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Rapid diffusion of the lipid phosphorus of phosphatidylglycerol liposomes through polycarbonate membranes is caused by the oxidation of the unsaturated fatty acids

Ching-Yi Chow, Timothy D. Heath *

School of Pharmacy, University of Wisconsin, 425 North Charter Street, Madison, WI 53706, USA

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Abstract

The lipid phosphorus of phosphatidylglycerol liposomes was found to diffuse extensively, after a lag time of 1 to 2 days, through a 0.1 μm pore size polycarbonate membrane in a two compartment system. Diffusion occurred when either multilamellar or large unilamellar vesicles were studied, even if they were sedimented to eliminate any smaller particles. The lipid of liposomes prepared under sterile conditions also diffused extensively. Diffusion appeared to be related to the age of the vesicles, and could be eliminated by incorporating antioxidants into the liposomes, or by using liposomes prepared from saturated phospholipids (C14 or larger). This indicated that diffusion was caused by phospholipid oxidation, which was confirmed by HPLC analysis. Phospholipid phosphorus that diffused through a membrane appeared more polar, as indicated by its capacity to distribute into the upper phase of a two phase extraction. Phospholipid phosphorus diffusion was preceded by the complete loss of liposomes contents, indicated by the complete diffusion of encapsulated carboxyfluorescein through the membrane. Oxidation of the lipid could be prevented by inclusion of either butylated hydroxytoluene or α-tocopherol in the membrane. The best retention of liposomal contents was achieved when both antioxidants and cholesterol were included in the liposome preparation. The antioxidant incorporated in the liposomes remained effective in protecting the phospholipids upon storage at 4°C for 2 months. The inclusion of EDTA in the suspension medium retarded the rapid oxidation, suggesting that the presence of trace amounts of heavy metal ions in the buffer catalyzed the oxidation. Phospholipid oxidation was most effectively inhibited by the presence of serum or chemically defined medium, suggesting that oxidation of liposomal lipids in a biological environment may be minimized if appropriate steps are taken.

Keywords: Liposome; Oxidation; Drug delivery; Polycarbonate membrane

1. Introduction

Liposomes have been widely studied as carriers for the delivery of drugs and macromolecules to cells [1]. In order to function effectively as a drug carrier, it is important for liposomes to remain sufficiently stable over a reasonable period of time. Liposomes can undergo both physical and chemical changes upon storage. The physical changes that can occur for phospholipid vesicles, especially small sonicated liposomes, include aggregation and fusion upon storage [2–4]. The chemical changes include hydrolysis of the ester bonds of phospholipids in aqueous liposomal dispersion [5,6], and oxidation of phospholipids containing unsaturated fatty acyl chains as well as oxidation of cholesterol [7–10]. All of the above transformations may cause encapsulated materials to leak. After in vivo administration, the stability of liposomes is also a major concern in the biological environment. It is known that liposome contents are released upon association with cells [11] and upon interaction with serum [12,13], even though the mechanism by which leakage occurs are still unclear.

Many pharmaceutical applications of liposomes have employed vesicles, whose lipid content is at least partly

Abbreviations: PG, egg phosphatidylglycerol; PC, egg phosphatidylcholine; DLPG, dilauroylphosphatidylglycerol; DMPG, dimyristoylphosphatidylglycerol; DSPG, distearoylphosphatidylglycerol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; BHT, butylated hydroxytoluene; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; CF, 5(6)-carboxyfluorescein; CH, cholesterol.

* Corresponding author: Fax: +1 (608) 2623397.

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composed of anionic phospholipid. Furthermore, negatively charged liposomes have been shown to deliver drugs to cells more efficiently than neutral liposomes [14]. Egg phosphatidylglycerol (PG) is a common anionic phospholipid for liposome preparation. In the present study, we show that an extensive diffusion of PG liposomes through a 0.1 μm polycarbonate membrane can occur. This unexpected phenomenon appears to be caused by oxidation of the acidic phospholipid, resulting in liposome degradation and extensive diffusion of the phospholipid phosphorus. This phenomenon also induces complete leakage of liposome contents prior to the diffusion of phospholipid components.

