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Stabilization and Degradation

Synthesis of Polymerizable Glycerophosphocholines and their Polymerized Vesicles

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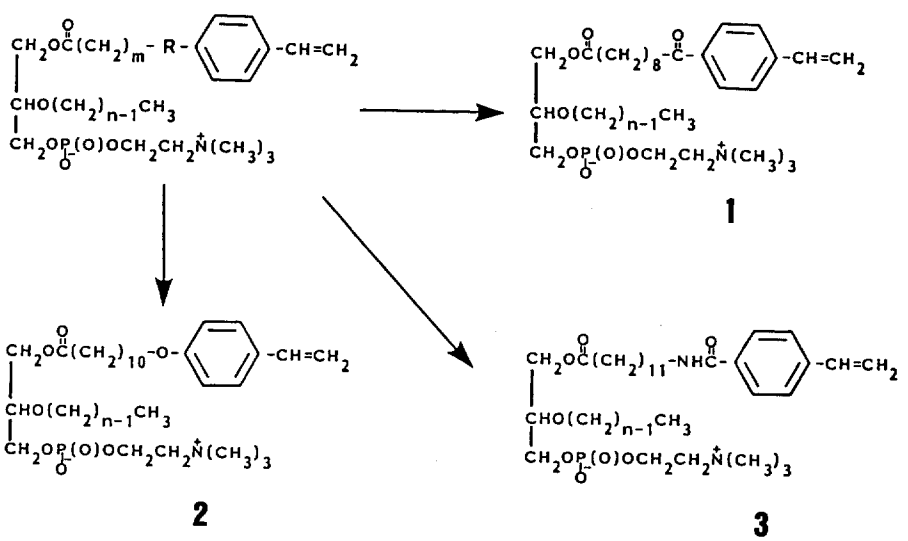
Summary

Three types of polymerizable glycerophosphocholines have been prepared. They have a styrene group through a spacer having a ketone, ether or amide group. The ketone- and ether-type compounds form nonpolymerized and polymerized vesicles depending on the chain length of the alkyl group at the 2-position of glycerol. The polymerized vesicles are stable for months.

Introduction

Phospholipid vesicles have made remarkable progress in their application as model biomembranes (1-3). But their mechanical unstability makes their use for limited purposes (4).

For stabilization of vesicles different ways have been reported (5-11). Among them recent developements by using polymerized vesicles are remarkable (2,7-11). One of them has displayed enhanced mechanical stability (12). Various polymerizable phospholipids having diyne, diene or methacrylate have been reported, but no styrene containing glycerophosphocholines. Recently the authors have found that the glycerophosphocholine having one styrene group with a spacer (1(n=18)) can form polymerized vesicles, but those having



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two styrene groups or one styrene group without a spacer can not (13).

In this paper three types of styrene containing glycerophosphocholines (1(n), 2(n) and 3(n), n=14, 16, 18 and 20) are synthesized and the effect of the R residue and the chain length of alkyl chain at 2-position on the vesicle formation is discussed.

Experimental

Apparatus ^{1}H and ^{13}C NMR, fast atom bombardment mass (FABMS), electronic absorption and infrared spectra were recorded on a JEOL JNM-FX100 spectrometer, a JEOL JMS-DX300 spectrometer, a Shimadzu UV-240 spectrophotometer and a Hitachi 260-50 spectrophotometer, respectively. Transmission electron microscopy was carried out using a Hitachi H-500 by the negative staining method (uranyl acetate). Gel and liquid-crystal phase transitions were measured by a Seiko SSC-560U differential scanning calorimeter (5°C/min).

Methyl 11-(p-vinylphenoxy)undecanoate

In a three necked flask equipped with a calcium chloride drying tube and a dropping funnel sodium hydride (60% in oil, 7.2 g) was washed with 50 ml of n-hexane three times and to the residue was added 300 ml of dry N,N-dimethylformamide (DMF). The mixture was cooled in a ice-cold water bath with stirring. To the solution was added dropwise 19 g (158 mmole) of p-hydroxystyrene dissolved in 300 ml of dry DMF for 25 min and the stirring was continued for further 10 min. 48.6 g (174 mmole) of methyl 11-bromoundecanoate dissolved in 160 ml of dry DMF was then added dropwise to the stirred solution for 30 min. After the reaction mixture was stirred for 90 min, 11 ml of absolute methanol was added. It was filtered and the filtrate was concentrated by evaporation under reduced pressure. The residue was purified by column chromatography on silica gel (benzene/petroleum ether=1/1) and the product was recrystallized from methanol to give 27.9 g of the title compound; yield 55%; TLC(silica gel, benzene) Rf=0.6; MS:318(M^+); IR(KBr): 1730(ester carbonyl), 1620 cm^{-1}(vinyl C=C); Anal. ($C_{20}H_{30}O_3$): C 75.20(75.43), H 9.58(9.49);m.p. 61-61.5°C.

