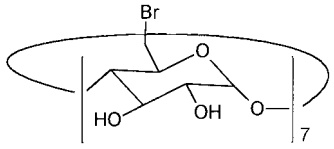
Cyclodextrin Bilayer Vesicles**

Bart Jan Ravoo and Raphael Darcy*

Over the past decade, several groups have reported amphiphilic cyclodextrin derivatives that display a range of lyotropic and thermotropic mesophases. Amphiphilic cyclodextrins have been shown to form monolayers at the air–water interface[1] and micelles in water.[2] Thermotropic liquid crystals of cyclodextrins were also described.[3] Amphiphilic cyclodextrins can be admixed in limited percentages to phospholipid monolayers[4] as well as liposomes,[5] and they can be dispersed as "nanoparticles" of pharmaceutical interest.[6] However, with few exceptions, these materials have poor water solubility. We have now prepared the first examples of bilayer vesicles composed entirely of amphiphilic cyclodextrins. These vesicles combine the properties of liposomes and macrocyclic host molecules, and create new possibilities for the development of advanced host–guest carrier and delivery systems. Some examples of vesicles composed of amphiphilic macrocycles such as calixarenes[7] and cryptands[8] were recently described.

Heptakis(6-alkylthio)-β-cyclodextrins[3] display thermotropic mesophases and form monolayers at the air–water interface,[1f,g, 3, 9] but are practically insoluble in water. The poor water solubility of these cyclodextrins most likely results from intramolecular hydrogen bonding of the secondary hydroxyl groups, as is the case for unmodified β-cyclodextrin. Attempts to "break" this hydrogen-bond network by per-substitution of the secondary side by methyl and acetyl groups only slightly improved the water solubility.[9] However, we demonstrate herein that substitution of the secondary side with hydrophilic hydroxyethyl groups dramatically improves the water solubility (as observed previously for random and low-degree substitution of β-cyclodextrin[10]), and provides cyclodextrins with pronounced amphiphilic character.

Cyclodextrins **4** and **5** were obtained in a three-step synthesis from β-cyclodextrin. β-Cyclodextrin was per-brominated at the C_6 positions,[11] and the resulting heptakis(6-bromo-6-deoxy)-β-cyclodextrin (**1**) yielded the 6-alkylthioethers **2** and **3** by reaction with potassium *n*-dodecylthiolate and *n*-hexadecylthiolate, respectively.[3] Subsequently, we developed an efficient modification of the hydroxyethylation process for β-cyclodextrin,[10] which uses ethylene carbonate as the alkylating agent. We found that **2** and **3** can be efficiently hydroxyethylated in tetra-*N*-methylurea (TMU) at 150 °C using an excess of ethylene carbonate and adding 10% of K_2CO_3 as base.[12] The elevated temperature is essential for rapid and extensive substitution. Cyclodextrin **4** was purified by size-exclusion chromatography and cyclodextrin **5** was purified by crystallization from methanol. The structures of **4** and **5** were confirmed using ^{1}H NMR and ^{13}C NMR spectroscopy, microanalysis, and electrospray-MS.[12] The most intense peaks in the elecrospray mass spectra correspond to 13-fold hydroxyethylation of **4** and 10-fold hydroxyethylation of **5**, with peaks for products with higher and lower degrees of substitution spaced 44 mass units apart, as expected for $(CH_2CH_2O)_n$. Line broadening of critical signals such as the anomeric proton in the ^{1}H NMR spectra and both C_1 and C_4 in the ^{13}C NMR spectra indicate that the cyclodextrin molecules lose their symmetry upon hydroxyethylation. Further details of the synthesis and characterization of these and similar cyclodextrins will be published in the near future.



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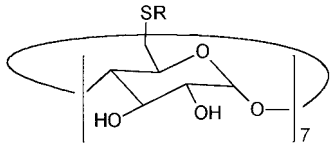
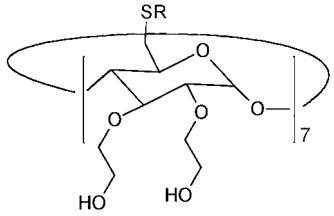


2: R = $C_{12}H_{25}$
3: R = $C_{16}H_{33}$



4: R = $C_{12}H_{25}$
5: R = $C_{16}H_{33}$

Cyclodextrins **4** and **5** were dispersed in water by sonication of a thin film (cast by slow rotary evaporation of a solution of the cyclodextrins in chloroform) by using a sonication bath at 50 °C for 1 h.[13] The solutions obtained in this way are rather turbid, which indicates the presence of cyclodextrin aggregates larger than 200 nm. At high concentrations (5–10 mg mL^{-1}), the cyclodextrins partly flocculate upon standing for 3–5 h at room temperature, but the precipitate is readily dispersed upon slight heating and agitation. No flocculation occurs if the cyclodextrin concentration is below 1 mg mL^{-1}.

