The influence of phenyl and phenoxy modification in the hydrophobic tails of di-*n*-alkyl phosphate amphiphiles on aggregate morphology†

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A series of di-*n*-alkyl phosphate amphiphiles containing phenyl and phenoxy groups in the hydrophobic tails were synthesised, and their aggregation behaviour was investigated using fluorescence spectroscopy, differential scanning calorimetry, and cryo-electron microscopy. The aggregates displayed a wide variety of aggregate morphologies. The incorporation of a phenyl group into the end or in the middle of the alkyl chain lowered the main phase transition temperature, resulting in closed vesicles only above the phase transition temperature. Introducing a phenoxy group at the end of the alkyl chain resulted in open bilayer structures and bicelles.

Introduction

The finding that sodium di-*n*-hexadecyl phosphate forms vesicles[1] led to various investigations of the properties of di-*n*-hexadecyl phosphate aggregates[2–4] Also the influence of changing the molecular structure of the di-*n*-alkyl phosphate on the morphology and properties of the aggregates in water has been investigated. The alkyl chain length was varied and also asymmetric di-*n*-alkyl phosphates, *i.e.* phosphates with two dissimilar alkyl chains, have been prepared.[5–7] Substituting the saturated chains by unsaturated ones changed the vesicular properties considerably.[8] Also less obvious structural changes have been applied. Various di(polyprenyl) phosphates,[9] macrocyclic phosphates,[10] and bolaform phosphates[11,12] have been studied.

Incorporating aromatic moieties into the alkyl chains of amphiphiles is expected to change the packing of the hydrophobic tails. Conformational changes of the hydrocarbon chain and π–π interactions are the most obvious effects to affect tail packing. Previous research has been performed on amphiphiles containing cyanobiphenyl units in both chains. The ammonium amphiphiles containing cyanobiphenyl units have a higher main phase transition temperature (T_m) than their aliphatic chain analogue dioctadecylammonium bromide ($T_m = 45$ °C)[13,14] and UV-VIS spectra of vesicular solutions showed the presence of H-aggregates.[15] Also azobenzene-containing phosphate amphiphiles have been studied. These amphiphiles form bilayer sheets instead of vesicles when dispersed in water and exhibit a blue shift in the UV-VIS spectrum, indicating H-aggregation.[16] A phosphate amphiphile containing a cyanobiphenyl moiety did not form vesicles.[15] The photophysical behaviour[17] and the phase behaviour[18] of phospholipid derivatives containing aromatic moieties have been studied.

In this article the synthesis, and the aggregation behaviour of di-*n*-alkyl phosphates with various structural modifications in their alkyl chains (Fig. 1) will be described. Care has been taken that the tail lengths of all these derivatives were approximately similar to that of di-*n*-tetradecyl phosphate. We find that incorporating a phenyl group at the end of the chain significantly disturbs the packing of the bilayer, resulting in a drastic reduction of the main phase transition temperature (T_m) and an increased permeability of the bilayer. This disturbance is even larger when a phenyl or phenoxy group is positioned in the middle of the chain. This disturbance is similar to the disturbance caused by a *cis* double bond.[8]

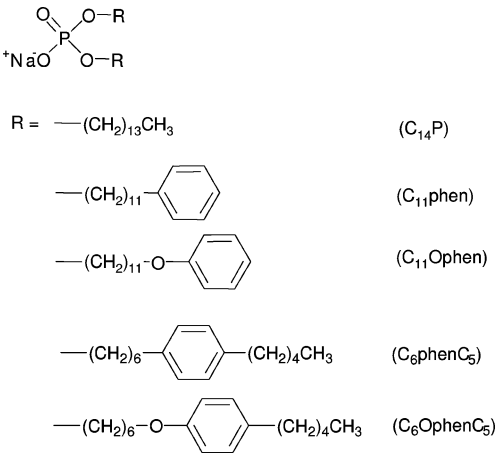

Fig. 1 Molecular structures of the di-*n*-alkyl phosphate derivatives.