11-(p-Vinylphenoxy)undecanoic acid

Methyl 11-(p-vinylphenoxy)undecanoate (27.7 g) was dissolved in dioxane (845 ml) and to this were added methanol (420 ml) and 2N NaOH (420 ml). It was stirred at room temperature under dark for 15 hr and then the solvents were removed by evaporation under reduced pressure. To the residue were added water (1000 ml) and conc. HCl (84.5 ml). The mixture was stirred for 30 min and the precipitate was collected, washed and dried. It was recrystallized from n-hexane to give 24.4 g of the title compound; yield 90%; m.p. 94-95°C; MS:304(M^+); ir(KBr): 1700 cm^{-1}(carboxylic acid C=O); Anal. ($C_{19}H_{28}O_3$): C 74.86(74.96), H 9.62(9.27).

1-O-[11-(p-Vinylphenoxy)undecanoyl]-2-O-octadecyl-rac-glycerol

In a flask equipped with a calcium chloride drying tube 0.92 g (3 mmole) of 11-(p-vinylphenoxy)undecanoic acid was dissolved in dry dichloromethane (40 ml). To this was added 0.62 g (3 mmole) of dicyclohexylcarbodiimide and the solution was stirred for 3 hr at room temperature under dark. It was filtered and the filtrate was directly added to the dichloromethane solution (100 ml) having 2.07 g (6 mmole) of 2-O-octadecyl-rac-glycerol (14) and 73 mg (0.6 mmole) of 4-(N,N-dimethylamino)pyridine (DMAP). It was stirred for

2 days under dark and then evaporated to dryness under reduced pressure. The residue was purified by column chromatography on silica gel (benzene/diethyl ether=10/1) to afford 0.7 g of the title compound; yield 37%; TLC(silica gel, benzene/diethyl ether=9/1): Rf=0.3; m.p. 54-55°C; MS:631(M+1); IR(KBr): 3430 (OH), 1740 cm^{-1} (ester carbonyl); Anal.($C_{40}H_{70}O_5$): C 76.38(76.14), H 11.19 (11.18).

The compounds with a different alkyl chain (2(n=14,16 and 20)) were prepared by the same procedure described above using the corresponding 2-O-alkyl-rac-glycerol derivatives. M.p., yield and MS data were 39-40°C, 58%, 574(M^+); 42-43°C, 48%, 602(M^+); and 51-52°C, 64%, 659(M+1) for n=14, 16 and 20, respectively.

1-O-[11-(p-Vinylphenoxy)undecanoyl]-2-O-octadecyl-rac-glycero-3-phosphocholine(2(n=18))

To a flask equipped with a calcium chloride drying tube were added 0.6 g (0.95 mmole) of 1-O-[11-(p-vinylphenoxy)undecanoyl]-2-O-octadecyl-rac-glycerol, 10 ml of dry benzene, 0.2 ml (1.4 mmole) of dry triethylamine and 18 mg of DMAP. To the stirred solution was added 0.16 g (1.4 mmole) of 2-chloro-2-oxo-1,3,2-dioxaphospholane (15) and the solution was stirred for 12 hr under dark at room temperature. It was filtered and the filtrate was concentrated under reduced pressure. The residue was dried *in vacuo*. It was dissolved in dry acetonitrile (20 ml) in a stainless steel pressure tube at 60°C for 6 hr. The reaction mixture was evaporated to dryness and the residue was purified by column chromatography on silica gel (chloroform/methanol/water=65/25/4). The desired fraction was collected, dried and then freeze-dried from dry benzene to afford 0.40 g of the title compound; yield 35%. It had the same Rf value on TLC (silica gel,chloroform/methanol/water=65/25/4) as purified egg yolk glycerophosphocholine (Sigma Chem. Co.). FABMS:796(M+1); IR(KBr):1720(ester C=O),1605,1505(phenyl C=C), 1625 cm^{-1} (vinyl C=C); Anal.($C_{45}H_{82}N_1O_8$): N 1.76(1.76); UV(methanol): λ_{max} 258 nm (ε_{max} 1.8×10^4 liter/mole.cm). The proton decoupled ^{13}C NMR spectral data, shown in Figure 1, are in complete agreement with the indicated structure.

