} [°C]
	¹ H NMR	MALDI-TOF	SEC (PDI) ^{b)}	
DCA(EG ₅) ₃	1070	1160	930 (1.09)	>90 ^{c)}
CA(EG ₃) ₄	1020	1010	940 (1.03)	–
CA(EG ₂) ₄	800	820	780 (1.08)	>90 ^{c)}
LCA(EG ₄) ₂	700	780	540 (1.08)	79

^{a)}The abbreviation DCA(EG_x)_y indicates that on a DCA core, y arms of PEG each of x repeating units are attached; ^{b)}SEC in THF, calibrated against PEG standards; ^{c)}Cloud point observed with heating the samples to 100 °C.

rate was adjusted at 0.2 °C · min⁻¹ and the detection wavelength was 400 nm. Transmittances were normalized by $T_{norm} = (T - T_{min}) / (T_{max} - T_{min})$, and plotted as a function of temperature. The mixing temperature T_{mix} was defined as the point at which the transmittance reached the maximum, and the cloud point T_{cp} was determined from the point where transmittance decreased to 50%. Samples were prepared by direct dissolution in water, followed by equilibration overnight at 5 °C prior to measurements. Saline solutions were prepared by adding a known amount of 0.5 or 5.0 M NaCl solution at room temperature before cooling the sample.

Differential scanning calorimetric (DSC) experiments were conducted on a TA Q2000 differential scanning calorimeter (TA Instruments). Indium was used for temperature and heat flow calibration. Helium was used as the purge gas (flow rate 25 mL · min⁻¹). The heating rate was 1 °C · min⁻¹ unless stated otherwise. Aqueous samples of 7–10 mg were measured in hermetic aluminum pans. Dynamic viscosities of aqueous samples were determined on a Cambridge Applied Systems viscometer (model VI-4100) at 25 °C. Polarized optical micrographs were recorded on a Zeiss Axioscope 2 optical microscope.

Results and Discussion

The anionic polymerization on the bile acid core proceeded well as demonstrated in our previous study on cholic acid derivatives.^[13] The star-shaped oligomers obtained in this study have very narrow molar mass distributions (Table 1), indicating equal or similar reactivity of the OH groups during the anionic polymerization process and the uniformity of the chain length.

Effect of Oligomer Structure on Their Association Behavior

LCSTs of PEGs in water are typically in the range of 98.7–175.6 °C for molar masses of 1 020 000–2 180 g · mol⁻¹, respectively, increasing with decreasing molar mass.^[15] PEG loses its thermosensitivity in aqueous solutions when

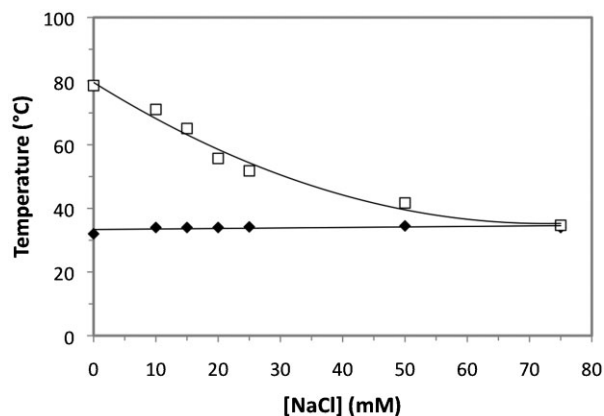


Figure 1. Salt-dependence of mixing temperature (T_{mix} , solid symbols) and cloud point (T_{cp} , open symbols) at constant concentration (20×10^{-3} M, 1.4 wt%) of LCA(EG₄)₂. Lines are added as a visual guide.

the degree of polymerization is lower than $DP=49$ ($\bar{M}_w = 2140 \text{ g} \cdot \text{mol}^{-1}$).^[16]

The pegylated bile acids, however, are more hydrophobic than PEGs, bringing their cloud points often below the boiling point of water at ambient pressure. Interestingly, cloud points were observed for some pegylated bile acids with arms as short as two repeating units (Table 1). Clouding was confirmed by simple visual observation of samples during heating to 100 °C, and observed for all the samples except for CA(EG₃)₄, which is the most hydrophilic, pushing its cloud point beyond the boiling point of water. The least sterically hindered two-armed lithocholate derivative, LCA(EG₄)₂, is insoluble in water but becomes soluble upon heating at ≈ 31 °C becoming immiscible again when heated to a cloud point at 79 °C (Figure 1). The entire process is reversible when the sample is cooled.