2. Materials and methods

2.1. Materials

Phospholipids (Avanti Polar Lipids, Pelham, AL) in chloroform were sealed in glass ampules (Wheaton, Millvile, NJ) under argon and stored at −20°C. Phospholipids used included egg phosphatidylglycerol (PG), egg phosphatidylcholine (PC), dilauroylphosphatidylglycerol (DLPG), dimyristoylphosphatidylglycerol (DMPG), and distearoylphosphatidylglycerol (DSPG). Cholesterol (CH; Sigma, St. Louis, MO) was recrystallized four times in methanol and stored in ampules as described for phospholipids. Water for all experiments was obtained by passing distilled water through a Barnstead PCS water purification system, containing one organic removal cartridge, two ultrapure mixed resin cartridges, a superorganic removal cartridge, and one 0.2 submicron filter. Effluent water was collected with a conductivity of 18 MΩ cm. 5(6)-Carboxyfluorescein (CF; Eastman Kodak, Rochester, NY) was purified by gel permeation chromatography with Sephadex LH-20 (Sigma, St. Louis, MO). Buffer species used included *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 2-(*N*-morpholino)ethanesulfonic acid (Mes; Sigma) and phosphate buffer. All buffers were used at a concentration of 50 mM, and were adjusted to pH 7.2 with 1 M NaOH, and to an osmolality of 290 mmol/kg with NaCl. Osmolality was measured using a vapor pressure osmometer (Wescor 5500XR, Logan, UT). Ethylenediaminetetraacetic acid (EDTA) disodium salt (Sigma) was included in one experiment to chelate heavy metal ions. CF solution for encapsulation was prepared by adding CF to the Hepes buffer described above. The final concentration of CF in the solution was 20 mM. Dulbecco's Modified Eagle Medium and fetal bovine serum were purchased from Gibco (Grand Island, NY). Butylated hydroxytoluene (BHT) and (±)-α-tocopherol were purchased from Sigma. The two compartment system comprised 6.5 mm transwells with 0.1 μm pore size membranes suspended in a 24-well plate (Costar, Cambridge, MA).

2.2. Liposomes preparations

Large unilamellar liposomes (LUV) were prepared in either Hepes buffer or CF solution by the reverse-phase evaporation method [15]. After preparation, the liposomes were passed through a 1 × 15 cm Sephadex G-50 (Pharmacia, Piscataway, NJ) column to remove the unencapsulated materials and any trace of remaining ether.

The phospholipid concentration of the liposome preparation was determined by a phosphorus assay according to Bartlett [16] with some modifications. Briefly, up to 0.1 μmol of phospholipid was mixed with 0.4 ml of 5 M H_2SO_4 in a 20 × 150 mm borosilicate glass tube (Pyrex®, Corning) and heated at 165–170°C on a heating block (Thermolyne, Dubuque, IA) for 30 min. After the mixture was cooled, 30 μl of 30% hydrogen peroxide (J.T. Baker, Phillipsburg, NJ) was added, and the samples were further heated for another 30 min. A 4.6 ml aliquot of 0.22% ammonium molybdate and 0.2 ml of Fiske-SubbaRow reagent (15.00 g sodium metabisulfite, 0.25 g 1-amino-2-naphthol-4-sulfonic acid, and 0.50 g sodium sulfite dissolved in 100 ml water) were added, immediately followed by thorough vortexing and heating for 7–10 min in a boiling water bath. Absorbance at 830 nm was measured on a Beckman DU®-64 spectrophotometer (Beckman, Fullerton, CA). The actual amount of phospholipid was calculated based on a standard curve, with a range of 0–4 μg phosphorus, prepared from KH_2PO_4 solution. The CF encapsulated in the liposomes was measured by diluting the liposome suspension with 1% Triton X-100. CF concentration was measured assuming a molar extinction coefficient of 70 000 at 493 nm.

2.3. Liposome diffusion studies

A liposome dispersion containing 1 μmol phospholipid in 0.15 ml Hepes buffer was placed in four individual transwells for each time point. In some studies, the liposomes contained CF encapsulated at a concentration of 20 mM. To minimize evaporation, experiments were only set up in the center eight transwells and wells in a 24-well plate. The surrounding 16 transwells and well were filled with 0.15 and 0.7 ml water, respectively. Then the plates were placed in a humidified, closed container, which, in turn, was placed on a shaker. At various times, the contents of wells and transwells were collected and used for CF and/or phosphorus determination. Any well solution clinging on the outside of each transwell was recovered by washing twice with 0.15 ml Hepes buffer, which was combined with the well contents. Similarly, each transwell was washed three times with 0.2 ml Hepes buffer after the contents were collected. One transwell and well pair for each time point was assayed first to estimate the amount of sample needed to give appropriate absorbance readings. Then the remaining three sets of transwells and wells were

assayed to determine the amount of phosphorus and/or CF present in the well and transwell (A_{well}, $A_{transwell}$). The results for both CF and phosphorus were expressed as percent diffusion, which is defined by the following equation:

$$\%\ \text{diffusion} = \frac{C_{well}}{C_{eq}} \times 100$$

where C_{well} is the concentration of phosphorus or CF found in the well, and C_{eq} is the concentration expected if the lipid or dye were uniformly distributed throughout the transwell and well. Percent diffusion was calculated from the following equation:

$$\%\ \text{diffusion} = \frac{A_{well}/0.70\,\text{ml}}{(A_{transwell} + A_{well})/0.85\,\text{ml}} \times 100$$

where A_{well} and $A_{transwell}$ are the amount of phosphorus or CF found in the well and transwell, respectively.

2.4. Phospholipid extraction

The procedure of Bligh and Dyer [17] was adapted to extract phospholipid from the liposome preparation. The mixture was thoroughly shaken and centrifuged at 2600 rpm for 10 min on a bench top centrifuge (IEC Model CL Centrifuge, Needham Heights, MA). The lower phase was carefully aspirated from under the upper phase, and the two phases were separately analyzed for their phosphorus content as described above.

