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Synthesis of and vesicle formation from phosphorylcholine amphiphiles with one symmetrically branched alkyl chain

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Abstract. Three phosphorylcholines with symmetrically branched alkyl chains $[(C_nH_{2n+1})_2CHOPO_3^-(CH_2)_2N(CH_3)_3^+]$ (**1a–c**) have been synthesized. The synthesis involves three steps: (1) reaction of the alcohol $(C_nH_{2n+1})_2CHOH$, (n = 6, 8, 10) with 2-chloro-1,3,2-dioxaphospholane in the presence of triethylamine; (2) oxidation of the resulting trialkyl phosphite with nitrogen dioxide and (3) ring opening of the cyclic phosphate triester by trimethylamine in acetonitrile. When suspended in water, these amphiphiles all form bilayer vesicles as revealed by electron microscopy and NMR spectroscopy. The vesicles have diameters of 300–1000 Å and are stable for more than a week.

Introduction

Membranes consisting of lipid bilayers are important building blocks of biological molecular organisation. They are directly involved in many fundamental biological functions of cells such as compartmentalisation, energy transduction and information transfer. Phospholipids are essential constituents of these membranes. In water, phospholipids spontaneously aggregate with formation of bilayers.

Pioneering work, particularly by *Kunitake*[1], showed that synthetic amphiphiles may also spontaneously assemble in water to form bilayer structures, which have the same structural characteristics as those of biolipids. This suggested that bilayer formation is a general physicochemical phenomenon that is not restricted to particular structures of biolipid molecules.

Since then many synthetic amphiphiles have been made which are able to form vesicles. The surfactant bilayers of these spherical aggregates successfully mimic important processes occurring in complex biological membranes. Therefore vesicles provide a way to examine single membrane processes in a chemically and physically well-defined environment. These processes include morphological changes[2,3], lateral diffusion and flip-flop movements of amphiphiles in the bilayer[4,5], phase transitions[6,7], osmotic activity[8] and membrane fusion[9] (merging of the bilayers of two or more vesicles). Consequently, the increasing interest in the development of new membrane models and drug delivery systems has resulted in the production of a large number of synthetic or semi-synthetic amphiphiles.

In attempts to rationalise the formation of surfactant aggregates of different morphologies, *Tanford*[10] and *Israelachvili*[11] introduced the packing-parameter concept. This is based on a purely geometrical consideration of the volume of the hydrophobic core and the cross-sectional surface area occupied by the head group, and should make it possible to predict which type of aggregate an amphiphile will form in aqueous solution. The packing parameter, P, is defined as follows:

$$P = \frac{V}{a_0 \cdot l_c}$$

where V is the hydrocarbon chain volume, a_0 the "optimal cross-sectional surface area" per head group and l_c the critical chain length of the alkyl chains. In the case of synthetic amphiphiles, few systematic studies have been performed to test the usefulness of the approach, but for aqueous solutions of conventional surfactants the analyses of aggregate morphologies in terms of the magnitude of P have been quite successful[12].

In this paper we describe the synthesis of a new type of surfactant (**1**), consisting of a single symmetrically branched alkyl chain, directly coupled to a phosphorylcholine head group. Interestingly, we find that these simple synthetic surfactants, carrying a phospholipid-type head group, form vesicles in aqueous solution which exhibit remarkable thermal stability.

Results and discussion

Several procedures for the synthesis of phosphatidylcholines from the corresponding alcohols have been described in the literature. The use of 2-bromoethylphosphodichloridate as a phosphorylating agent, first described by *Hirt* and *Berchthold*[13], leads to poor yields and numerous by-products, which makes purification difficult[14,15]. Another approach employs 2-chloro-1,3,2-dioxaphospholan-2-one[16,17,18], which has been successfully applied in the coupling of glycerols to the phosphate

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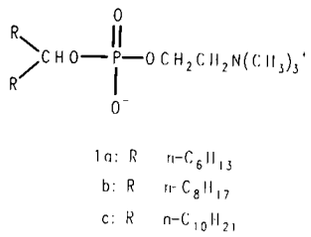

Figure 1.