Results

Synthesis

Di-*n*-alkyl phosphates can be synthesised from the corresponding alkanol and P_2O_5 or $POCl_3$.[19] Alternatively, the mono-*n*-alkyl phosphate can be alkylated with the corresponding bromide using tetramethylammonium hydroxide.[6] A mild method to synthesise dialkyl phosphates was published recently.[16] We used PCl_3 for the synthesis of the phosphoric acid esters, because it is more reactive than $POCl_3$ and it was expected that the functional groups in the chains would withstand the subsequent oxidation step.

A slightly modified literature procedure[9] was used for the synthesis of the phenyl and phenoxy substituted di-*n*-alkyl phosphates (Scheme 1). First a di-*n*-alkyl phosphite was synthesised from two

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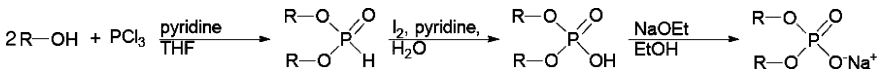
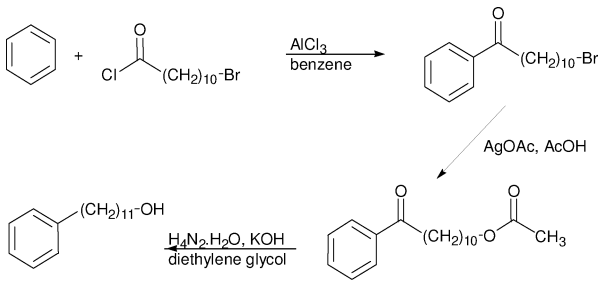


Scheme 1 Synthesis of sodium di-*n*-alkylphosphate analogues.

equivalents of alkanol and pyridine and one equivalent of PCl_3 in tetrahydrofuran. After evaporation of the solvent, the ester was oxidised in 98% (v/v) aqueous pyridine with an excess of iodine. This usually gave overall yields of around 30%.

Alcohols with an aromatic moiety connected *via* an ether linkage were synthesised by refluxing the corresponding aromatic alcohol, and the ω-bromoalkanol, or ω-bromoalkanoic acid in acetone with K_2CO_3 followed in the case of the alkanoic acid by reduction with $NaBH_4$–I_2 in THF. 11-Phenylundecan-1-ol was synthesised by attaching a phenyl group to 11-bromoundecan-1-oyl chloride *via* a Friedel–Crafts acylation in benzene, followed by converting the bromide into the acetate ester and, subsequently removing the carbonyl group by a Wolf–Kishner reduction, during which hydrolysis of the acetate ester to form 11-phenylundecan-1-ol took place (Scheme 2). The synthesis of the derivatised alcohols is described in the supplementary material.†



Scheme 2 Synthesis of 11-phenylundecan-1-ol.

Aggregation behaviour and colloidal stability

The colloidal stability of aggregates formed from these compounds (Fig. 1) was compared by monitoring the absorbance of a 0.5 mM amphiphile solution at 400 nm as a function of time.

Aggregate solutions in pure water or at 50 mM ionic strength were stable over two days. Solutions of aggregates formed from C_{11}phen in 150 mM NaCl precipitated within one hour, but solutions of C_6phenC_5, C_6OphenC_5, and C_{14}P in 150 mM NaCl were stable for more than two days.

Phase behaviour

The aggregate solutions were studied using differential scanning calorimetry (DSC). The main phase transition temperature (T_m) measured for C_{14}P is 54.5 °C, in agreement with previous studies.[7] Replacement of three methylene units at the end of the hydrophobic tail by a phenoxy group leads to a slight increase of T_m to 56.3 °C. When a phenyl group replaces the terminal methylene groups, the T_m is drastically lowered to 28.5 °C (Fig. 2). Aggregates formed from C_6OphenC_5 and C_6phenC_5 do not show a phase transition between 0 and 100 °C. Phosphatidylcholine derivatives with chains that contain aromatic units also do not exhibit a phase transition.[18]

The change in steady-state fluorescence anisotropies upon variation of temperature was measured for 1,6-diphenyl-(*E*),(*E*),(*E*)-1,3,5-hexatriene (DPH) in the bilayers of C_{11}phen, C_6phenC_5, and

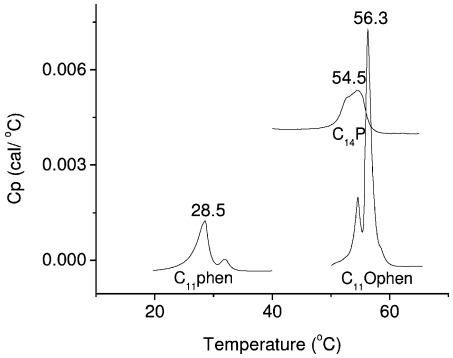


Fig. 2 DSC enthalpograms of C_{11}Phen, C_{14}P and C_{11}Ophen.