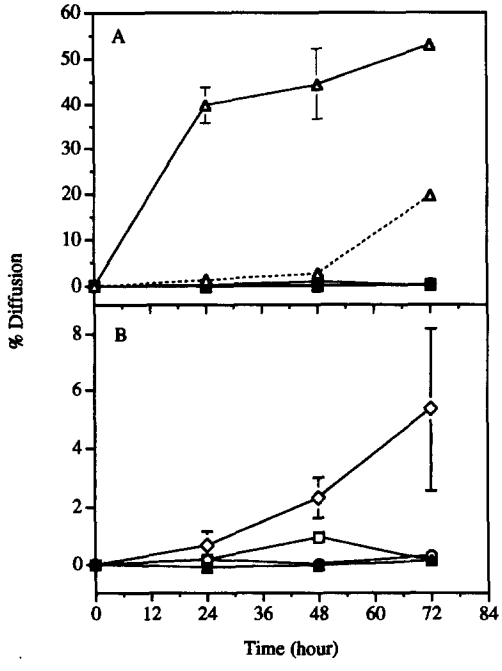

Fig. 1. Diffusion of phospholipid across polycarbonate membranes. (A) Liposome preparations tested include PG/CH (2:1) (△), PC/CH (2:1) (▲), DLPG/CH (2:1) (◇), DMPG/CH (2:1) (○) and DSPG/CH (2:1) (□). (B) The values for PC, DMPG and DSPG liposomes were shown on an enlarged scale. Each value plotted is the average of three determinations and the error bars represent the standard deviation.

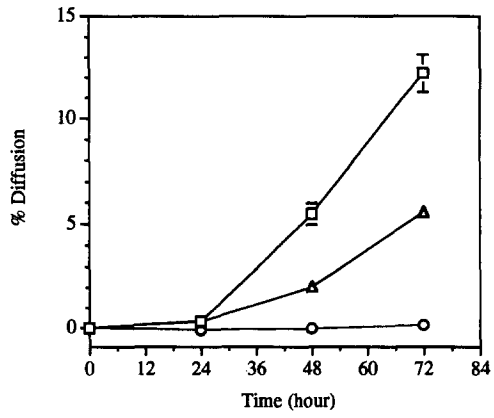

Fig. 2. Diffusion of mixtures of PG and PC. Liposomes were prepared from PG/CH (2:1) (□), PG/PC/CH (1:1:1) (△) and PC/CH (2:1) (○). Each value plotted is the average of three determinations and the error bars represent the standard deviation.

2.5. Thin-layer chromatography (TLC)

Extracted lipids were spotted on 3.5 × 8 cm silica gel TLC plates, 0.2 mm layer thickness (EM Science, Gibbstown, NJ). To separate lysophospholipds from phospholipids, $CHCl_3/CH_3OH/NH_4OH/H_2O$ (69:27:1.5:2.5, v/v) was used as the solvent system. To separate fatty acids, cholesterol and phospholipids, petroleum ether/diethyl ether/acetic acid (60:40:1, v/v) was used. The separation was carried out at room temperature in a 5 × 10 cm glass jar saturated with 10 ml of developing solvents. After the plates were dried with a stream of hot air, the lipids were visualized by exposing the plates to iodine vapor.

2.6. Reverse-phase high-performance liquid chromatography

All HPLC analyses were performed on a Beckman HPLC system including a NEC PC-8300 controller, a System Gold Programmable Solvent Module 116, a System Gold Scanning Detector Module 167, equipped with an Altex 210A injector with 50 μl sample loop.

The mobile phase used to elute phospholipids was composed of 20 mM choline chloride in methanol/water/acetonitrile (90.5:7:2.5, v/v) [18]. Methanol and acetonitrile were HPLC grade OmniSolv® solvents (EM Science, Gibbstown, NJ), and choline chloride was reagent grade (Fisher, Fair Lawn, NJ). The mobile phase was filtered through a 0.45 μm membrane filter (Alltech Associates, Deerfield, IL), degassed under vacuum, and sonicated in a bath-type sonicator (Branson, Shelton, CT) prior to use.

Liposomes, in capped polystyrene tubes, were con-

stantly shaken at 120 rpm in a 37°C incubator. Aliquots of liposome solution were taken at different time points. Extracted phospholipids were dried under reduced pressure on a rotavapor, reconstituted with 250 μl chloroform, and flushed with argon.

Phospholipid extracts were separated on a Beckman 4.6 × 250 mm Ultrasphere® ODS column packed with 5 μm spherical particles, 80 Å pore size, at room temperature, and eluted isocratically with mobile phase at a flow rate of 1.5 ml/min.

Phospholipids were detected by their UV absorbance at 205 and 233 nm with a range of 0.2 AUFS. The two profiles were recorded 1 cm apart from each other simultaneously at a chart speed of 1 mm/min with 10 mV full scale on a Kipp and Zonen BD 41 chart recorder.