Dynamic light scattering of solutions of **4** and **5** in water indicated the presence of cyclodextrin aggregates with an average diameter of 170 nm.[14] These aggregates were also examined by transmission electron microscopy.[14] Spherical vesicles with diameters of 50–300 nm were observed using

[*] Dr. R. Darcy, Dr. B. J. Ravoo
Department of Chemistry
University College Dublin
Belfield, Dublin 4 (Ireland)
Fax: (+353) 1-7062127
E-mail: raphael.darcy@ucd.ie

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uranyl acetate as a negative staining agent (Figure 1 A). A monodisperse solution of spherical vesicles with an average diameter of 60 nm was obtained upon prolonged sonication (9 h) of a dispersion of **4** in water (Figure 1 B). The structure of the vesicles is reminiscent of that of conventional liposomes, which can be prepared in a similar manner, and which show a similar correlation of particle size and sonication time.[13]

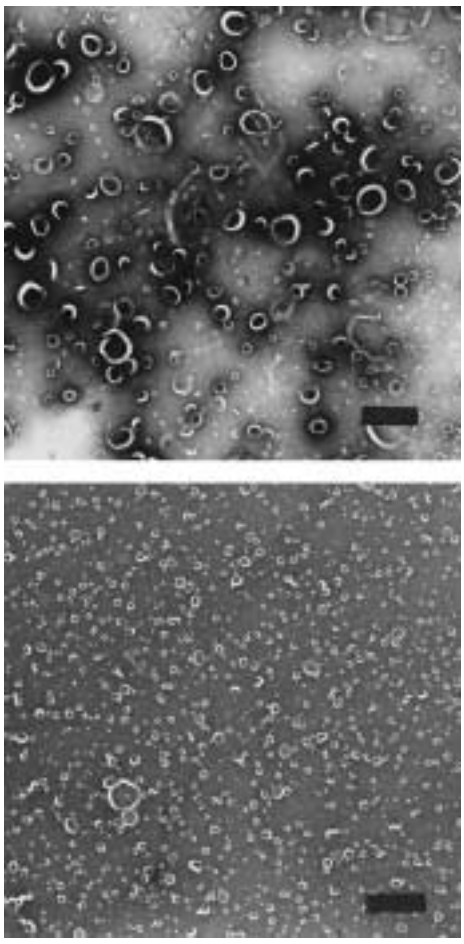

Figure 1. Electron micrographs of vesicles of **4** and **5**. Top: Vesicles of cyclodextrin **5** obtained after 2 h sonication at 50 °C. Bottom: Vesicles of cyclodextrin **4** obtained after 9 h sonication at room temperature. The scale bars represent 500 nm.

Further evidence for the formation of vesicles of **4** and **5** was obtained by encapsulation of the fluorescent dye carboxyfluorescein (CF) in the aqueous interior of the vesicles.[13] Vesicles of **4** and **5** were prepared in a buffered solution of CF at a self-quenching concentration. The CF entrapped in the vesicles of **4** and **5** was separated from free CF by gel filtration on Sephadex G25. The elution profiles are shown in Figure 2 A. The results are presented as the ratio of fluorescence intensity after (F_{TX}) and before (F_{init}) the addition of 0.1 % of Triton TX-100 (which solubilizes the cyclodextrin vesicles and causes release of their contents), which is a direct measure of the presence of entrapped CF. Independent absorbance measurements indicated that "empty" vesicles of **4** and **5** elute at 5 – 8 mL, whereas free CF elutes beyond 16 mL. The