C_6OphenC_5 (Fig. 3) to obtain more information on the phase behaviour and orientational order in the bilayer aggregates.[20,21] C_6phenC_5 and C_6OphenC_5 showed no sudden increase in anisotropy, indicating that no cooperative transition from the liquid-crystalline to the gel phase took place. Instead a gradual increase of polarisation (*P*) from 0.12 to 0.34 (for C_6OphenC_5) and from 0.12 to 0.28 (for C_6phenC_5) was observed, which has been described as a non-cooperative phase transition.[8] An alternative description is that the phase transition occurs below 0 °C, similar to di-oleylphosphatidylcholine (DOPC). The polarisation of DPH in DOPC bilayers also shows a gradual increase, although the values are lower and range from 0.05 to 0.18.[22] The anisotropy of C_6OphenC_5 is higher over the whole temperature range than that for C_6phenC_5, which might point to a more ordered bilayer for C_6OphenC_5.

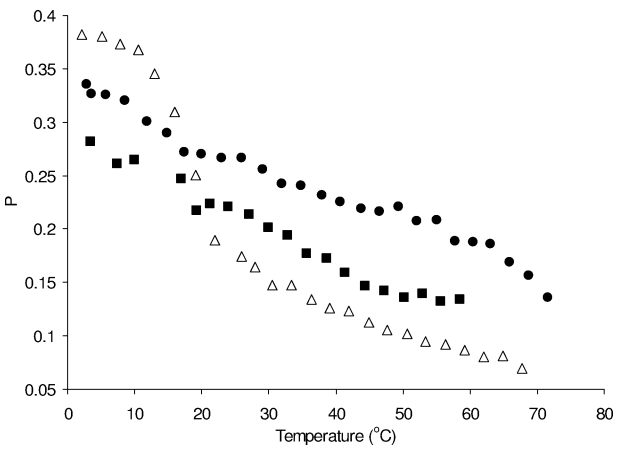


Fig. 3 Temperature dependent fluorescence depolarisation of DPH in C_{11}phen (△), C_6phenC_5 (■) and C_6OphenC_5 (●) aggregates.

The T_m found for C_{11}phen is 22 °C, which is in reasonable agreement with the value determined with DSC (28.5 °C). When the DPH polarisation values of C_{11}phen aggregates at temperatures below the T_m (0.38) and above the T_m (0.08), are compared to those of C_{14}P (0.38 and 0.13), it seems that the liquid crystalline phase of C_{14}P is slightly more ordered than that of C_{11}phen. The depolarisation values in sodium di-dodecyl

phosphate vesicles (0.39 and 0.09)[23] agree more with those of C_{11}phen, also its T_m, 33 °C,[7] is more comparable to that of C_{11}phen than the T_m of C_{14}P. Bearing in mind that a linear relationship exists between the T_m and chain length, the 'effective chain length' of C_{11}phen is somewhat less than 12 methylene moieties.

Encapsulation and leakage

A vesicle can encapsulate a water-soluble dye in its aqueous compartment. The ability of the aggregates of the different dialkyl phosphates to entrap the fluorescent dye carboxyfluorescein (CF) was investigated to determine whether the amphiphiles formed effectively closed vesicular aggregates. The relation between amphiphile structure and leakage over the membrane was simultaneously studied.

C_{11}Ophen aggregates did not encapsulate CF. Aggregates formed from C_6OphenC_5, C_6phenC_5, and C_{11}phen did encapsulate CF. Leakage studies of C_6OphenC_5 and C_6phenC_5 could be performed at 25 °C (Fig. 4). For C_{11}phen a temperature of 70 °C, well above T_m, was needed to perform the measurements. Below this temperature instantaneous leakage occurred, indicating rupture of the membrane. Also the encapsulations and the separation of the extra-vesicular CF had to be carried out at this temperature.