3. Results

Fig. 1 shows the diffusion of egg PG through 0.1 μm pore size polycarbonate membranes. Contrary to expectations, a significant amount of the phospholipid phosphorus appears to diffuse through the polycarbonate membranes. There is considerable batch to batch variation in the extent of diffusion. In addition, a lag time, whose length can vary between 1 and 3 days, is often observed. In contrast, virtually no diffusion of lipid phosphorus is observed for liposomes prepared from egg PC, which contains the same fatty acid composition as egg PG (Fig. 1). Similarly, neither DMPG nor DSPG diffuse, indicating that the variable but extensive diffusion phenomenon is specific, not only to the PG headgroup but also to the fatty acid composition of the egg yolk phospholipid. Interestingly, DLPG does exhibit significant diffusion through the membranes.

Fig. 2 shows the diffusion of PG, PC, and PG/PC 1:1, all suspended as 1:1 mixture with cholesterol. As before, the PG diffusion is extensive, while PC diffusion is minimal. Diffusion of PG/PC 1:1 is about half as extensive as the diffusion of PG alone, showing that the extent of diffusion appears to be proportional to the PG content. Various factors were investigated and ruled out as causes of the extensive diffusion of lipid. They will be described here, although, for brevity, the data will not be shown. The diffusion was not appreciably affected by varying the batch size of the liposome preparations. Extensive diffusion was also observed when the cholesterol content of the liposomes was between 0 and 50 mol/100 mol phospholipid. Therefore, rapid diffusion of the phospholipid was not appreciably affected by the presence or absence of cholesterol. Most experiments involved placing the lipid in

Table 1
The distribution of phosphatidylglycerol phosphorus in two-phase extraction following incubation in transwell chambers

	Percent of total phosphorus					
	3 days			7 days		
	well	transwell	total	well	transwell	total
Aqueous	14.04	25.74	39.78	35.13	12.83	47.96
Lipid	1.98	58.24	60.22	11.15	40.89	52.04
Total	16.02	83.98	100.00	46.28	53.72	100.00

1 μmol of PG/cholesterol (2:1, mol/mol) liposomes were placed in transwells and allowed to diffuse for 3 or 7 days. Individual well and transwell contents were extracted by the method of Bligh and Dyer. Results are the mean of three extractions, and are expressed as the percent of total phosphorus in both the well and transwell.

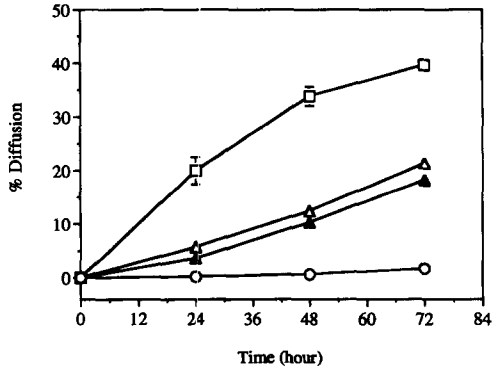

Fig. 3. Effects of shaking, incubation and storage on lipid diffusion. Fresh PG/CH (2:1) LUV liposomes (○) were pre-shaken 7 days (△), pre-incubated 7 days (▲) or stored at 4°C for 2 months (□) before they were subjected to diffusion assays. Each value plotted is the average of three determinations and the error bars represent the standard deviation.

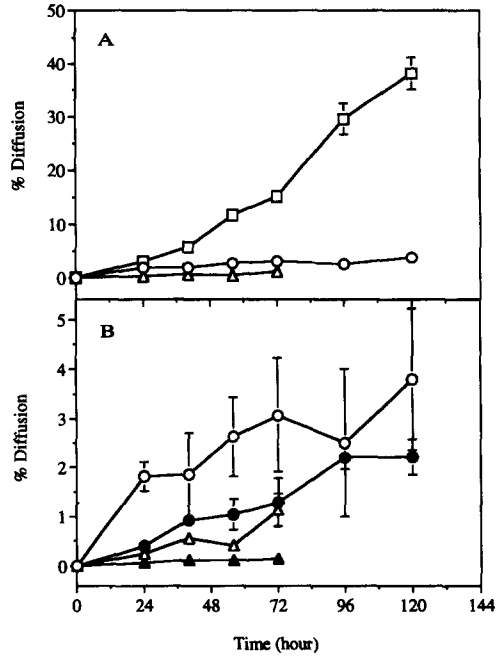

Fig. 4. Effects of antioxidants and cholesterol incorporated in PG liposomes on diffusion studies. PG/α-tocoperol (6:1) liposomes (○), PG/BHT (20:1) liposomes (△), PG liposomes (□), PG/CH/α-tocoperol liposomes (6:3:1) (●), and PG/CH/BHT (20:10:1) liposomes (▲). Each value plotted is the average of three determinations and the error bars represent the standard deviation.