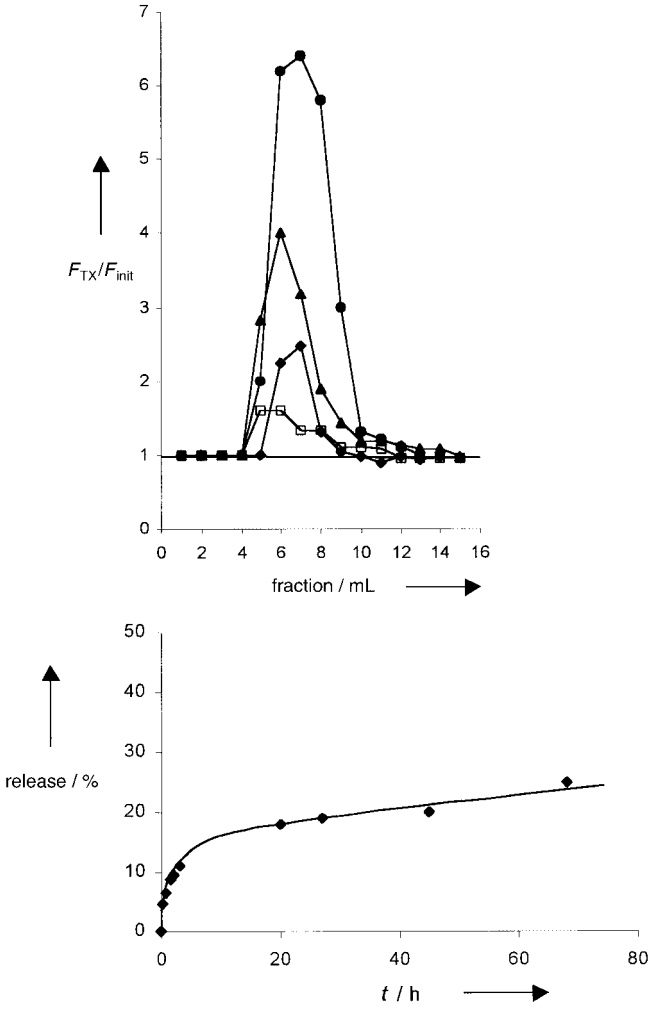

Figure 2. Encapsulation and release of carboxyfluorescein (CF) from vesicles of cyclodextrins **4** and **5**. Top: Elution profiles of vesicles of cyclodextrins **4** and **5** loaded with CF on a 18 × 1 cm Sephadex G25 column (void volume 4 mL). The relative fluorescence intensity scale F_{TX}/F_{init} indicates encapsulated CF only, as explained in the text. ●: **5** at 20 mg mL^{-1}; ▲: **5** at 8 mg mL^{-1}; ◆: **5** at 5 mg mL^{-1}; □: **4** at 5 mg mL^{-1}. Bottom: Leakage of CF from vesicles of cyclodextrin **5**.

coincidence of the peak of entrapped CF with the elution of vesicles of **4** and **5** confirms the existence of an aqueous inner compartment within the vesicles. As anticipated, the amount of entrapped CF in vesicles of **5** correlates with the concentration of **5**. The encapsulation of CF in vesicles of **4** was much less efficient, which may be a consequence of the shorter alkyl chains in **4** compared to in **5**. The spontaneous release of CF from vesicles of **5** was measured over time (Figure 2 B): CF leakage is limited, and the vesicles still retain more than 75 % of CF after three days. Finally, vesicles of **5** were prepared in a solution of fluorescein-labeled dextran with an average molecular weight of 4000 daltons. Encapsulated (3 – 5 %) and free dextran were separated by gel filtration on Sephadex G50. We contend that the encapsulation procedure can be optimized and extended to larger and more significant solutes, such as proteins.

Vesicles of **5** were further characterized by a study of the temperature dependence of the fluorescence polarization of diphenylhexatriene (DPH) absorbed in vesicles of **5**.[13]

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Diphenylhexatriene has been extensively used to probe the main thermotropic phase transition of lipid bilayers.[13] This L_β-L_α transition is typical of lamellar lyotropic phases, and its occurrence provides strong evidence for the formation of bilayers. The fluorescence polarization of DPH in vesicles of cyclodextrin **5** shows a sharp decrease from 0.38 to 0.27 around 50 °C. This decrease is most likely the result of an L_β-L_α transition of the bilayer of **5**. Lipids of similar hydrophobic chain length display this transition at comparable temperatures.[15] The DPH fluorescence remains rather high above 50 °C, possibly because of partial inclusion of DPH in the hydrophobic cavity of **5** which restricts the DPH mobility.

