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Characterization of polymerized vesicles derived from polymerizable 1,3-diacylglycero-2-phosphocholines: formation of large unilamellar vesicles by ultrasonication*

Etsuo Hasegawa[1], Kiyoshi Eshima[1], Yoh-ichi Matsushita[1], Hiroyuki Ohno[2], and Eishun Tsuchida[2]

[1]Tokushima Research Institute, Taiho Pharmaceutical Co., Ltd., Tokushima, 771-01 Japan

[2]Department of Polymer Chemistry, Waseda University, Tokyo, 160 Japan

Summary

A series of polymerizable lipids, 1,3-bis(alkana-2,4-dienoyl)-rac-glycero-2-phosphocholines,and 1-(alkana-2,4-dienoyl)-2-O-alkyl-rac-glycero-3-phosphocholine were synthesized. Their vesicle formation and photopolymerization to form polymerized and single walled vesicles were elucidated. The acyl chain length remarkably affected features of liposome formation. 1,3-Bis(dodeca-2,4-dienoyl)-rac-glycero-2-phosphocholine gave large unilamellar vesicles only by ultrasonication of the suspended powders in water, but the lipids with longer acyl chain(s) formed normal and small unilamellar vesicles.

Introduction

There is an increasing interest in vesicles (liposomes) of natural and synthetic amphiphiles as models for biomembrane and as carriers for biologically active materials (2-4). Fixation and/or stabilization of the liposomal structure by introducing polymerizable group(s) into lipids followed by polymerization in the bilayer have made them useful for practical applications (5).

Different methods of vesicle preparation are known (6,7). For unilamellar liposome preparation, a simple method by the use of ultrasonication is widely used because of the simple and quick methodology (8). This normally gives small unilamellar vesicles (SUV) 20 to 50 nm in diameter with a small amount of multilamellar vesicles (MLV). Large unilamellar vesicles (LUV) are prepared by the special methods, which are injection of etheral lipid solutions into water (9), removal of detergent from mixed detergent/lipid micelles by dialysis (10) and evaporation of reverse phases (11). These give LUV 50 to 200 nm in diameter. Although it has been reported the sponteneous formation of LUV of acidic phospholipids, phosphatidic acids, without sonication (12), no LUV formation has been achieved on neutral phospholipids, phosphatidylcholines, induced by only ultrasonication.

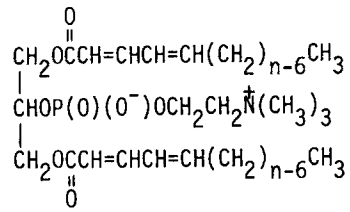
In this communication, we report the synthesis of a series of polymerizable lipids, 1,3-bis(alkana-2,4-dienoyl)-rac-glycero-2-phosphocholines 1(n) and 1-(alkana-2,4-dienoyl)-2-O-alkyl-rac-glycero-3-phosphocholine 2(n,m), and the sizing of their polymerized vesicles which are prepared by sonication followed by uv irradiation.

Results and Discussion

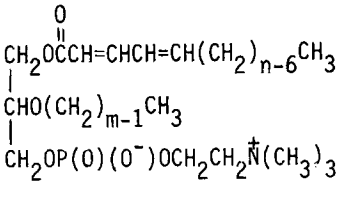
Synthesis of Polymerizable Lipids

Here, two types of polymerizable lipids have been prepared. 1,3-Diacylglycero-2-phosphocholines, 1(n) (n=12,14,16,18), having two polymerizable groups, were synthesized by phosphocholination of 1,3-diacylglycerols which were prepared by acylating glycerol with the corresponding alkana-2,4-dienoyl chlorides in the presence of quinoline. The 1,3-diacylglycerols could be

* See reference 1



1(n) (n=12,14,16,18)



2(n,m) (n=m=18)

purified by column chromatography on silica gel and no contamination of 1,2-diacylglycerol was found by ^{13}C-NMR spectral and TLC measurements of the products. The ^{13}C-NMR data also supported the trans-2,trans-4-dienoyl structure of the acyl chains. 1(n) was sensitive to oxygen and might be polymerized at room temperature. Therefore they were stored under N_2 at -80°C.