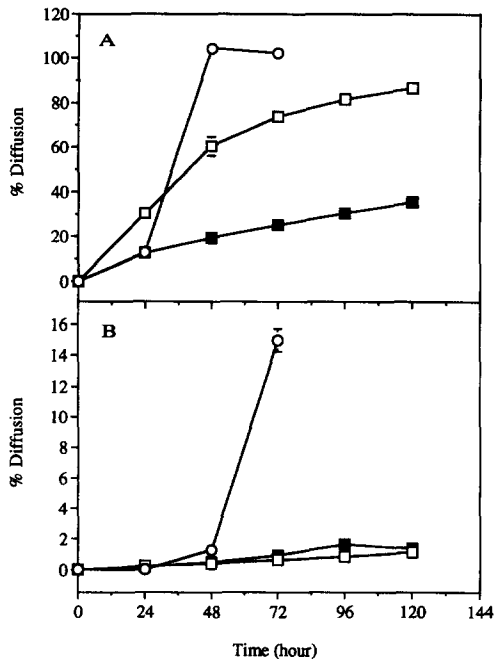

Fig. 5. Retention of carboxyfluorescein in liposomes. Carboxyfluorescein at a concentration of 20 mM was encapsulated in PG/CH (2:1) (○), PG/α-tocopherol (6:1) (□), and PG/CH/α-tocopherol (6:3:1) (■) liposomes. (A) Percent diffusion of encapsulated carboxyfluorescein was determined by its absorbances at 493 nm with the molar absorptivity of 70000 liter/mol per cm after the samples from wells and transwells at different time points were solubilized with 200 μl 1% Triton X-100. (B) Percent diffusion of phosphorus was obtained by phosphorus assays. Each value plotted is the average of three determinations and the error bars represent the standard deviation.

the transwell, but similarly extensive diffusion into the transwell was also observed if the liposomes were placed in the well. Preparation of liposomes by hand shaking of a lipid film with suspension medium to produce MLV also resulted in liposomes, which exhibited high levels of lipid diffusion. Centrifugation of LUV or MLV at 200000 × *g* for 1 h, and the use of the pellet, thereby eliminating any small liposomes, also had little effect on diffusion rates. Finally, if the liposomes were prepared under sterile conditions, high levels of diffusion were also observed. Therefore, diffusion in no way required degradation of the lipid by contaminating organisms.

Having ruled out a number of possible factors, we established the conditions that could affect diffusion. The lag time and the extent of lipid diffusion do appear to depend on the age of the liposomes, as shown in Fig. 3. Liposomes stored at 4°C for two months exhibited more extensive lipid diffusion than freshly prepared liposomes. In addition, liposomes incubated at 37°C for 7 days exhibited more extensive lipid diffusion than freshly prepared liposomes, although the extent of the diffusion was not further increased if the liposomes were shaken at 120 rpm. These results suggest that the extensive diffusion of the lipid is mainly caused by its storage in aqueous medium, and is accelerated by elevated temperature, but not by mechanical agitation.

Table 1 shows the phosphorus distribution of samples obtained from well and transwell compartments at day 3 and day 7 in a Bligh and Dyer extraction. Phospholipid phosphorus is exclusively present in the lower (chloroform) phase when phospholipids are extracted from freshly prepared liposome preparations. However, in this case significant amounts of phosphorus were detectable in the upper (aqueous) phase. Furthermore, the phosphorus was especially abundant in the upper phase for samples obtained from the well compartment. The well content is the material that has diffused through the polycarbonate membrane. These results suggest that the phospholipid has been

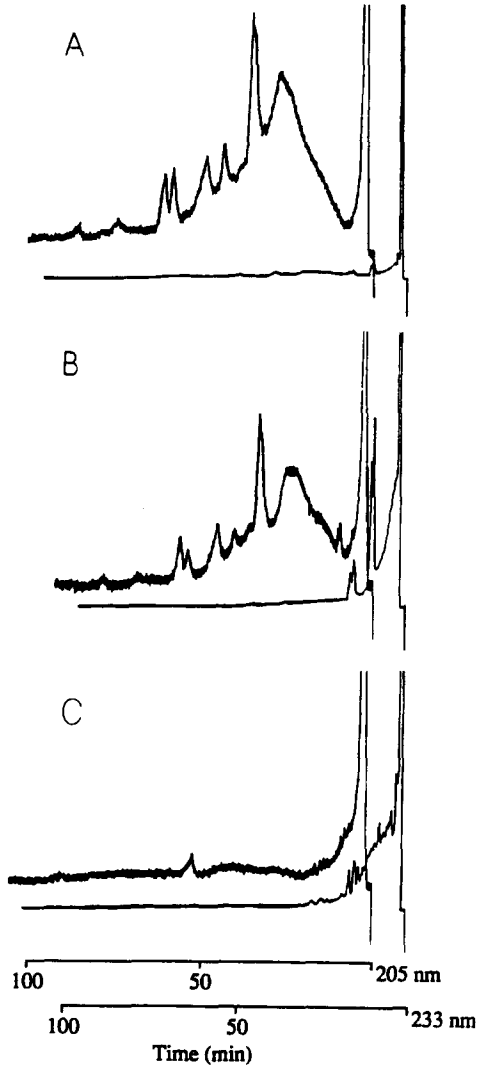

Fig. 6. HPLC chromatographs of PG from PG liposomes in Hepes buffer. PG extracted from LUV liposomes freshly made (A), after 3 (B) and 7 (C) days of incubation at 37°C. PG, separated on an 4.6×250 mm ODS column, was eluted with 20 mM choline chloride in methanol/water/acetonitrile (90.5:7:2.5, v/v) at flow rate of 1.5 ml/min, detected by their UV absorbances at λ = 205 and 233 nm with 0.2 AUFS.

degraded to more polar and highly diffusible phosphorus-containing materials. Moreover, the degradation mostly occurred during the first 3 days of the experiment.