The compounds (2(n=14,16 and 20)) with a different alkyl chain were prepared by the same procedure described above except for the use of the corresponding 1-O-[11-(p-vinylphenoxy)undecanoyl]-2-O-alkyl-rac-glycerol derivatives. FABMS, analytical (N) data and yield were 740(M+1), 1.88(1.89), 59%; 768(M+1), 1.76(1.82), 57%; and 824(M+1), 1.69(1.70), 42% for n=14, 16 and 20, respectively. All of the compounds had the same Rf values as purified egg yolk glycerophosphocholine and showed almost the same ^{13}C NMR spectral data as 2(n=18).

1-[9-(p-Vinylbenzoyl)nonanoyl]-2-O-alkyl-rac-glycero-3-phosphocholine (1(n))

The compounds (n=14, 16, 18 and 20) were prepared according to the previous paper (13) using the corresponding 2-O-alkyl-rac-glycerols. FABMS and analytical (N) data were 724(M+1), 1.97(1.93); 752(M+1), 1.82(1.86); and 808(M+1), 1.74(1.73) for n=14, 16 and 20, respectively.

Preparation of nonpolymerized vesicles and their photopolymerization

Glycerophosphocholine was suspended in H_2O, D_2O or phosphate buffer (pH7.0) presaturated with N_2 gas and the mixture was ultrasonicated under N_2 for 15 min in a ice-cold water bath. The almost clear solution was transferred into a quartz NMR tube or UV cell throgh membrane filter (0.45 μm) and then it was sealed under N_2. The vesicle solution thus prepared was irradiated by UV lights using a low vacuum mercury lamp (30w) in a water bath at 50°C for 10-30 min.

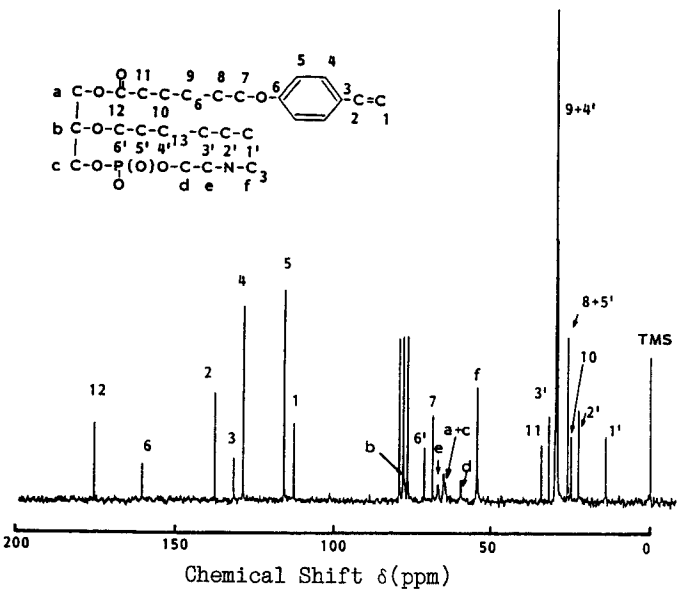
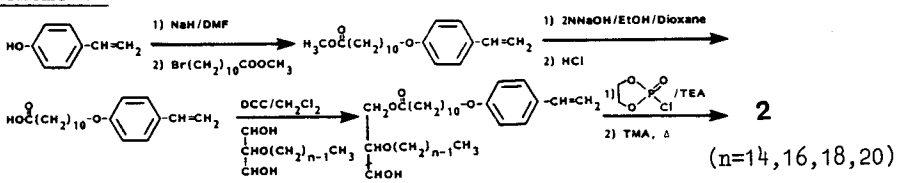
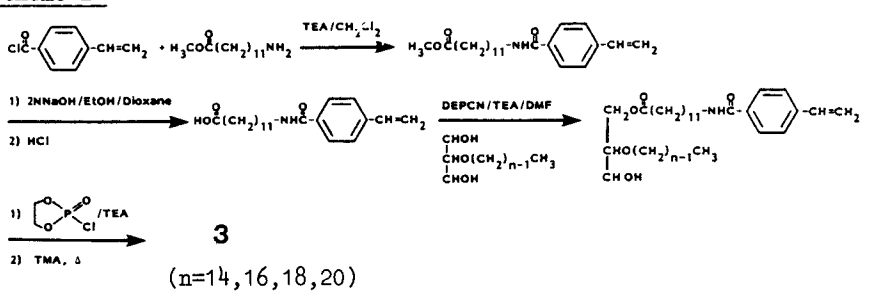