Fig. 4 shows that C_{11}phen vesicles at 70 °C are more permeable towards carboxyfluorescein than vesicles formed from C_6OphenC_5 and C_6phenC_5 at 25 °C. Apparently the phenyl group at the end disturbs the packing more significantly, leading to an increased trafficking of the fluorescent probe across the bilayer.

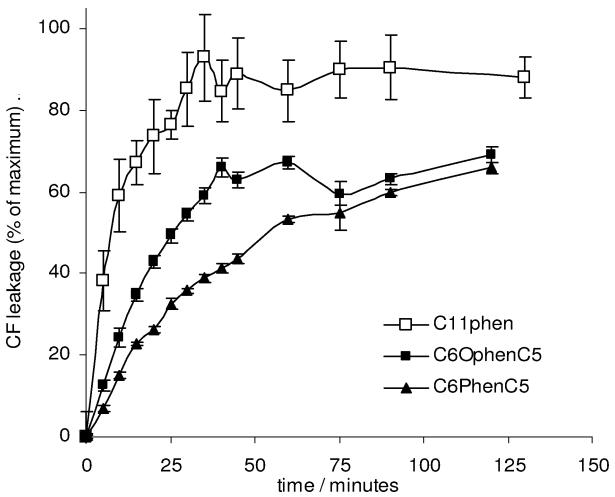


Fig. 4 CF leakage from vesicles composed of C6OphenC5 (■), C6phenC5 (▲), and from vesicles and C_{11}phen (□) at 70 °C.

Aggregate morphology

In order to obtain more insight into the morphology of the aggregates, cryo-electron microscopy (cryo-EM) was performed on aqueous aggregate solutions of C_6OphenC_5, C_6phenC_5, C_{11}phen, and C_{11}Ophen.

In sonicated aqueous solutions of C_6phenC_5 (Fig. 5) smooth unilamellar spherical vesicles are observed. The vesicles display a large variation in size, from many small vesicles with diameters of 15 to 35 nm to a smaller number of larger vesicles. These larger vesicles have diameters ranging between 65 nm and 105 nm.

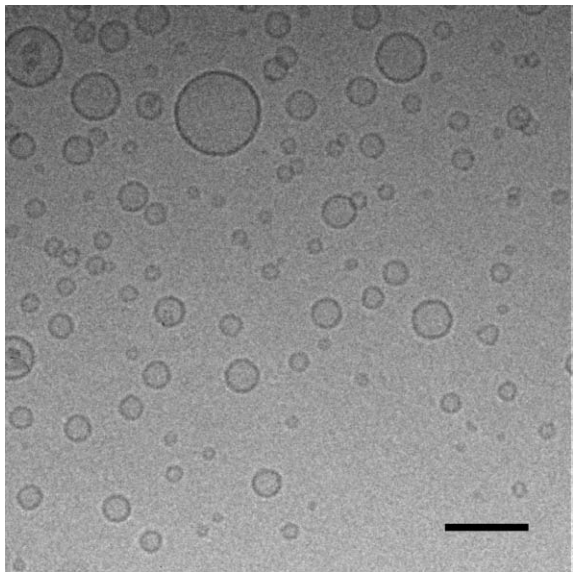


Fig. 5 Cryo-EM micrographs of vesicles composed of C_6phenC_5 after sonication. Bar represents 100 nm.

The samples of C_6OphenC_5 and C_6phenC_5, prepared after sonication and repeated freeze–thaw and extrusion through 200 nm polycarbonate membranes, showed bilayer vesicles with various inclusions (Fig. 6). When this preparation method is used for phospholipids, usually unilamellar vesicles are observed.[24]

The inclusions in the vesicles composed of both C_6OphenC_5 and C_6phenC_5 consist of small vesicles and non-spherical bilayer fragments. In giant liposomes, the formation of endo-vesicles has been observed previously and is usually induced by temperature changes or addition of surfactant molecules that can influence the membrane curvature.[25,26] Also as a result of osmotic effects invagination can be found in unilamellar vesicles.[27] Here the lateral mobility of the phosphate molecules seems to be hindered in the bilayers, due to the phenyl or phenoxy substituents.

