Polymerized Liposomes Formed under Extremely Mild Conditions[1]

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Abstract: This paper describes an efficient synthesis of 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (**1**) and its use in the construction of highly stable polymerized liposomes under extremely mild conditions. Esterification of glycerophosphorylcholine with 12-(tetrahydropyranyloxy)dodecanoic acid affords the corresponding phosphatidylcholine; subsequent deprotection furnishes 1,2-bis(12-hydroxydodecanoyl)-*sn*-glycero-3-phosphocholine (**2**). Esterification of **2** with DL-1,2-dithiolane-3-pentanoic (lipoic) acid anhydride produces a 43% overall yield of **1**. Injection of an ethanolic solution of **1** into 10 mM borate buffer (pH 8.5) and treatment with 10 mol % dithiothreitol at 27 °C yields polymerized liposomes which are completely stable in the presence of 1% sodium dodecylsulfate.

Over the past 6 years, substantial synthetic effort has focused on the preparation of polymerized forms of lipid bilayer vesicles.[3] Phospholipid-based systems (liposomes[4]), in particular, have attracted special attention because of their close structural relationship to biological membranes.[5] In order to maximize their utility for biomechanistic studies (membrane modeling) and for biomedical applications (drug delivery), polymerized liposomes should be prepared under the mildest conditions possible, so that sensitive comembrane and entraped components can be incorporated.

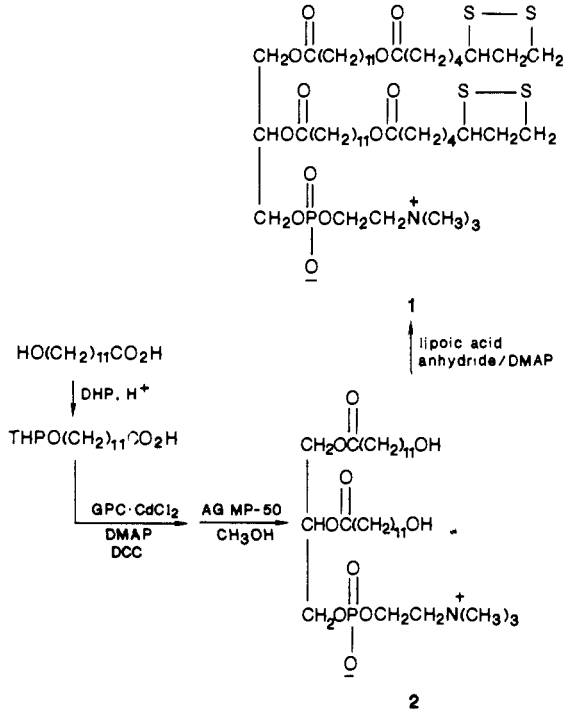
We have recently shown that 1,2-bis(11-mercaptoundecanoyl)-*sn*-glycero-3-phosphocholine can be polymerized in vesicle form via oxidation with hydrogen peroxide.[6] We have also shown that its macrocyclic disulfide analogue is polymerized in the vesicle state through ring-opening polymerization, initiated by using a catalytic amount of dithiothreitol (DTT).[7] In both cases, polymerization proceeds to completion within 4 h at 50 °C, affording *linear* polymers having a maximum number average degree of polymerization of 28. While polymerization results in improved vesicle shelf life, it does not yield membranes that can withstand lysis by sodium dodecylsulfate (SDS).[6]

In this paper we report an efficient synthesis of 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (**1**) and demonstrate its utility in the formation of polymerized liposomes. On the basis of its ease of synthesis, its ability to form highly stable polymerized liposomes under extremely mild conditions, and its potential biodegradability, **1** promises to become *the polymerizable lipid of choice* for a wide variety of mechanistic and practical applications.

Results and Discussion

Synthesis of 1. Scheme I summarizes the synthetic route which has been employed in the preparation of **1**. Esterification of the cadmium chloride complex of glycerophosphorylcholine (GPC·$CdCl_2$) with 12-(tetrahydropyranyloxy)dodecanoic acid, using dicyclohexylcarbodiimide (DCC) as the condensing agent and 4-(dimethylamino)pyridine (DMAP) as the catalyst, produced the corresponding phosphatidylcholine; subsequent deprotection, via ion-exchange-catalyzed methanolysis, afforded a 48% yield of 1,2-bis(12-hydroxydodecanoyl)-*sn*-glycero-3-phosphocholine (**2**). Esterification of **2** with DL-1,2-dithiolane-3-pentanoic (lipoic) acid anhydride in the presence of DMAP resulted in a 90% isolated yield of **1**.