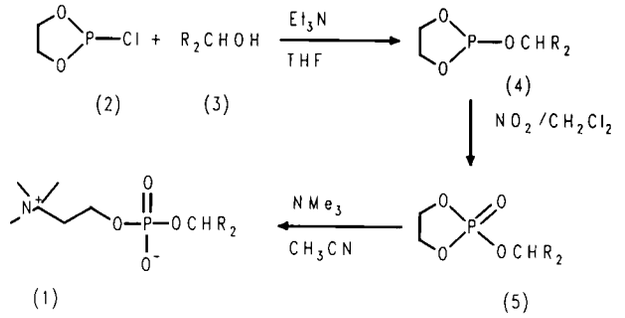

Figure 2.

moiety. However, this reagent is unreactive towards sterically hindered alcohols[19,20,21]. This also proved to be the case for the secondary alcohols used in this study, which only couple slowly and not completely. Furthermore, long reaction times lead to product decomposition, which may be due to the presence of some unreacted triethylamine used in the last step of the synthesis. Therefore we applied the very reactive phosphite analogue, first used in lipid synthesis by *Ni'fantev* et al.[22,23] (Figure 2). In the first step, the branched alcohol is coupled with 2-chloro-1,3,2-dioxaphospholane. The resulting trialkyl phosphite is oxidised with NO_2 and finally the resulting unstable cyclic phosphate is ring-opened by trimethylamine. The alkylphosphorylcholine can be readily purified by crystallisation from acetonitrile.

Based on a rough estimation of the packing parameter, these compounds are expected to form bilayer structures. This was indeed borne out in practice as indicated by electron microscopy and NMR spectroscopy. Using fluorescence depolarisation and differential scanning calorimetry (DSC), no phase transition was observed in the temperature range of 0° to 90°C for any of the compounds. This is in agreement with the estimation made according to *Cevc*'s model[24], which predicts phase transitions far below 0°C for vesicles formed from these relatively short-chain surfactants.

Synthesis

Three symmetrically branched secondary alcohols (**3a–c**) were converted into the corresponding alkylphosphorylcholine zwitterions, **1a–c**, following the sequence of reactions shown in Figure 2. In the first step the alcohol is coupled to the chlorophosphite **2** at −40°C in THF. The reaction proceeds in virtually quantitative yield within 2 h, as indicated by the appearance of one singlet at δ −85.9 ppm in the ^{31}P-NMR spectrum. The alkyl phosphite **4** was isolated but not purified. Subsequent oxidation with nitrogen dioxide in dichloromethane at −40°C could also be accomplished within two hours, as indicated by the appearance of a singlet in the cyclic phosphate region (δ 17.9) in the ^{31}P-NMR spectra. The resulting cyclic phosphate **5** is extremely vulnerable to hydrolysis and should not be purified but used as soon as possible for the next reaction. The compound is ring-opened by reaction with trimethylamine (excess) in acetonitrile at 65–70°C for 24 h; the alkyl phosphorylcholine precipitates upon cooling to 0°C. Generally, the reaction mixture can be filtered and the residue crystallised from acetonitrile to provide pure **1a–c** in 35–45% yield. The alkylphosphorylcholines are extremely hygroscopic and **1b** retains water even after prolonged drying in vacuum. The reactions can easily be monitored by ^{31}P-NMR and this makes it possible to keep the reaction time of the last step as short as possible to minimise product decomposition. This method is fast and the absence of by-products facilitates the purification considerably.

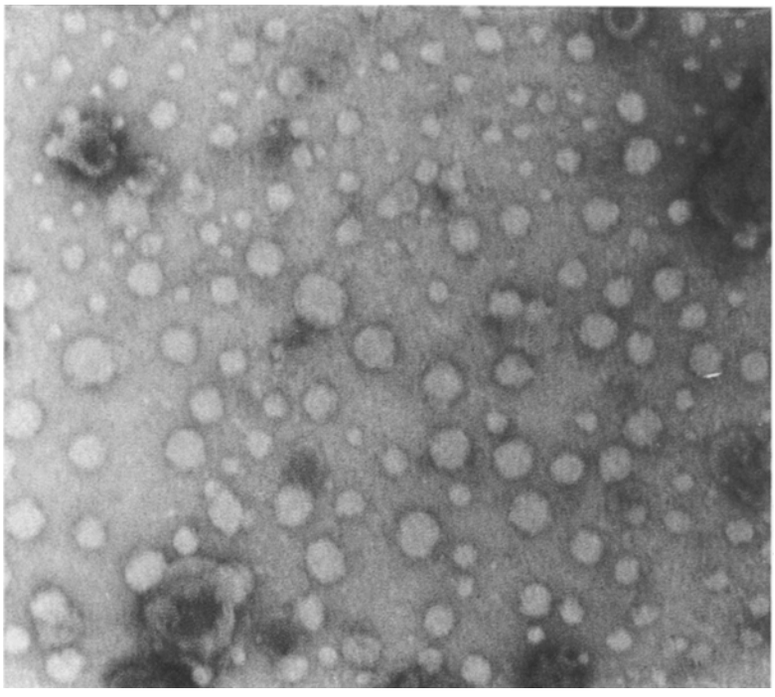

Figure 3. Negative strain electron micrograph of vesicles of ***1a****, prepared by the ethanol injection method (1 cm represents 50 nm).*