2(n,m) having one alkana-2,4-dienoyl group was synthesized by phosphocholination of 1-acyl-2-O-alkylglycerol with the corresponding alkana-2,4-dienoyl chloride in the presence of triethylamine.

The structures of 1(n) and 2(n,m) were determined by ^{13}C-NMR, IR and fast atom bonbardment (FAB) mass measurements and N-analysis. All supported the expected structures.

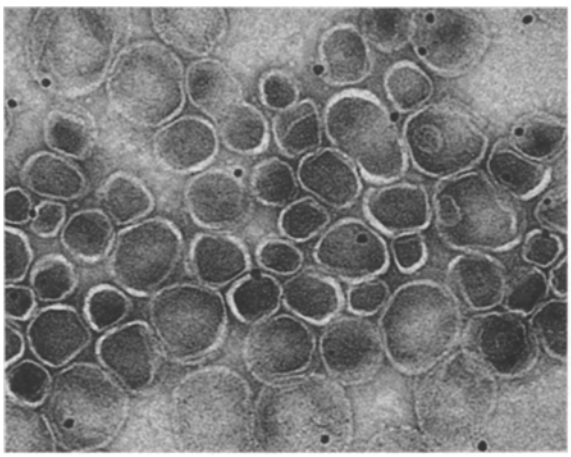
Characterization of Lipid Dispersions and their Polymerized Liposomes

Freeze-dried powder of lipids was dispersed in H_2O or D_2O by ultrasonication using a probe-type microtip under Ar atmosphere. All of the lipid dispersions (4-5 w/v%) was not viscous and could be taken a following examination.

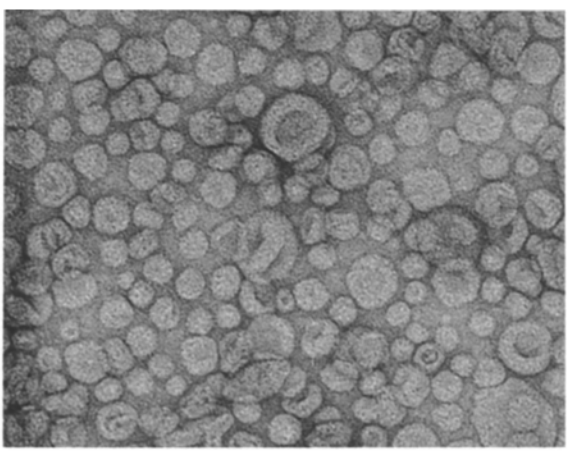
Polymerization was carried out at 50°C under Ar in a quarz cell by irradiation of uv lights.Completion of polymerization was confirmed by the disappearance of the uv absorption at 261 nm corresponding to the dienoyl groups

Characterization of liposomes and their polymerized ones was made by DSC, ^{1}H-NMR and particle size measurements. DSC measurements on non-polymerized lipid dispersions indicated that the gel to liquid crystal phase transition was found on 1(18) (Tc=19.5°C) and 2(18,18) (Tc=40.3°C), but no distinct transitions on other lipid dispersions. ^{1}H-NMR spectral measurements at 50°C, where Eu ions were used as shift reagent, showed the change of the single resonance peak due to the choline methyl groups to the double peak by the addition of the Eu ions for the dispersions of 1(18), 1(16) and 2(18,18), supporting the presence of outward and inner face lipids and no possibility of the Eu ions moving across the lipid bilayers even in their liquid crystalline state. Polymerized 1(n) did not show the signal splitting after addition of Eu ions, while 2(18,18) did.