Scheme I



Liposome Formation–Polymerization. Injection of a 20 mM ethanolic solution of **1** into 10 mM borate buffer (pH 8.5), which was 140 mM in NaCl and 2 mM in NaN_3, at room temperature, produced a vesicle dispersion having an average diameter ranging, typically, between 270 and 400 Å (dynamic light scattering); a relatively small population, appearing at ca. 1000 Å, is presumed to be due to vesicle aggregation. Analysis of this dispersion by thin-layer chromatography (TLC) indicated negligible polymerization; i.e., only trace amounts of lipid remained at the origin, and nearly all of the lipid moved with an R_f which was identical with that of **1**. Treatment with 10 mol % dithiothreitol (DTT) resulted in complete polymerization after 4 h at 27 °C. All of the lipid remained at the origin of the TLC plate. Polymerization

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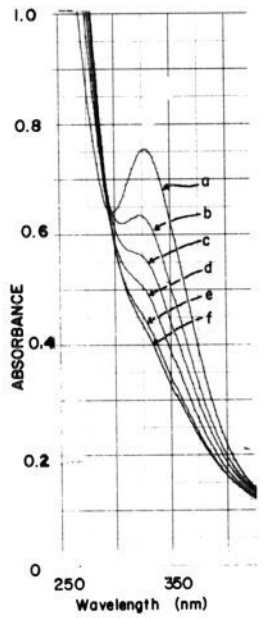

Figure 1. UV spectral changes accompanying the DTT-inititated polymerization (27 °C) of vesicular **1**, as a function of time (min): a, 0; b, 20; c, 50; d, 65; e, 120; f, 220.

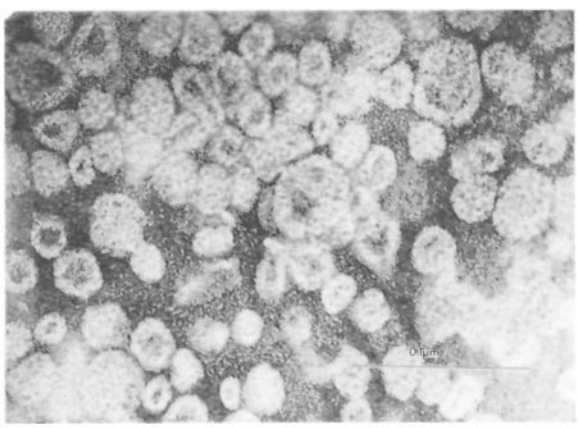

Figure 2. Transmission electron micrograph (2% uranyl acetate) of DTT-polymerized liposomes of **1**; bar represents 1000 Å.

was further supported by the loss of the UV absorption at 333 nm, which is characteristic of the five-membered ring cyclic disulfide (Figure 1).[8] This decrease in absorbance obeyed clean first-order kinetics ($k_{obsd} = 3.9 \times 10^{-4}$ s^{-1}) and was essentially complete (>95%) within 4 h. In the absence of DTT, extensive (but incomplete) polymerization was observed after 72 h at 23 °C or 6 h at 50 °C.

Examination of the polymerized dispersion by light scattering indicated a slight reduction in particle size; i.e., the apparent diameters ranged between 240 and 370 Å. Electron micrographs confirmed the presence of closed vesicles having an average diameter of 320 ± 50 Å; the apparent thickness of the vesicle membrane is estimated to be 75 ± 15 Å (Figure 2). Space filling models (CPK) for **1** predict a maximum lipid length of ca. 38 Å, when the α and β chains are fully extended. Thus, electron microscopy provides strong support for the expected bilayer structure. Gel filtration of these polymerized liposomes, using a Sephadex G 50 column, resulted in a 92% lipid recovery in the void volume (phosphorus analysis).