Figure 1. Proton decoupled ^{13}C NMR spectrum of 2(n=18) in $CDCl_3$ (internal standard: TMS)

Scheme 1



Scheme 2



1-O-[12-(p-Vinylbenzamido)dodecanoyl]-2-O-alkyl-rac-glycero-3-phosphocholine (3(n))

The same procedure described for 2(n) using 12-(p-vinylbenzamido)dodecanoic acid in place of 11-(p-vinylphenoxy)undecanoic acid was applied for preparing 3(n) (n=14, 16, 18 and 20). FABMS and analytical (N) data were 781(M+1), 3.48(3.59); 809(M+1), 3.26(3.46); 837(M+1), 3.23(3.35); and 865 (M+1), 3.13(3.24) for n=14, 16, 18 and 20, respectively. UV(methanol): λ_{max} 264 nm(ε_{max} 2.1x10^4 liter/mole.cm); ^{13}C NMR($CDCl_3$,TMS)δ(ppm): 115.7,136.0 (vinyl), 126.2,127.2,133.9,140.3(phenyl), 167.1(amide carbonyl), 173.8 (ester carbonyl), 54.3(choline methyl).

Results and Discussion

Synthesis of styrene containing glycerophosphocholines

Diacylglycerophosphocholines having long acyl chains can form bilayer and the carbon number of acyl chains should be more than 12 (16). Preliminary study on the ketone type styrene containing glycerophosphocholines revealed that those having one styrene group with a spacer can form vesicles but those having two styrene groups or one styrene group without a spacer cannot (13). Therefore, glycerophosphocholines should be designed to have only one styrene residue with a spacer. And it is interesting to elucidate the effect of the R residue situated between a terminal styrene and an acyl chain on vesicle formation. In this communication, therefore, three types of styrene containing glycerophosphocholines are synthesized.

Glycerophosphocholines 2(n) and 3(n) (n=14, 16, 18 and 20) were prepared according to Scheme 1 and Scheme 2, respectively. The ketone type derivatives 1(n) (n=14, 16, 18 and 20) were also synthesized according to the previous paper (13).

All glycerophosphocholines have been characterized by ^{13}C NMR, ir, fast atom bombardment mass and/or N analyses. They are soluble in chloroform and methanol and have the same Rf values on TLC as purified egg yolk glycerophosphocholine.

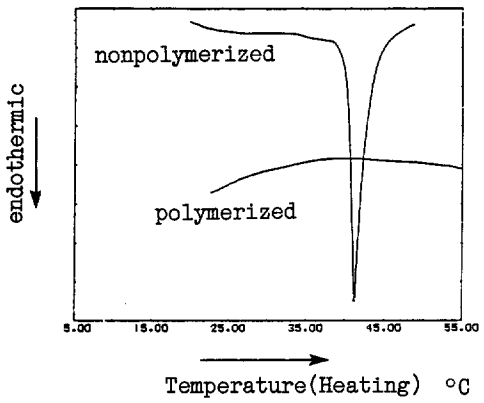


Figure 2. Differential scanning calorimetry (DSC) curves of nonpolymerized and polymerized vesicles of 2(n=18) in H_2O

Nonpolymerized vesicle formation

Vesicle formation of 1(n), 2(n) and 3(n) was studied by measuring the properties of their water dispersions prepared by ultrasonication. For example, 2(n), after ultrasonication, gave nonviscous and almost clear dispersions in H_2O and DSC measurements showed the critical temperature (Tc) due to the gel and liquid-crystal phase transitions (Figure 2) for n=16, 18 and 20, but not for n=14. Tc was 33.1°C, 41.2°C and 41.6°C for n=16, 18 and 20, respectively.

The formation of unilamellar and single-walled vesicle is supported by ^{1}H NMR spectral measurements (13,17,18). The singlet peak characteristic of the choline methyl groups was split by the addition of Eu^{3+} ions (data are not shown, but ^{1}H NMR spectra of polymerized 2 are shown in Figure 4). But no splitting was observed in the case of 2(n=14).

Gel permeation chromatography on Sepharose 4B eluted by the phosphate buffer (pH7.0) (detected by measuring absorbance at 280 nm) supported the formation of small particles (diameter:200-400 A) (19).

From these results, it was found that 2 can form unilamellar vesicles when n is more than 16. By the same measurements on 1, it was found that 1 can form vesicles (n ≧12). But the dispersions of 3(n) (n=14, 16, 18 and 20) (0.5-5 wt/vol%) were viscous and neither ^{1}H NMR spectral nor electron microscopic measurements supported vesicle formation.