The size analysis was carried out by three different methods: electron microphotography for negatively stained liposomes by uranyl acetate, gel filtration chromatography by a high performance liquid chromatograph and a quasi elastic light scattering (QELS). The electron microphotos of the polymerized 1(n) as shown in Figure 1(a) and 1(b) for n=12 and n=14, respectively, clearly support the formation of spherical microparticles having a membrane structure of about 5 nm thickness, being equal to those of the well-known lipid bilayers (13). The results of size determinations are summarized in the Table. We used the Coulter N4D size distribution processor which was based on the CNTIN program for constrained solutions of linear equation (14). The results are represented as weight-averaged values, which are more reliable than number-averaged ones. Gel filtration chromatograph was carried out by the use of a combination of two columns(TSK G5000PW and 6000PW).TSKG5000PW was known



(a) ⟵⟶ 100 nm



(b) ⟵⟶ 100 nm

Figure 1. Electron microphotos of negatively stained lipid dispersions of polymerized 1(12) (a) and 1(14) (b).

to be effective to determine radii of dextran up to 20 nm (15). Therefore we used the column combination of TSK G5000PW and TSK6000PW for determination of larger particles. However, larger particles like polymerized 1(12) may be overestimated. But the agreement between the three methods was satisfactory. Polymerized 2(18,18) gave the particles 35 nm in diameter determined by QELS.

Table Average diameters of polymerized liposomes derived from 1(n)

n	Average diameters (nm) ± S.D.		
	Electronphotograph	GFC	QELS
12	104.6±32.1	140.0	85.7±18
14	29.4±13.5	29.5	23.4± 7
16	39.0±17.2	31.0	29.4±10
18	30.1±10.5	31.7	35.3± 7

Electron microphotos of the polymerized 1(n) were analyzed in terms of the size distribution and the results are represented as the bar histogram in Figure 2. The Table and Figures support that 1(n) (n=14,16,18) form particles 20 to 40 nm in diameter which are similar to those of normal SUV, while 1(12) formed larger particles 50 to 200 nm in diameter and the particles had one membrane layer. These support the formation of LUV.

In conclusion, it was found that the 1,3-diacylglycero-2-phosphocholines can form polymerized liposomes (microcapsules) and that 1(12) can form LUV while 1(n=14,16,18) and 2(18,18) can form SUV. But the membranes of polymerized 1(n) are distorted during polymerization allowing Eu ions moving across them. Polymerized 1(12) , LUV, is interesting as carriers of water soluble larger compounds like proteins or enzymes because it can be prepared by a simple method, i.e. by sonication followed by polymerization.

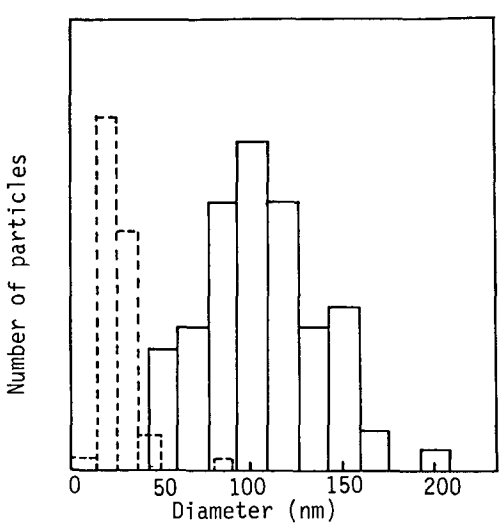


Figure 2. Bar histogram derived from electron microphotos of polymerized 1(12) (———) and 1(14) (-----).

Experimental

Reagents Glycerol was distilled under reduced pressure and the middle fraction was collected. Chloroform was distilled from P_2O_5 immediately before use. Tetrahydrofuran was distilled from sodium/benzoquinone under Ar. Others were synthesized according to the previous papers (16-18).

Apparatus Proton decoupled ^{13}C-NMR, DSC, FAB ms and IR measurements were carried out as described (16-18). A Hitachi H-500 type electron microscope was used. A laser particle analyzer (Coulter N4D, Coulter Electronics) was used for measuring diameters of particles by a quasi elastic light scattering method. Gel filtration chromatographic measurements were carried out by a model LC-4A high performance liquid chromatograph (Shimadzu Co., Kyoto) having the gel set of TSK Gel PWH (7.5x7.5 mm), G5000PW (7.5x600 mm) and G6000PW (7.5x600 mm) by modifying the method of Ollivon et al (19). Standards were purchased from Sigma Chem. Co.: tyroglobulin (diameter 17.0 nm), apoferritin (12.2 nm) and bovine serum albumin (7.0 nm).