Solubility, Stability, and Permeability Properties. In contrast to liposomes comprised of low molecular weight disulfide-based phospholipid polymers,[6] freeze-dried polymerized liposomes of **1** are insoluble in chloroform and chloroform/methanol (1/1, v/v). This insolubility provides strong indirect evidence for extensive cross-linking; i.e., each liposome is presumed to be *one* polymer molecule. Moreover, unlike the former, which are readily lysed in 0.6% SDS, polymerized liposomes derived from **1** are completely stable in 1% SDS, even after brief heating at 60 °C (light scattering); nonpolymerized vesicles of **1** are readily destroyed with 0.05% SDS at room temperature. Particle size analysis (Nicomp 270) of the polymerized liposomal dispersion showed no detectable change in size distribution after 3 months of storage at room temperature. Entrapment of [^{14}C]sucrose within polymerized liposomes of **1**, and subsequent dialysis against distilled water, using standard procedures,[9] revealed 61% retention of the marker after 4 h at 23 °C. Under identical conditions, nonpolymerized vesicles retained 32% of the entrapped sucrose.

Experimental Section

General Methods. Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification. House-deionized water was further purified by using a Millipore Milli-Q filtering system containing one carbon and two ion-exchange stages. *sn*-Glycero-3-phosphorylcholine (GPC) was prepared from egg lecithin[10] and converted into its $CdCl_2$ complex (GPC·$CdCl_2$) by using established methods.[6] [^{14}C]Sucrose (400 mCi/mmol, 20% ethanol solution) was obtained from ICN Laboratories. Lipoic acid (Sigma), dihydropyran (Aldrich), and dicyclohexylcarbodiimide (Aldrich) were used as obtained. 12-Hydroxydodecanoic acid (Aldrich) and 4-(dimethylamino)pyridine (DMAP, Aldrich) were recrystallized once from toluene prior to use. Tetrahydrofuran was purified by distillation over sodium benzophenone ketyl. Vesicle dispersions were prepared in 10 mM borate buffer (pH 8.5) containing 140 mM NaCl and 2 mM NaN_3. Chloroform and methanol used for chromatography were reagent grade (Fisher). Dichloromethane (Aldrich, Gold Label) was used as obtained. AG MP50 was obtained from Bio-Rad Laboratories and was purified by extraction with methanol prior to use. ^{1}H NMR, IR, and UV spectra were recorded on JEOL FX 90Q, Beckman Acculab 7, and Bausch & Lomb Spectronic 2000 spectrometers, respectively. Chemical shifts are reported relative to tetramethylsilane. Elemental analyses were performed by Robertson Laboratory, Inc. (Florham Park, NJ). Chromatographic separations were carried out by using precoated Merck 0.25-mm silica gel 60 TLC plates (with fluorescent indicator) and Merck 70-230 ASTM silica gel with the following eluting solvents mixtures: (A) $CHCl_3/CH_3OH$ (9/1, v/v); (B) $CHCl_3/CH_3OH/H_2O$ (4/5/1, v/v/v); (C) $CHCl_3/CH_3OH/H_2O$ (65/25/4, v/v/v). Detection on TLC plates was made by using iodine vapor or phosphomolybdic acid (10% in ethanol). Vortex mixing was carried out by using a VWR Scientific mixer (Model K-550 G). Specific procedures used for electron microscopy were similar to those previously described;[6] 2% uranyl acetate was used as the staining agent. Electron micrographs were recorded by using a Philips 300 microscope. Freeze drying of vesicle dispersions was carried out by using a Virtis freeze dryer. Liquid scintillation was performed with a Beckman instrument, Model LS 5801, using a liquid scintillation cocktail comprised of 70% 1,2,4-trimethylbenzene plus 30% surfactant ("Mini Blend", ICN Laboratories). Dynamic light scattering measurements were carried out by using a Nicomp 270 submicrometer particle analyzer, equipped with a helium–neon laser (632.8 nm, scattering angle of 90°) and a computing autocorrelator. Samples were filtered by using a 0.45-μm HV4 Millipore filter prior to analysis. Phosphorus analysis was performed by using established procedures.[6]