Although the process is reversible, interestingly, the demixing temperature where the oligomer precipitates upon cooling (T_{demix}) is lower than the mixing temperature of dissolution upon heating (T_{mix}). In addition, T_{demix} depends strongly on the cooling rate, indicating the kinetic nature of the demixing process. Increasing the cooling rate from 0.2 to 2.0 °C · min⁻¹ lowers the value of T_{demix} from 23 to 15 °C. Faster cooling affords less time for the rearrangement of interacting domains, resulting in a lower apparent temperature of phase transition. In contrast, T_{mix} is nearly constant at different heating rates with a broad mixing transition, whereas, the demixing occurs within a narrower temperature range. The concentration of LCA(EG₄)₂ has only a minor effect on the mixing/demixing transition. A drop in concentration from 74.0 to 0.3 wt.-% increases the T_{mix} from 29.0 to 31.2 °C. T_{demix} is 18.7 ± 0.4 °C in the concentration range of 8.5–79.1 wt.-% (at a cooling rate of 1.0 °C · min⁻¹). These transition temperatures were much higher than the glass transition temperature of the oligomer ($T_g = -45$ °C

for LCA(EG₄)₂. No melting point was observed.). The Figures showing the effects of cooling rate and polymer concentration are presented in Supporting Information (Figure S1 and S2).

The mixing/demixing behavior of LCA(EG₄)₂ is similar to the Krafft boundary typical for both ionic and non-ionic surfactants in aqueous solutions. This composition-dependent temperature limit corresponds to the equilibrium between micelle solution (above the limit) and hydrated crystalline surfactant (below the limit), and is relatively independent of the surfactant and salt concentrations above the critical micellization concentration (CMC), although salt may increase slightly the value of the boundary even for non-ionic surfactants.^[17,18] The upper limit of the Krafft boundary increases with increasing hydrophobicity, as a higher temperature is needed to dissolve the compound.^[18] In more hydrophilic systems, the micellar state may persist even on cooling to 0 °C, and in some cases cooling may lead to gelation instead of precipitation.^[17] The persistence of micelle state can also be associated with more effective steric stabilization, as CA(EG₂)₄ with similar number of ethylene glycol units to LCA(EG₄)₂ does not show demixing above 0 °C.

Effect of Salt on Phase Transitions of LCA(EG₄)₂

Figure 1 shows the “salting-out” effect upon the addition of NaCl, narrowing the miscible range in the middle between T_{mix} and T_{cp} by lowering the cloud point. Examples of turbidimetric plots are presented in Figure 2 to demonstrate the effect of added salt. Since LCA(EG₄)₂ is relatively hydrophobic, even a small amount of NaCl has a drastic effect on its solubility. Figure 2 also shows that T_{cp} becomes very close to T_{mix} at a NaCl concentration of 75×10^{-3} M. The mixing temperature remains in the proximity of 30 °C,

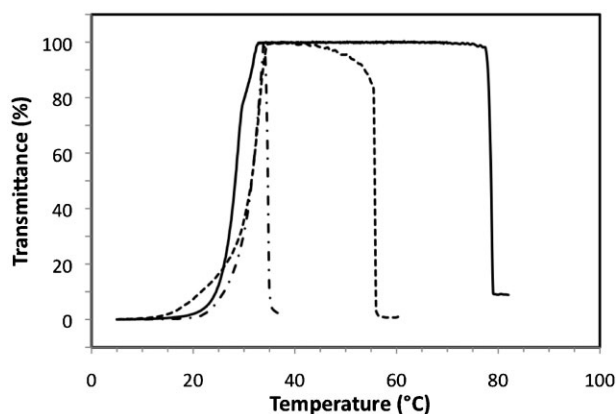


Figure 2. Heating curves for 20×10^{-3} M (1.4 wt.-%) LCA(EG₄)₂ in water at different NaCl concentrations: $[\text{NaCl}] = 75 \times 10^{-3}$ M (dash-dots), 25×10^{-3} M (dashes), and 0 M (solid line). Heating rate was 0.2 °C · min⁻¹.

increasing only by 2–4 °C upon the addition of the salt, in stark contrast to the changes of the cloud point, from ca. 80 to 35 °C. This may be an indication of the different mechanistic origins of these two phenomena.