Alkana-trans-2,trans-4-dienoic acids

These compounds were prepared according to the literature method for hexadecadienoic acid (20), except the intermediates (esters) being purified by chromatography on silica gel eluted with hexane/ether (15/1) to separate a trans,trans isomer from other stereo isomers. Octadeca-trans-2,trans-4-dienoic acid gave the following analytical data: mp:69°C; IR(KBr): 1690, 1610 and 1635 cm^{-1}; EI ms: 280 (M^+) ($C_{18}H_{32}O_2$, MW 280); ^{13}C-NMR ($CDCl_3$, TMS) δ(ppm): 10.91(1),19.5(2),25.5(12),26.4-26.0(4-11),28.7(3),29.9(13), 115.0(17),125.0(15),143.1,144.4(14,16) and 169.5(18).

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          17  15 13          2
O-CO-C=C-C=C-CCCCCCCCCCCCC
  18   16  14           3 1
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In the similar manner, hexadecadienoic acid (mp:67-68°C), tetradecadienoic acid (53-54°C) and dodecadienoic acid (48.5-50°C) were prepared.

1,3-Bis(alkana-trans-2,trans4-dienoyl)glycerol

Octadecadienoic acid (40.6 g, 145 mmol) was allowed to react with oxalyl chloride (290 ml, 381 mmol) at 60°C for 2.5 h. It was evaporated under reduced pressure and the residue was dried in vacuo.

Anhydrous glycerol (6.67 g, 72.5 mmol) and dry quinoline (36.3 ml) were dissolved in dry chloroform (15 ml) and the solution was cooled to 13-15°C. A dry chloroform solution of the acid chloride (70 ml) was added dropwise to the stirred solution for 1 hr. It was stirred overnight at room temperature and then diluted with ether (2.5 liter). It was filtered and the filtrate was concentrated under reduced pressure. The residue was chromatographed on silica gel (7 liter) eluted with hexane/ether (3/1). The desired fraction was collected, evaporated and dried. It was recrystallized from hexane to give 16.5 g (37%) of 1,3-bis(octadeca-2,4-dienoyl)glycerol: TLC (silica gel, hexane/ether=3/1): Rf=0.27; mp 69-70°C; EI ms 616(M^+); IR(KBr): 3450, 1710, 1610 and 1635 cm^{-1}; Anal.($C_{39}H_{68}O_5$): C 76.07(75.92), H 11.79 (11.11); ^{13}C-NMR ($CDCl_3$,TMS) δ(ppm): 14.1(1),22.7(2),28.7(12),29.7-29.2 (4-11), 33.1(13),31.9(3),65.2,68.5(a,b),118.2(17),128.2(15),145.6,146.2(14, 16) and 167.3(18).

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                O                          2
                ||
O-C-(C-O-C-C=C-C=C-CCCCCCCCCCCCC)2
  b  a    18  16  14           3 1
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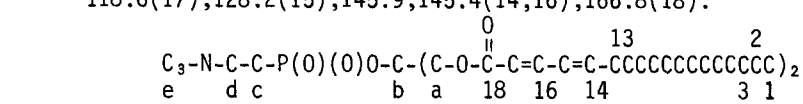
In the same manner, 1,3-bis(hexadeca-2,4-dienoyl)glycerol (mp:60-61°C), 1,3-bis(tetradeca-2,4-dienoyl)glycerol (49-50°C) and 1,3-bis(dodeca-2,4-dienoyl)glycerol (40-41°C) were prepared.

1,3-Bis(alkana-trans-2,trans-4-dienoyl)-rac-glycero-2-phosphocholines 1(n)