Vesicle formation

Using the ethanol-injection method, it was found that the phosphorylcholines **1a–c** all form vesicles as evidenced by electron microscopy (*vide infra*). The vesicle suspensions of **1b** and **1c** were opaque, while **1a** gave a colourless clear solution. The concentration was 8–11 mmol. Using the sonication method, **1a** only forms vesicles at relatively high concentration (~ 10 mmol). Apparently, the solubility of monomeric **1a** in water is rather high, due to the relatively short alkyl chain.

Estimation of the packing parameter

Generally, amphiphiles with a single hydrocarbon chain per polar head group tend to form micellar aggregates and amphiphiles with two hydrocarbon chains per head group tend to form bilayer structures. These findings can be explained in terms of the packing-parameter concept introduced by *Tanford*[10] and *Israelachvili*[11] (*vide supra*). Single-chain surfactants adopt a cone-shape geometry and the packing parameter P will be between 0 and 0.5; double-chain surfactants adopt a more cylindrical shape and P will be between 0.5 and 1.
For the estimation of P for **1a–c**, V and l_c were calculated from the equations of *Tanford*[11], and for the value of a_0 we have taken the calculated value of 60–70 Å^2 for hydrated choline head groups in phosphatidylcholine vesicles[11,32]. The estimated P value is 0.6–0.7 for compounds **1a–c**, which is consistent with the bilayer-forming properties of these amphiphiles.

Electron microscopy

Using the ethanol-injection method, all three compounds form unilamellar vesicles with diameters of 300–1000 Å. An electron micrograph of vesicles of **1a** is shown in Figure 3. There is no clear relation between the size of the vesicles formed and the alkyl-chain length. Aggregated vesicles were observed to some extent for **1b** and **1c**, possibly due to the presence of uranyl acetate, which is a strongly dehydrating agent[31]. The diameter of the vesicles formed from **1a** was 400–600 Å. Little or no aggregation was observed for these vesicles. The vesicles formed from **1a–c** were stable for more than a week at room temperature as indicated by electron microscopy. There was no visible change in turbidity of the vesicle solutions. This high thermal stability was not anticipated as intervesicular coulombic interactions are relatively weak because of the zwitterionic nature of the head group. Perhaps flocculation and crystallisation is hampered by the branched alkyl chains of **1a–c**. Vesicles formed from monoalkyl phosphate amphiphiles carrying branched alkyl chains are also quite stable[33]. The relatively high stability may considerably facilitate future experiments with these bilayer systems.

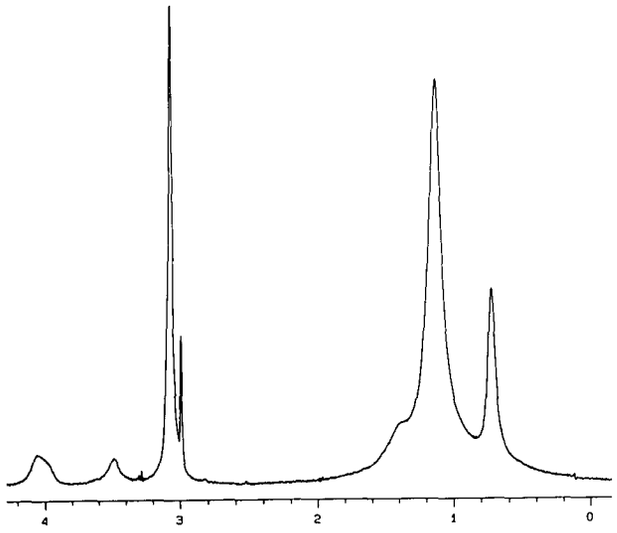

Figure 4. 300-MHz ^{1}H NMR spectrum of 11-heneicosylphosphorylcholine (5 mM) in D_2O at 25°C.