Samples from an aggregate solution of C_{11}phen (prepared after stirring the amphiphile in water above T_m followed by extrusion through 200 nm polycarbonate filters) were prepared for cryo-EM by vitrification above T_m (Fig. 7, left) and below T_m (Fig. 7, right). Micrographs of aggregate solutions of C_{11}phen above T_m show closed bilayer vesicles with inclusions similar to those observed with phenyl and phenoxy substituents in the middle of the alkyl chain. Upon cooling below the phase transition temperature the vesicles become faceted and burst open. Below T_m the hydrophobic chains loose part of their flexibility, which makes the bilayer apparently too rigid to allow accommodation of the required curvature to form a vesicle. As a result the vesicle bursts open and forms faceted open bilayer fragments with a low curvature. This is in contrast to phospholipid vesicles, which change from spherical to faceted upon going from a temperature above to a temperature below T_m, but stay in one piece.[28] The hindered mobility of the phenyl containing phosphate in combination with increased rigidity, likely causes the breakage of the vesicles. It is known that vesicles prepared from certain synthetic amphiphiles, including di-*n*-hexadecyl phosphate, do not form closed spherical vesicles below T_m.[4,29,30] The current observations are in accordance with the leakage studies (Fig. 4). Aggregates formed from C_{11}phen

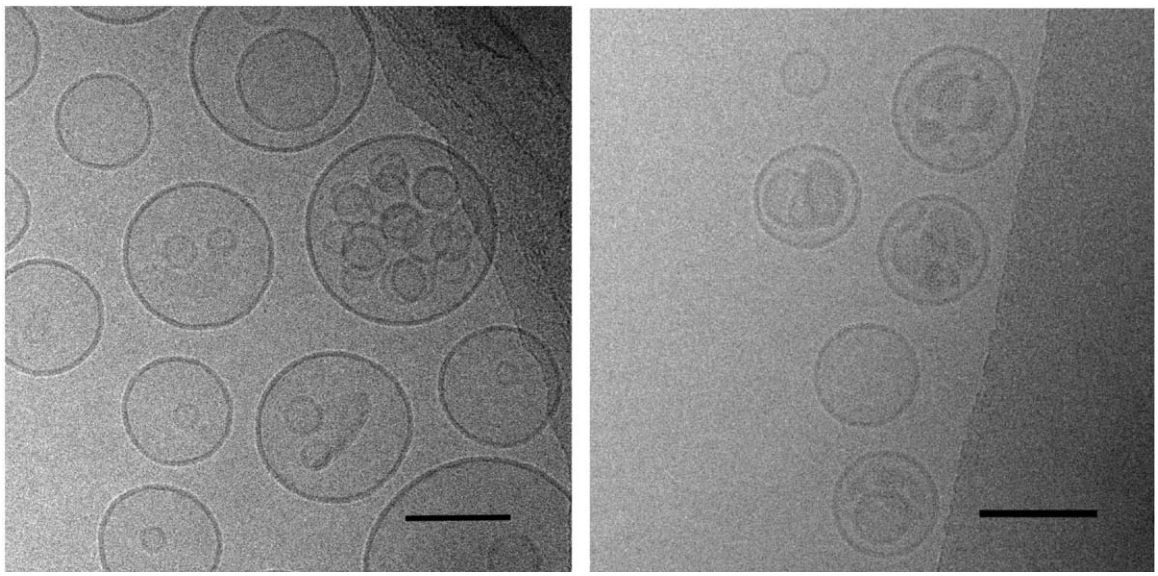


Fig. 6 Cryo-electron micrographs of vesicles (after freeze–thawing and extrusion through 200 nm polycarbonate filters) composed of $C_6OphenC_5$ (left), C_6phenC_5 (right). Bar represents 100 nm.