In response to the observations described above, the possible chemical degradation of the phospholipid was examined in a number of ways. TLC analysis of the phospholipids after incubation showed no distinct spots corresponding to lysophospholipids, diglyceride or fatty acids (data not shown). This suggests that hydrolysis was not the main cause of the high levels of phosphorus diffusion. However, plates developed to detect lysophospholipids showed a distinct 'tailing' of the PG spot for the aged material, suggesting that oxidation had occurred. Fig. 4 shows the effects of including the antioxidants, α-tocopherol or BHT, in the liposomes. The lipid diffusion rates were greatly decreased by the incorporation of antioxidant, and further suppressed with the presence of cholesterol. These results suggest that the degradation of the lipid to diffusible phosphorus-containing components is caused by oxidation of the lipid.

In order to examine the fate of both the lipid and the encapsulated contents, a series of liposome preparations were made that contained CF. Fig. 5 shows the diffusion of both the phosphorus and the CF of these preparations through polycarbonate membranes. PG/CH liposomes containing α-tocopherol showed, as one would expect, virtually no phosphorus diffusion. CF was observed to diffuse through the membrane at a rate equal to about 12% of the total captured dye per day, indicating the leakage rate of liposomes associated with this composition. When cholesterol was omitted in the formulation, the diffusion of phosphorus was still negligible, but the initial diffusion rate of CF increased to about 30% per day, implying that the leakage of CF was reduced by the presence of cholesterol in the membrane. For liposomes prepared from PG and cholesterol, the leakage rate of CF was identical to that of PG/CH/α-tocopherol liposomes for the first day. However, between day 1 and day 2 there was a very rapid diffusion, which resulted in a complete diffusion of CF to equilibrium by day 2, suggesting a total release of the dye from the liposomes. In contrast, the diffusion of phosphorus was still insignificant by day 2, and only reached approx. 15% by day 3. These data suggest that the oxidative degradation of lipids resulted in destabilization of the lipid bilayer between day 1 and day 2, such that all contents were released.

Based on the length of the fatty acyl chain and the degree of unsaturation, it is possible to separate phospholipids on an ODS column. Methods have been established to detect simultaneously the underivatized phospholipids and their oxidized products by measuring the absorbance at 205 and 233 nm. Absorbance at 205 nm detects the phospholipids by virtue of the presence of the unsaturated fatty acid, while absorbance at 233 nm detects the oxidation products. Fig. 6 shows the chromatographs of PG extracted from liposomes shaken at 37°C for between 0 and 7 days. PG obtained directly from an ampule and PG extracted from freshly made PG liposomes gave identical profiles (data not shown), indicating that extensive oxidation did not occur during the liposome preparation process. As the liposome incubation period increased, there was a progressive loss of the 205 nm peaks, and by 7 days little

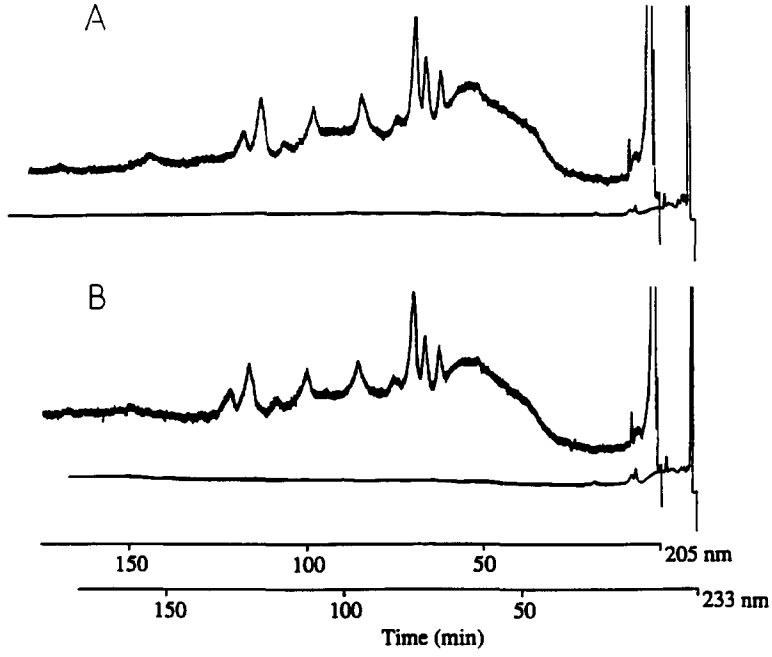

Fig. 7. HPLC chromatographs of PC from PC liposomes in Hepes buffer. PC extracted from LUV liposomes freshly made (A) and after 7 days (B) of incubation at 37°C. PC, separated on an 4.6 × 250 mm ODS column, was eluted with 20 mM choline chloride in methanol/water/acetonitrile (90.5:7:2.5, v/v) at flow rate of 1.5 ml/min, detected by their UV absorbances at λ = 205 and 233 nm with 0.2 AUFS.