NMR spectroscopy

Figure 4 shows a ^{1}H-NMR spectrum of **1c** in D_2O (25°C, 5 mM). The typical line broadening of the proton resonances is a strong indication of the presence of vesicles[34–36]. The extent of the line broadening is similar to that observed for phospholipid vesicles in other studies. The ^{31}P-NMR spectrum of the same vesicle solution showed one symmetrical signal with a peak width of 25.8 Hz.

Experimental

General

NMR spectra were recorded on a Varian Gemini-200 or Varian VXR-300 spectrometer. The solvent was $CDCl_3$ or D_2O. Chemical shifts are reported in δ units. ^{31}P chemical shifts were determined relative to hexachlorotriphosphatriazine (+19.9 ppm downfield from 85% H_3PO_4) as an external reference. Ether, THF, CH_2Cl_2, and $CHCl_3$ were distilled from P_2O_5. Acetonitrile and benzene were dried over 4 Å molecular sieves. Et_3N was distilled from and stored on KOH pellets. PCl_3 was freshly distilled and 1,2-ethanediol was distilled from CaH_2 and stored on 3 Å sieves. 2-Chloro-1,3,2-dioxaphospholane (**2**) was prepared according to literature procedures[25,26] and was stored under argon at −20°C. A solution of NMe_3 in CH_3CN was prepared by dropwise addition of a concentrated NaOH solution to a well-stirred solution of NMe_3 in water (45%) and leading the gaseous trimethylamine through a column filled with KOH pellets into strongly cooled acetonitrile. The solution was stored at −20°C. 7-Tridecanol, 9-heptadecanol and 11-heneicosanol were prepared in an analogous way to the procedure for the synthesis of 5-nonanol[27]. The alcohols were dried azeotropically by distillation with benzene and kept in a drying pistol at reduced pressure. All the syntheses involving cyclic phosphorus derivatives were carried out in an inert argon atmosphere in a Schlenk vessel.

*7-Tridecanol (**3a**)*

A solution of 1-bromohexane (100 g, 0.606 mol) in ether (200 ml) was added dropwise to magnesium (activated with a crystal of iodine) under spontaneous reflux. After the addition was completed, the mixture was refluxed until all magnesium had disappeared. A solution of ethyl formate (22.42 g, 0.30 mol) in ether (50 ml) was added dropwise and the mixture was refluxed for one more hour. Under cooling and vigourous stirring water (40.5 ml) was added slowly followed by a solution of concentrated sulphuric acid (34.5 g) in water (160 ml). The mixture was stirred for 90 min until two almost clear layers were formed. The two layers were separated and the aqueous phase extracted with ether (four times 100 ml). The combined ethereal solutions were evaporated and the residue was refluxed with 30 ml 15% KOH solution for 3 h. The mixture was extracted with ether. After evaporation of the solvent the residue was crystallised from petroleum ether (b.p. 40–60°C) to yield 42.4 g (94%) of **3a**, m.p. 42–43°C (lit.[27] m.p. 42.5–43°C); ^{1}H NMR ($CDCl_3$) δ: 0.88, t, $J_{1,2}$ 6.6 Hz, 6 H; 1.28–1.47, m, 20 H; 3.58, p, $J_{6,7,8}$ 6.1 Hz, 1 H. ^{13}C NMR δ: 72.00, C7.

*9-Heptadecanol (**3b**)*

M.p. 59°C (lit.[28] 60.8–61.2°C). ^{13}C NMR δ: 71.95, C9.

*11-Heneicosanol (**3b**)*

M.p. 71.3–72.5°C. ^{13}C-NMR δ: 71.99, C11.

2-Chloro-1,3,2-dioxaphospholane (2)

^{1}H NMR δ: 4.34, m, OCH_2CH_2O. ^{31}P NMR δ: −54.7.