The cloud point of PEGs and the oligomers based on lithocholic acid reflects a local structural transition involving water molecules surrounding the compound, and the hydrophobic effect plays an important role in their interactions with water.^[19] Hydrophobic groups favor phase separation thus lowering the LCST. The addition of a salt such as NaCl disrupts the hydrogen bonds and reduces the hydration of micelles, resulting in a further decrease in the cloud points.^[18,20,21] The effect of hydrophobe content and of added salt are well-known also for non-ionic surfactants.^[5,22] The effect of added salt on LCST depends on the molar mass of PEG, and hence the amphiphilic systems stabilized sterically by high-molar-mass PEG are more resistant against salting-out effect than their low-molar-mass counterparts.^[20] The results above indicate that also the number of arms has a similar effect, as a higher number of PEG chains provides a more effective shielding for the hydrophobic core from unfavorable interactions with the aqueous phase.

Aggregation of LCA(EG₄)₂ at High Concentrations

A 8.5 wt.-% sample of LCA(EG₄)₂ was cloudy and viscous below the mixing temperature, suggesting a high degree of intermolecular association. However, shaking the sample vial or shearing the sample in a viscometer during the measurement decreased the viscosity η to 1.75 cP at 25 °C, which is close to that of DCA(EG₅)₃, CA(EG₃)₄, and CA(EG₂)₄ (η = 1.46, 1.46, and 1.33 cP, respectively), accompanied by the disappearance of cloudiness, both reflecting the labile nature of the self-assemblies. Cloudiness and high viscosity were restored in 30 min when the sample was allowed to stand unperturbed at 5 °C. The shear-thinning effect was also observed at 5.0 °C, although the viscosity was higher (η = 3.54 cP).

The high viscosity of LCA(PEG₄)₂ below the demixing temperature indicates strong intermolecular interactions, and is common for large aggregates such as wormlike micelles or liquid crystalline structures. Shearing the sample leads to the fragmentation of elongated species or ordered domains, and thus to a lower viscosity.^[23–26] Indeed, optical anisotropy indicating liquid crystalline behavior was observed by polarized optical microscopy for the LCA(PEG₄)₂ sample (Figure S3 in Supporting Information). Fibrillar structures shown by micrographs are similar to those of presented in literature for bile salts, assigned by La Mesa and coworkers to lyotropic liquid crystallinity^[27] Shear-thinning character has been described for alkaline suspensions of sodium lithocholate, arising from the orientation of entangled nanotubes or fibers.^[28] Such

nanotubes exhibit thermosensitivity, disintegrating above a threshold temperature of 61 °C.^[29] Lithocholic acid is poorly soluble at physiological pH or below it, but its deprotonated form dissolves in alkaline conditions, which are unnecessary for the solubilization of PEGylated bile acids. Fibrillar aggregates have also been observed for a group of bile acid derivatives with gelating properties in organic solvents^[30,31] or in aqueous solutions.^[32] However, a common feature for these gelators is a large number of interacting hydroxyls, amide groups, or charges,^[33] which is not the case with LCA(PEG₄)₂. Further investigations are necessary to elucidate the structure of the self-assembled lithocholate derivative.

Conclusion

The thermal behavior of amphiphilic star-shaped oligo(ethylene glycol)s with a constant size of hydrophobic core but varying number of hydrophilic arms in aqueous solutions depends on the number rather than the length of the arms. The two-armed lithocholate derivative showed the strongest tendency for association and exhibited the lowest cloud point of the oligomers made, as well as a thermosensitive phase separation close to room temperature, represented by Krafft boundary. The latter transition was not observed for other oligomers, reflecting their more stable micellar state in aqueous solutions even when the stabilizing chains are very short. Due to its low solubility, lithocholic acid is the least studied bile acid, but recent reports on its complex self-assembly^[28,34,35] have shown that its behavior in aqueous solutions is still far from being well understood. The “double thermosensitivity” arising both from the salt-dependent LCST of oligo(ethylene glycol) and the temperature-responsive self-assembly of neutral amphiphilic bile acid derivative provides an interesting concept for bile acid-based smart materials, and thus warrants further studies on the self-assembling characteristics of lithocholate-based oligomer.

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