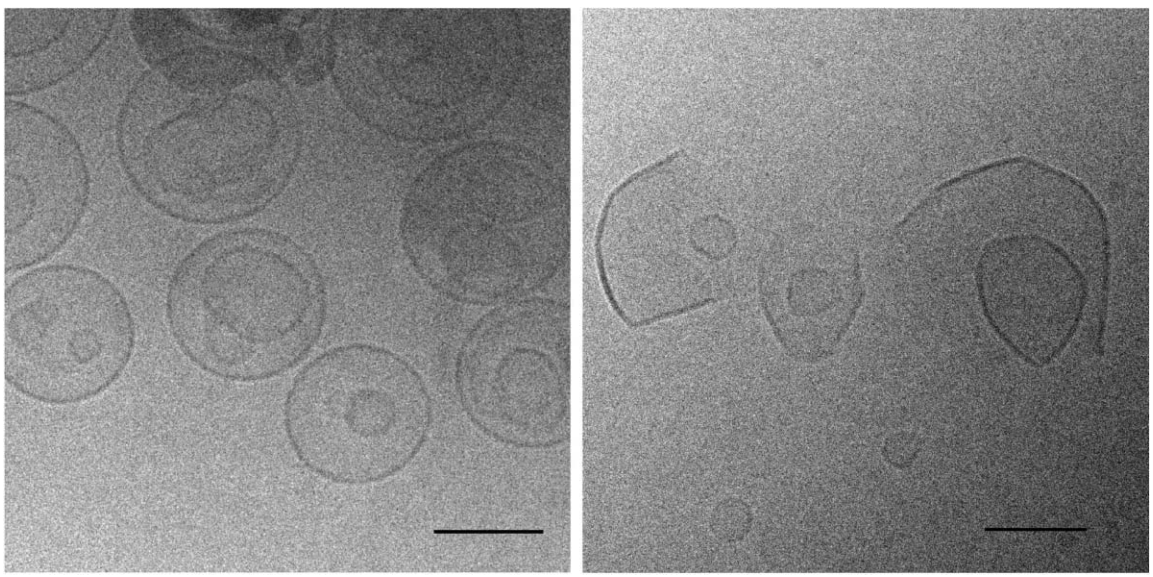


Fig. 7 Cryo-electron micrographs of vesicles (after extrusion through 200 nm polycarbonate filters) composed of C_{11}phen above T_m (left), and below T_m (right). Bar represents 100 nm.

could not entrap carboxyfluorescein at temperatures below T_m, in contrast to vesicles of C_{11}phen above T_m.

In aggregate solutions of C_{11}Ophen curved bilayer fragments were observed (Fig. 8). From these structures it is not surprising that C_{11}Ophen cannot entrap carboxyfluorescein, even at temperatures above T_m. Surprisingly, bicelles were also observed in C_{11}Ophen suspensions. Bicelles are bilayered aggregates that have a discoid shape. Usually bicelles are observed in lipid mixtures, often composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), or DMPC and 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxypropanesulfonic acid (CHAPSO), a zwitterionic bile salt derivative.[31,32] Recently it was found that bicelles are only formed below the T_m of DMPC.[33] Pure DHPC and CHAPSO both form spherical micelles.[34,35] It is believed that in bicelles DMPC forms the bilayered part of the bicelle while DHPC or CHAPSO is predominantly situated at the rim of the bicelle. This rim has a high curvature. To accommodate this curvature, the lipids must have a small packing parameter. This is presumably not the case for C_{11}Ophen, so the reason for C_{11}Ophen to aggregate into bicelles is not obvious (*vide supra*).

Discussion and conclusions

The cryo-EM results show that the phosphates with phenyl and phenoxy substituents in the middle of the alkyl chain form spherical, closed bilayer vesicles at room temperature when they are in the liquid-crystalline phase. Above T_m, also the phosphate