(1-Decylundecyl)phosphorylcholine (1c)

A solution of 1.75 g (13.8 mmol) 2-chloro-1,3,2-dioxaphospholane (**2**) in 10 ml THF was added to a solution of 4.3 g (13.8 mmol) 11-heneicosanol (**3b**) and 1.9 ml Et_3N in 30 ml THF over 20 min at −40°C in an argon atmosphere. After stirring for 2 h at a temperature below −10°C, ^{31}P NMR (δ −85.9) showed that complete coupling had occurred. The precipitate of $Et_3N \cdot HCl$ was removed by filtration and THF was removed by distillation in vacuum. A few ml of CH_2Cl_2 were added and a cooled, dilute solution of NO_2 in CH_2Cl_2 was added dropwise at −40°C. The addition was continued until the acquired blue colour turned to yellowish. The ^{31}P-NMR spectrum showed that the oxidation was complete (one singlet at δ 17.1). The solvent was removed in vacuum and the crude residue was characterised by NMR. ^{1}H NMR ($CDCl_3$) δ: 0.91, t, 6 H, 2 CH_3; 1.29–1.70, m, 36 H, $(CH_2)_9$; 4.31–4.58, m, 5H, OCH_2CH_2O, OCH. ^{13}C NMR ($CDCl_3$) δ: 82.38, d, ^2J(P–C) 7.0 Hz, POCH; 65.97, s, 2 C, OCH_2CH_2O; 35.22, d, ^3J(P–C) 4.3 Hz, 2 C, $POCH(CH_2\text{–}R)_2$. This compound should be used as soon as possible for the next reaction. The phosphate triester was diluted with a few ml of CH_3CN and injected into a pressure bottle and cooled to 0°C. NMe_3 (10 ml of a 40 w/w % solution) in CH_3CN (large excess) was added at once and the mixture was heated in an oil bath at 65°C for 30 h. Upon cooling to 0°C the phosphorylcholine (**1c**) precipitates. The precipitate was further purified by crystallisation from acetonitrile. Subsequent drying in a drying pistol at reduced pressure and 70°C yielded 2.96 g (45%) of a white, very hygroscopic solid; m.p. 164–172°C. ^{1}H NMR ($CDCl_3$) δ: 0.88, t, 6 H, 2 CH_3, 1.25, m, 32 H, $(CH_2)_8$; 1.51, m, 4 H, $OCH(CH_2)_2$; 3.42, s, 9 H, $N(CH_3)_3$; 3.82, m, 2 H, CH_2N^+; 4.11–4.29, m, 3 H, CH_2OP, R_2CHOP. ^{13}C NMR δ: 75.90, d, ^2J(P–C) 6.0 Hz, POCH; 66.34, d, ^2J(P–C) 5.8 Hz, $POCH_2$; 59.73, d, ^3J(P–C) 4.8 Hz, CH_2N; 54.30, s, $N(CH_3)_3$; 35.26, d, ^3J(P–C) 3.2 Hz, $OCH(CH_2)_2$. ^{31}P NMR δ: −0.88. Anal. calcd. for $C_{26}H_{58}NO_4P$: C 65.37, H 11.82, N 2.93, P 6.48; found: C 65.46, H 11.93, N 3.13, P 6.31%.

(1-Hexylheptyl)phosphorylcholine (1a)

M.p. 219–224°C. Anal. calcd. for $C_{18}H_{40}NO_4P$: C 59.15; H 11.03, N 3.83, P 8.47; found: C 58.65, H 10.96, N 3.80, P 8.20%.

(1-Octylnonyl)phosphorylcholine (1b)

M.p. 209–212°C. Anal. calcd. for $C_{22}H_{48}NO_4P$: C 62.67; H 11.48, N 3.32, P 7.35; found: C 62.01, H 11.57, N 3.40, P 7.24%.

Vesicle preparation

Vesicles can be prepared by a variety of standard methods[29]. The present alkylphosphorylcholine vesicles were prepared by the ethanol-injection method[30]. Thus, the alkylphosphorylcholine (5 mg) was dissolved in 50 μl of 96% ethanol. Using a Hamilton microsyringe, small aliquots (40 μl for electron microscopy) of this solution were injected into 1 ml distilled water under stirring.
For the NMR measurements vesicles were prepared by dissolving the surfactant in $CHCl_3$, removing organic solvent under a stream of N_2, and then evacuating all remaining traces of solvent at low pressure for at least 4 h. After addition of 1.0 ml D_2O, the mixture was sonicated for about 10 min at room temperature using a Branson Type B15 sonifier cell disrupter, under a N_2 stream. The mixture was then centrifuged for 30 min in an Eppendorf Type 5414 centrifuge, at 12000 rpm, to precipitate titanium particles originating from the sonifier (pH 5.0–6.0).

Electron microscopy

The samples were examined with a Philips EM 201 electron microscope operating at 60 kV. Carbon-coated Formvar grids, pretreated by glow discharge in 1-aminopentane were used as matrices. Aliquots of solutions containing vesicles were stained with a 1% (w/v) solution of uranyl acetate.

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