205 nm absorbing material remained. The disappearance of the 205 nm peaks during the course of experiment was accompanied by the concomitant appearance of rapidly eluting materials with absorbance at 233 nm, which corresponds to oxidation products. In accordance with diffusion results, all the HPLC profiles of PC at different time points remained the same (Fig. 7), showing no evidence of PC oxidation when PC liposomes were incubated at 37°C for up to 7 days. Liposomes with incorporated antioxidant were also analyzed by HPLC after 0, 3 and 7 days incubation at 37°C. The chromatographs of PG/BHT are shown in Fig. 8. BHT was eluted following the solvent front. The lipid peaks remained unchanged after 7 days of experiment. Similarly, the lipid peaks obtained from PG/α-tocopherol liposomes also remained, although α-tocopherol had a longer retention time and therefore co-eluted with some of the phospholipid peaks (data not shown).

Studies were carried out to discover if other factors could affect lipid oxidation, and the results are summarized in Table 2. All experiments described to this point were carried out in Hepes buffer. As Fig. 6 and Table 2 show, oxidation of PG in Hepes buffer was significant after 3 days and complete after 7 days. Substitution of a phosphate buffer did not prevent PG oxidation, although the rate of oxidation is somewhat slower. Substitution of Mes/Hepes buffer for Hepes alone did substantially reduce PG oxidation, which is surprising in view of the oxidation observed in Hepes alone. Mes may protect the lipid by itself being susceptible to oxidation. Oxidation was also substantially reduced by the inclusion of 1 mM EDTA in Hepes buffer. The oxidation of PG was also investigated in cell culture medium, both with 10% serum and with a defined (serum-free) supplement that was previously developed for drug delivery studies [19]. No oxidation of PG was observed over 7 days in either media.

Table 2
Oxidation of phosphatidylglycerol liposomes

Incubation medium	Extent of oxidation	
	3 days incubation	7 days incubation
Hepes buffer	++	++++
Phosphate buffer	–	+++
Mes/Hepes	–	+
Hepes/EDTA	–	+
Medium + 10% serum	–	–
Defined medium	–	–

Liposomes were incubated for 3 or 7 days before HPLC analysis to determine oxidation and residual phospholipid content. No change in chromatograph as compared to freshly made liposome, –; detectable oxidation at 233 nm, +; significant increase in 233 nm peaks and decrease in 205 nm peaks, ++; extensive oxidation with only 3 peaks at 205 nm remaining, +++; extensive oxidation with only 1 peak at 205 nm remaining, ++++.