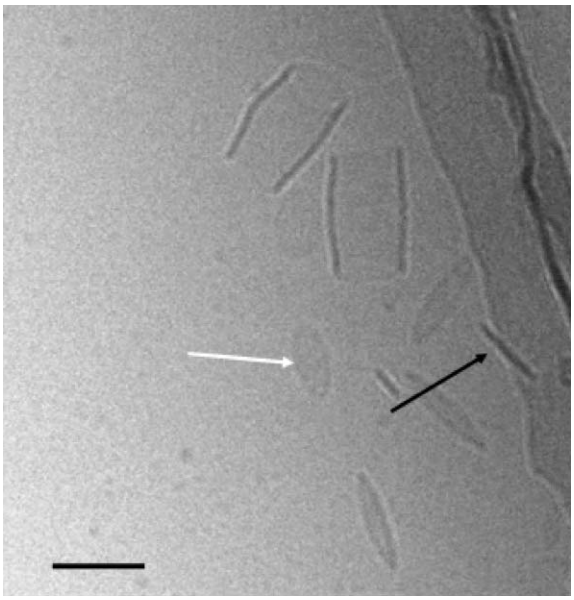


Fig. 8 Cryo-electron micrographs of aggregates (after extrusion through 200 nm polycarbonate filters at a temperature above T_m) composed of C_{11}Ophen. White arrow indicates top view of the bicelle, black arrow indicates side view of a bicelle. Bar represents 100 nm.

with a phenyl substituent at the end of the alkyl chain forms closed vesicles. This is consistent with the inability of C_{11}phen to entrap CF below their T_m and also with cryo-EM results for di-*n*-hexadecyl phosphate aggregates below T_m.[4] Striking is the presence of inclusions in the larger vesicles. The preparation method that was deployed usually yields unilamellar vesicles in the case of phospholipids.[24] The shapes of the vesicles and the inclusions bear a resemblance to vesicular shapes observed in giant liposomes.[26] The cause of these inclusions is not clear. The inclusions are probably inherent to the amphiphiles used. We believe that the tails in the substituted di-*n*-alkyl phosphate molecules in the inner and outer leaflet cannot move as independently from each other as compared to phospholipid molecules in their bilayer environment. This hampers the ability to accommodate the required bilayer curvature. In a phospholipid molecule the tails are connected to each other *via* three bonds. In many synthetic amphiphiles the tails are connected *via* two bonds. This means that a phospholipid molecule has one rotational degree of freedom more than the synthetic amphiphiles. During preparation of the vesicles a certain curvature stress and a certain ratio between the surface and volume of the vesicle is induced. In phospholipid vesicles relaxation takes place, but in our vesicles this apparently does not occur.[36] Mathematical modelling studies predicted vesicle shapes corresponding to minimal isotropical bending energies for different values of the normalised average mean curvature.[4] We have observed these vesicular shapes in our electron microscopic studies. We believe that due to the inflexibility of the chains, no unilamellar vesicles can be formed.

From NMR and molecular dynamics simulations, it is known that the conformational order at the interface is high, and that the order in the hydrophobic core is low.[37,38] An already low order cannot be perturbed much further, whereas an ordered situation can be perturbed more easily.

Micrographs of aggregate solutions of C_{11}Ophen showed only curved bilayer structures and bicelles. Apparently this amphiphile is able to accommodate the high curvature at the rim of the bicelle. This could occur by bending the less hydrophobic phenoxy back to the interface, thereby changing the packing parameter. Back bending has been recently also proposed for single-tailed surfactants with two ester modifications in the alkyl tail.[39] Another possibility is that this bending is not necessary, because the substituted tails could better interact with water at the edge of the flat bilayer than the corresponding alkyl tails. The polar phenoxy functionality will also decrease the hydrophobic interactions. The hydration sphere of the polar functionality prevents a complete development of hydrophobic hydration shells of the CH_2-groups next to the polar moiety, and therefore limits the availability of these CH_2-groups for hydrophobic interactions. The influence of hydrophilic hydration on the apparent hydrophobicity has been investigated using kinetic medium effects on hydrolysis reactions in dilute aqueous solution.[40,41]

Overall, it can be concluded that the phenyl rings that are incorporated in the alkyl chain disrupt the packing of the hydrophobic tails in a bilayer. A phenyl or phenoxy substituent in the middle of the alkyl chain results in a drastic lowering of the phase transition temperature and leads to the formation of bilayered vesicles with hindered lateral movement. A phenyl or phenoxy substitution at the end of the alkyl chain results in a less drastic lowering (phenyl) or no change in the phase transition temperature (phenoxy). The alkyl phosphates with a terminal phenoxy substituent do not form vesicles but curved bilayer fragments and bicelles.