12-(Tetrahydropyranyloxy)dodecanoic Acid. 12-Hydroxydodecanoic acid (2.16 g, 10.0 mmol) was suspended in 20 mL of dry THF. After addition of 1.39 g (16.5 mmol) of dihydropyran, and the solution was stirred for 10 min at room temperature, 20 mg (0.105 mmol) of crystalline *p*-toluenesulfonic acid monohydrate was then added. The mixture became clear immediately, and was stirred for an additional 2 h at room temperature. Evaporation of solvent under reduced pressure afforded a crude product which was purified by flash chromatography ($CHCl_3$) to yield 2.40 g (80%) of 12-(tetrahydropyranyloxy)dodecanoic acid as a colorless oil: R_f 0.5 [silica; $CHCl_3/CH_3OH$ (20/1, v/v)]; IR (neat) $\nu_{C=O}$ 1701, ν_{C-O} 1031 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.25 (s, 14 H, CH_2), 1.40–1.80 (br s, 10 H, CH_2), 2.32 (t, 2 H, $CH_2C{=}O$), 3.38–4.10 (m, 4 H, CH_2O), 4.6 (br s, 1 H, CH), 10.0 (br s, 1 H, CO_2H).

1,2-Bis(12-hydroxydodecanoyl)-*sn*-glycero-3-phosphocholine (2). A mixture of 3.79 g (12.6 mmol) of freshly prepared 12-(tetrahydro-

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pyranyloxy)dodecanoic acid, 1.381 g (3.15 mmol) of GPC·$CdCl_2$, 0.854 g (7.0 mmol) of 4-(dimethylamino)pyridine, and 1.648 g (8.0 mmol) of dicyclohexylcarbodiimide was suspended in 15 mL of dry dichloromethane and stirred under nitrogen in the dark for 40 h. After removal of solvent in vacuo, the residue was dissolved in 50 mL of CH_3OH/H_2O (95/5, v/v) and stirred in the presence of 8.0 g of AG MP-50 (23 °C, 2 h) to allow for complete deprotection of the hydroxyl groups (monitored by thin-layer chromatography).[11] The resin was then removed by filtration and the solution concentrated under reduced pressure. The crude product (2.75 g), obtained after drying [12 h, 23 °C (0.05 mm)], was then subjected to chromatographic purification by using a 30-g (4 × 4 cm) silica gel column, eluting with solvents A and C, to yield 0.990 g (48%) of **2**: R_f 0.25 (solvent C); IR (KBr) ν_{OH} 3390, $\nu_{C=O}$ 1728, $\nu_{N(CH_3)_3}$ 967, 1055, 1090 cm^{-1}; ^1H NMR (CD_3OD) δ 1.30–1.65 (br s, 36 H, CH_2), 2.32 (t, 4 H, $CH_2C{=}O$), 3.22 (s, 9 H, $N(CH_3)_3$), 3.52 (t, 4 H, CH_2O), 3.60–4.55 (m, 8 H, CH_2O, $N(CH_2)$), 4.85 (s, 2 H, OH), 5.2 (m, 1 H, CH).

Lipoic Acid Anhydride.[12] A mixture of lipoic acid (1.03 g, 5.0 mmol) and dicyclohexylcarbodiimide (0.65 g, 3.0 mmol) was stirred in 15 mL of dry methylene chloride for 20 h at room temperature under a nitrogen atmosphere. The product mixture was filtered in order to remove the urea which had formed. Examination of the filtrate by IR revealed the presence of lipoic acid anhydride (1735 and 1805 cm^{-1}) and the absence of the parent carboxylic acid ($\nu_{C=O}$ 1701 cm^{-1}). This solution was used directly in the synthesis of **1** described below.