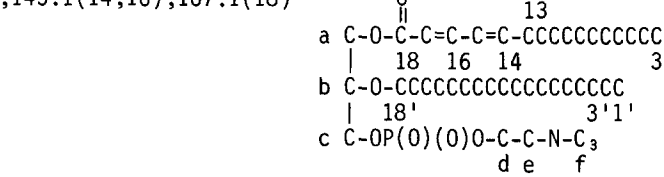
1,3-Bis(octadeca-2,4-dienoyl)glycerol (16.3 g, 26.5 mmol) and dry triethylamine (3.21 g, 32 mmol) were dissolved in dry benzene (380 ml). The solution was cooled in an ice-water bath with stirring. To this was added dropwise 2-chloro-2-oxo-1,3,2-dioxaphospholane (4.56 g, 32 mmol) dissolved in dry benzene (20 ml) for 30 min. N_2 gas was bubbled through the solution and the mixture was stirred overnight at room temperature under dark. A precipitate was removed by suction and the filtrate was evaporated to dryness. The residue was dried in vacuo. This was dissolved in dry benzene/dry acetonitrile (150 ml/150 ml) and was allowed to react with dry trimethylamine (100 ml) in a pressure bottle at 60°C for 4.5 hr, cooled and poured into dry acetone (2 liter). A precipitate was collected and dried. This was dissolved in chloroform/methanol (100 ml/100 ml) and passed through the mixed bed resin column (Bio Rad Lab., AG 501-X8(D), 500 ml). The eluate was evaporated and the residue was freeze-dried from dry benzene to give 13.2 g (64%) of 1,3-bis(octadeca-2,4-dienoyl)-rac-glycero-2-phosphocholine: TLC(silica gel, chloroform/methanol/water=65/25/4): The same Rf value was observed as that of commercial 1,3-dipalmitoyl-rac-glycero-2-phosphocholine; FAB ms: 782$(M+1)^+$; ^{13}C-NMR ($CDCl_3$, TMS) δ(ppm): 14.1(1),22.7(2),28.8(12), 29.7-29.4(4-11),31.9(3),33.1(13),54.3(e),59.5(c),63.3(a),66.4(d),70.5(b), 118.6(17),128.2(15),145.9,145.4(14,16),166.8(18).



In the same manner, 1,3-bis(hexadeca-2,4-dienoyl)-rac-glycero-2-phosphocholine (Anal.($C_{40}H_{72}NO_8P$): N 1.90(1.93); FAB ms: 726 $(M+1)^+$), 1,3-bis-(tetradeca-2,4-dienoyl)-rac-glycero-2-phosphocholine ($C_{36}H_{56}NO_8P$: 2.05(2.09), 670) and 1,3-bis(dodeca-2,4-dienoyl)-rac-glycero-2-phosphocholine ($C_{32}H_{56}NO_8P$: 2.28(2.28); 614) were prepared. They may be purified by silica gel column chromatography (chloroform/methanol/water=65/25/4), if necessary.

1-(Alkana-trans-2,trans-4-dienoyl)-2-O-alkyl-rac-glycero-3-phosphocholine 2(n,m)

2(n,m) was synthesized by applying the method (16,17) to alkana-2,4-dienoyl chloride. The product was freeze-dried from dry benzene. The yield and analytical data of 2(18,18) were as follows: yield: 36% based on 2-O-octadecylglycerol; TLC(silica gel, chloroform/methanol/water=65/25/4): Rf value was the same as that of purified egg yolk phosphatidylcholine; FAB ms: 772$(M+1)^+$; IR(KBr); 1710,1640,1620,1250,1090 and 970 cm^{-1}; Anal. ($C_{44}H_{86}NO_7P$, MW 772.14): N 1,70(1.81); ^{13}C-NMR ($CDCl_3$, TMS) δ(ppm): 14.1 (1,1'),22.7(2,2'),26.1(17'),28.8(12),29.8-29.4(4-11,4'-16'),31.9(3,3'),33.1 (13),54.3(f),59.5(d),64.4(a,c),66.3(e),70.6(18'),76.6(b),118.8(17),128.2(15), 145.6,145.1(14,16),167.1(18)



Preparation of lipid dispersions and photopolymerization

A freeze-dried powder of the polymerizable lipid (200 mg) was added to D_2O or H_2O (5 ml) and the mixture was ultrasonicated by a probe-type microtip (7 mm φ) with a US-600 type ultrasonicator (Nippon Seiko Co.) at 50 W for 10 min under Ar. It was filtered through a membrane filter (0.45 μm, Millex-HA, Millipore Co.). The filtrate was annealed for 30 min at 50°C and then irradiated in a quarz tube under Ar by uv lights at 50°C (16.17).

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