Experimental

Syntheses are described in the supplementary material.† All water used in the experiments was bidistilled in an all quartz apparatus or purified by a Millipore system.

Colloidal stability

Vesicles were prepared by dissolving the appropriate amount of amphiphile in water by heating above T_m and vigorous vortexing. Then the amphiphile solution was extruded 21 times through a polycarbonate filter of 200 nm. The time dependence of the optical density at 400 nm was monitored using a Perkin Elmer λ5 spectrophotometer.

Differential scanning calorimetry

Vesicles were prepared by dissolving the appropriate amount of amphiphile in water by heating above T_m and vigorous vortexing. Then the amphiphile solution was extruded 21 times through a polycarbonate filter of 200 nm. The DSC enthalpograms were recorded on a VP-DSC apparatus (Microcal, Northhampton, MA). The reference cell was filled with water. The concentration of the amphiphiles was 2.0 mM. The samples were degassed before measurements. Heating scans were performed from 20 to 100 °C at a rate of 1 °C min^{-1} (C_{11}Ophen), from 10 to 100 °C (C_{11}phen) or from 0 to 100 °C (C_6phenC_5 and C_6OphenC_5), the time in between runs was 60 minutes.

Fluorescence polarisation

The measurements were performed with an SLM Aminco SPF 500 C spectrofluorometer equipped with a thermostated cell holder. 1,6-Diphenyl-*E*,*E*,*E*-1,3,5-hexatriene (DPH) (5×10^{-8} M) was excited at 360 nm. The emission wavelength was 428 nm (bandpass 5 nm). The fluorescence polarisation was calculated from the intensities of the emitted light parallel and perpendicular to the direction of the excitation radiation using $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$. The amphiphile concentration was 5×10^{-5} M. Measurements involved heating scans with 3 °C intervals. The samples were allowed to equilibrate for 10 minutes after each temperature increase. Reported values for *P* are average values of six independent measurements.

Carboxyfluorescein leakage assay

250 μL of a 10 mM solution of the appropriate amphiphile in methanol was evaporated to form a film on the wall of a glass tube. This was kept at vacuum for several hours. 100 μL of a stock solution of carboxyfluorescein (100 mM) and 0.49 mL of a buffer solution (5 mM HEPES, 5 mM sodium acetate, 1 mM EDTA) were added. This solution was heated to 70 °C (C_{11}phen) and sonicated for 30 seconds at this temperature. The non-encapsulated carboxyfluorescein was removed by passing the vesicle solution over a column of Sephadex G-75 at 65 °C (C11phen) or at 25 °C (C6phenC5 and C6OphenC5) with an elution buffer (5 mM HEPES, 5 mM sodium acetate, 1 mM EDTA and 20 mM NaCl, preheated to 65 °C or 25 °C). The fraction containing the vesicles was collected in a preheated glass tube. 20 μL of this solution was injected into a vigorously stirred cuvet containing 3 mL elution buffer at 70 °C (C_{11}phen) or at 25 °C (C_6phenC_5 and C_6OphenC_5). The carboxyfluorescein emission intensity ($\lambda_{ex} = 490$ nm, bandpass 2 nm, $\lambda_{em} = 520$ nm, bandpass 4 nm) was measured on a SLM-Aminco SPF-500 C spectrofluorometer equipped with a thermostatted cell holder and magnetic stirring device. CF leakage was calculated using $\% = (I - I_0)/(I_{max} - I_0) \times 100$, where % is the leakage in percentage of the maximum leakage, I_0 is the emission intensity at $t = 0$, I_t is the emission intensity at $t = t$ and I_{max} is the maximum emission intensity after disruption of the vesicles with 150 μl of a Triton X-100 solution (10% w/v).

Cryo-electron microscopy

Aliquots of 5 mM di-*n*-alkyl phosphate solutions were deposited on holey carbon grids. Filter paper was used to blot off excess solution. The samples were vitrified by plunging into liquid ethane. The samples from C_{11}phen above and below T_m were placed in a temperature controlled environment at 100% humidity and plunged into ethane from this environment.[42] The grids were transferred to a Gatan model 626 cryo holder at −170 °C in a Philips CM120 microscope operating at 120 kV. Micrographs were recorded under low dose conditions.

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