1,2-Bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine (1). 1,2-Bis(12-hydroxydodecanoyl)-*sn*-glycero-3-phosphocholine (0.04 g, 0.06 mmol) was added to 2.0 mL of a 0.15 M solution of lipoic acid containing 16 mg (0.13 mmol) of 4-(dimethylamino)pyridine. After the mixture was stirred for 6 h under nitrogen at room temperature, thin-layer chromatography (silica, solvent C) indicated complete conversion to **1**. The product mixture was then filtered and concentrated under reduced pressure. The residue was dissolved in 5 mL of solvent B and passed through a 1.2 × 1.5 cm AG MP-50 cation-exchange column in order to remove 4-(dimethylamino)pyridine. The filtrate was concentrated under reduced pressure, dissolved in a minimum volume of absolute ethanol, and then concentrated again. Chromatographic purification of the residue on a silica gel column (0.9 × 6 cm), eluting first with solvent A and then with solvent C (compound **1** elutes on silica as a single yellow band), afforded, after drying [10 h, 22 °C (0.05 mm)], 0.055 g (90%) of **1** as a yellow solid: R_f 0.45 (solvent C); IR (KBr) $\nu_{C=O}$ 1732, $\nu_{N(CH_3)_3}$ 970, 1050, 1090 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.25 (s 28 H, CH_2), 1.40–2.05 (m, 20 H, lipoic-CH_2, CH_2CH_2O, $CH_2CH_2CO_2$), 2.3 (t, 8 H, $CH_2C{=}O$), 2.20–2.65 (m, 4 H, CH_2–lipoic ring), 3.15 (t, 4 H, CH_2SS), 3.40 (s, 9 H, $N(CH_3)_3$), 3.55 (m, 2 H, CHSS), 4.08 (t, 4 H, $CH_2OC{=}O$), 3.80–4.6 (m, 8 H, CH_2O, NCH_2), 5.20 (m, 1 H, $CH(CH_2O)$). Anal. Calcd for $C_{48}H_{88}O_{12}NPS_4$: C, 55.95; H, 8.61; N, 1.36; P, 3.01; S, 12.44. Found: C, 53.85; H, 8.58; N, 1.19; P, 3.01; S, 11.99.

Upon drying, a small and unavoidable percentage (less than 10%) of lipid **1** becomes polymerized on the walls of the glass flask. For storage purposes, the lipid should be dissolved in dichloromethane (0.017 M), filtered (0.2-μm FG Millipore filter), and kept at 0 °C in the dark.

Polymerized Vesicle Formation. Typically, 1 mL of a 0.017 M dichloromethane solution of **1** was concentrated under a stream of nitrogen and dried under vacuum [0.5 h, 23 °C (0.05 mm)]. The lipid was then dissolved in 0.85 mL of absolute ethanol. An aliquot of this solution (90 μL, 1.8 μmol) was then rapidly injected into 0.75 mL of a 10 mM borate buffer (140 mM NaCl, 2 mM NaN_3, pH 8.5) by using a 100-μL Hamilton syringe (22 S gauge). The dispersion was incubated at 30 °C for 30 min under a nitrogen atmosphere, with brief vortex mixing. Polymerization was carried out by injecting an aqueous solution of DTT (17 μL of a 0.01 M solution) directly into the dispersion. To ensure complete polymerization, samples were normally allowed to stand at room temperature for 16 h.

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Silyl Ketone Chemistry.[1] Preparation and Reactions of Silyl Allenol Ethers. Diels–Alder Reactions of Siloxy Vinylallenes Leading to Sesquiterpenes[2]

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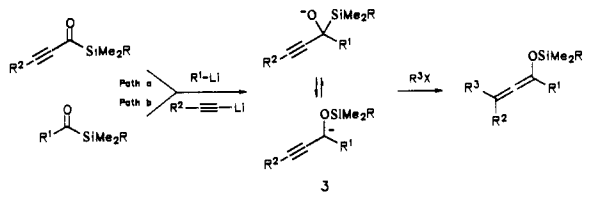
Abstract: Allenol silyl ethers **1** with various substitution patterns were prepared. The allene functionality was introduced either by the alkylation of siloxypropargyllithium reagents **3** (Scheme I) or the β-elimination of 2-halo-1-siloxyallyllithium reagents **4** (Scheme II). In each case the lithium reagent was formed by a [1,2]-sigmatropic rearrangement of a suitable α-silyl alkoxide, which in turn was prepared by addition of alkynyl, vinyl, or other lithium reagents to silyl ketones. The allenol silyl ether **22a** could be halogenated, selenenylated, and cleaved to the lithium allenolate **24**. This vinylic enolate reacts with aldehydes to give aldol products. Vinylallenol ethers **27b** and **28b** were easily prepared by the above methods. Intramolecular Diels–Alder reactions of **27b** and **28b** were key steps in the synthesis of dehydrofukinone (**31**) and selina-4(14),7(11)-dien-8-one (**32**).

Enol silyl ethers are widely used as enol and enolate equivalents both for the purpose of performing chemical reactions and for the masking of carbonyl groups.[3] This is because enol silyl ethers participate in many useful reactions and are in general more easily

Scheme I



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