Thus, the vesicle formation of the styrene containing glycerophosphocholines merkedly depends on both the structure of R and the chain length of an alkyl group at the 2-position of glycerol.

Polymerized vesicles

The nonpolymerized vesicles of 2 and 1 (n=16, 18 and 20) were irradiated by UV lights. The polymerization was followed by measuring UV absorption spectra. As shown in Figure 3, the absorption band of the nonpolymerized vesicles of 2(n=18) at 254 nm is shifted to 225, 277 and 285 nm after irradiation. The new bands are consistent with those of the model compound 4, i.e. 1-[11-(p-ethylphenoxy)undecanoyl]-2-O-octadecyl-rac-glycero-3-phosphocholine(λ_{max}(ε_{max}) :224nm(1.9×10^4liter/mole.cm),277(1.6×10^3),284(1.4×10^3)).

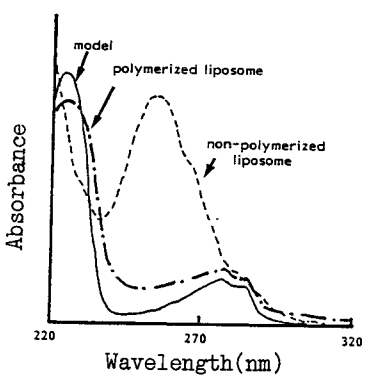


Figure 3. Ultraviolet absorption spectra of the vesicle of 2(n=18) before (-----) and after(— · —) irradiation by UV lights and of the vesicle of the model compound 4 (———) in H_2O

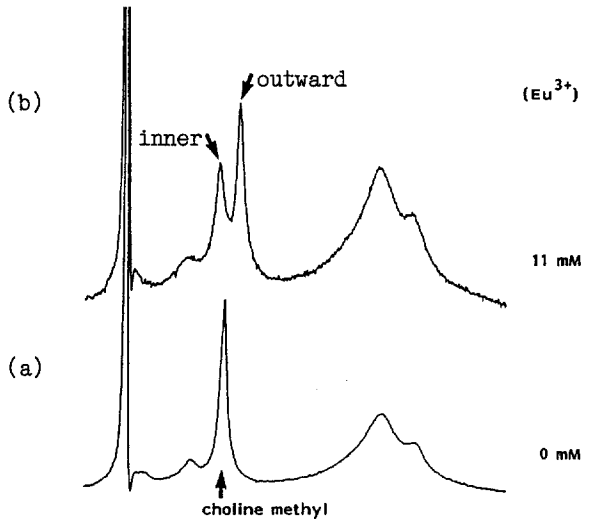


Figure 4. ^{1}H NMR spectra of the polymerized 2(n=18) (a) in the absence and (b) in the presence of $Eu(NO_3)_3$ in D_2O at 50°C. [lipid]=4 wt/vol%

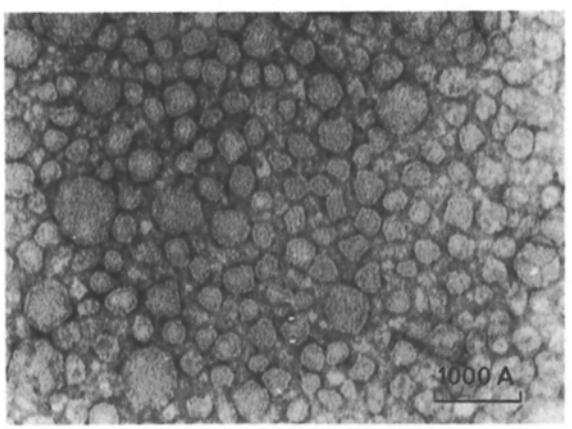


Figure 5. Electron microscopic photograph of the polymerized 2(n=18) negatively stained with uranyl acetate

The photopolymerization of nonpolymerized vesicles of 1(n) (n=16 and 20) was supported from the UV spectral change of the absorption band from 265 to 251 nm as reported for 2(n=18) (13).

The ^{1}H NMR spectral measurements, which showed the outward and inner cholines, supported the formation of unilamellar and single-walled vesicles of the polymerized 2 (Figure 4).

Due to the formation of styrene polymers in bilayer, no phase transition was found in the polymerized vesicles (Figure 2).

The sizes were estimated by negative stain (uranyl acetate) electron microscopy. The electron micrograph of the polymerized 2(n=18), for example, showed the formation of closed particles with average diameters of 340 A (Figure 5).

The suspensions of polymerized 2(n=18 and 20) and 1(n=16, 18 and 20) were stable without any phase separation and the sizes of the particles were constant for months.

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