Finally, a sample of aqueous cyclodextrin **5** was analyzed by differential scanning calorimetry (DSC).[16] This technique enables the quantification of heat effects that accompany the L_β-L_α transition. Indeed, the heating scan in the DSC study displays a reproducible endothermic phase transition of a 10% (w/w) dispersion of **5** in water. The transition occurs around 48–49 °C and the enthalpy of transition amounts to 59 kJ mol^{-1} for cyclodextrin. The transition temperature coincides with the decrease in the DPH fluorescence polarization. The enthalpy of transition is high compared to the typical transition enthalpy for phospholipid bilayers (30–40 kJ mol^{-1}).[15] However, it is much lower when calculated per mole of hydrocarbon chain: 8.5 kJ mol^{-1} for cyclodextrin **5** (with seven hexadecyl chains) compared with 15–20 kJ mol^{-1} for a phospholipid (with two hexadecyl chains). The lower transition enthalpy may indicate the rigidity of the macrocyclic amphiphile relative to a phospholipid in an L_α bilayer.

In summary, we have developed novel non-ionic amphiphilic hydroxyethylated cyclodextrins that form bilayer vesicles in water. The encapsulation of solutes in such vesicles creates exciting new possibilities for the use of cyclodextrins as carrier materials.

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[12] Synthesis of **4**: Compound **2** (500 mg; 200 μmol), K_2CO_3 (50 mg) and ethylene carbonate (1.00 g) were mixed in TMU (5 mL). The reaction mixture was stirred at 150 °C for 4 h. Analysis by thin-layer chromatography (silica, $CHCl_3$/MeOH/H_2O 50/10/1) indicated complete conversion of **2** ($R_f = 0$) into a single product ($R_f = 0.5$). The solvent was removed by rotary evaporation at 100 °C. The crude product was taken up in methanol (2 mL) and purified by size-exclusion chromatography through a column of lipophilic Sephadex LH 20–100 using methanol as eluent. **4** was isolated in 89% yield as a yellow wax. ^{1}H NMR (270 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 5.05$ (br s, 7H, H_1), 3.4–4.0 (m, 80H, H_2-H_5 and $(OCH_2CH_2O)_{13}$), 3.00 (m, 14H, H_6), 2.60 (m, 14H, SCH_2), 1.60 (m, 14H, CH_2), 1.27 (br s, 126H, CH_2), 0.89 (t, 21H, CH_3); ^{13}C NMR (270 MHz, $CDCl_3$, 25 °C, TMS): $\delta = 13.9$ (CH_3), 22.4 (CH_2), 28.8 (CH_2), 29.2 (CH_2), 29.5 ($(CH_2)_n$), 31.7 (CH_2), 33.4 (CH_2S), 33.4 (C_6), 61.2 (CH_2OH), 71.0–72.0 (C_3, C_5), 72.2 (CH_2O), 81.0 (C_2, C_4), 100.7 (C_1); ES-MS: *m/z* (%): 3067 (42) [M^+_{14HE}+Na], 3023 (100) [M^+_{13HE}+Na], 2979 (74) [M^+_{12HE}+Na], 2934 (69) [M^+_{11HE}+Na], 2890 (50) [M^+_{10HE}+Na], elemental analysis calculated for $(C_{22}H_{42}O_6S)_7$: C 60.83, H 9.68, S 7.37; found: C 60.12, H 9.38, S 7.62.

[13] Preparation of cyclodextrin vesicles, encapsulation of carboxyfluorescein, and fluorescence polarization measurements of DPH were carried out by using conventional experimental procedures used for liposomes: R. C. C. New, *Liposomes: a practical approach*, Oxford University Press, Oxford, **1990**.

[14] Dynamic light scattering measurements were carried out at room temperature using Malvern instrumentation. The solutions contained 0.1 mg mL^{-1} of cyclodextrin and were filtered through a 1.0 μm microfilter to remove dust. Samples for electron microscopy were prepared on 200 mesh formvar/carbon-coated copper grids. A drop of cyclodextrin solution (0.5 mg mL^{-1}) was incubated on the grid for 2 min, and then gently blotted with filter paper. The specimen was negatively stained with a drop of 2% (w/w) UAc solution, incubated for 5 min, then gently blotted. The samples were examined in a JEOL 2000 electron microscope operating at 80 kV.

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[16] DSC was performed on a Rheometric Scientific Instruments calorimeter. **5** (20 mg) was sonicated in water (200 μL) for 2 h at 50 °C. The suspension (50 μL) was sealed in pressure-resistant DSC cups and scanned against an empty reference cup. The sample was equilibrated at 24 °C, then heated to 80 °C at 1 °C min^{-1}. After cooling the sample to room temperature, the measurement was repeated.

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