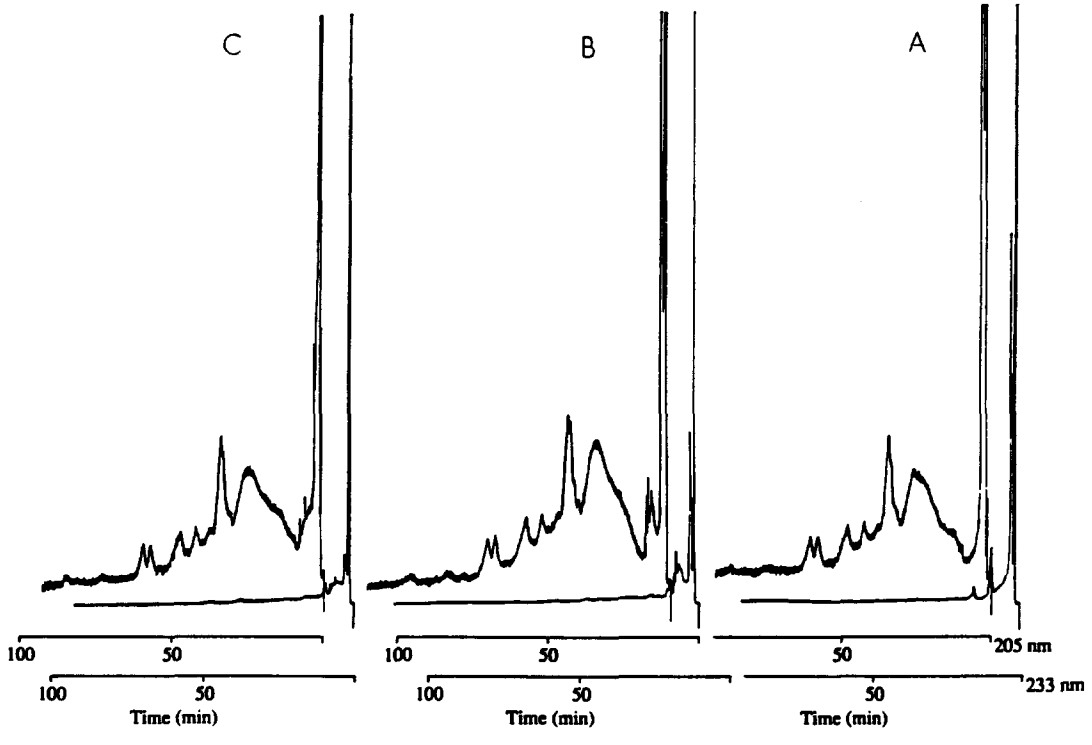

Fig. 8. HPLC chromatographs of PG from PG/BHT liposomes in Hepes buffer. PG extracted from freshly made PG/BHT (20:1) LUV liposomes (A), and from liposomes after 3 (B) and 7 days (C) of incubation at 37°C. PG, separated on an 4.6 × 250 mm ODS column, was eluted with 20 mM choline chloride in methanol/water/acetonitrile (90.5:7:2.5, v/v) at flow rate of 1.5 ml/min, detected by their UV absorbances at λ = 205 and 233 nm with 0.2 AUFS.

4. Discussion

The study here described demonstrates that the lipid phosphorus of liposomes prepared from phosphatidylglycerol is apparently capable of diffusing extensively through 0.1 μm pore size polycarbonate membranes. Based on the known size of such preparations, it seems unlikely that such liposomes would diffuse intact through the membranes. Moreover, such diffusion disagrees with the observations of Ng and Heath [20]. Our experiments have shown that the level of lipid diffusion does not appear to be affected by the mechanical agitation of liposomes, ruling out the possibility of physical instability as a cause for the substantial diffusion. In addition, the polarity of the phospholipid is somehow increased as demonstrated by the appearance of phosphorus in the upper phase of a Bligh–Dyer extraction. These facts indicate a possibility of chemical degradation of the phospholipid, which may result in a breakdown of liposome structure. TLC studies show no evidence for the production of lysophospholipids or diglycerides, but do show a characteristic 'tailing' of the phospholipid spot. Therefore, degradation presumably involves the fragmentation of the fatty acyl chains through oxidative degradation of the double bonds.

Experiments to determine the diffusion of both the phospholipid and the encapsulated contents show clearly that the liposome bilayer becomes permeable to aqueous contents at least 24 h prior to the measurable diffusion of phospholipid. This is demonstrated by the complete loss of contents from PG/CH liposomes between days 1 and 2, while lipid only begins to diffuse in the same experiment by day 3. However, the exact mechanism of destabilization, and the form in which the lipid diffuses through the membrane is unclear. We envisage two possible mechanisms. In the first, the chemical degradation of the phospholipid increases its critical micelle concentration, allowing it to diffuse out of the bilayer and through the polycarbonate membrane. Its diffusion out of the bilayer would destabilize the latter and allow for leakage of contents. In the second mechanism, the chemical degradation of the phospholipid to shorter chain lipids may result in a transformation of the phospholipid structures from a liposomal to a micellar form. This transformation would also destabilize the bilayer and produce smaller, diffusible particles. Electron microscopy of PG liposomes that have been stored at 4°C and shaken at 37°C for up to 7 days shows the presence of 0.1–0.5 μm liposomes together with much smaller particles of about 10–20 nm in diameter (data not shown). These smaller particles are not present in fresh liposome preparations. This observation seems to favor the second possible mechanism, although it does not rule out a possible involvement of lipid in the monomeric form. Interestingly, DLPG, a saturated lipid that cannot be oxidized, does diffuse appreciably, suggesting that oxidized PG whose chain length is reduced to 12 carbon or less may exhibit diffusion.

Oxidation of phospholipids has been extensively studied, although most studies have focussed on phosphatidylcholines [21–26]. The extreme sensitivity of PG to oxidation as compared to a PC of identical fatty acid composition has not previously been observed and seems surprising. The observation that the use of EDTA, serum, or defined medium, eliminates the oxidation would appear to suggest that the oxidation is brought about by heavy metal ion contamination. If this is so, the sensitivity of the negatively charged PG to oxidation may result from the binding of metal ions to the headgroup of the phospholipid, thereby increasing the effective concentration of the radical-forming metal ions. Heavy metal ion contamination most likely comes from the reagents used to prepare buffer solutions, rather than the water or lipids themselves. The latter two sources seem unlikely, because the water is of known high purity, and the lipid shows no oxidation on storage for extended periods at −20°C.

Whatever mechanism may be involved, these results point to a need for caution in formulating liposomes for drug delivery. Even when prepared as mixtures with PC, PG appears to be susceptible to oxidation and loss from the liposomes. This will have an adverse effect on the stability and properties of commonly used liposome formulations containing unsaturated PG, and possibly other unsaturated anionic phospholipids, if they are not appropriately protected. The results of lipid oxidation are not only deleterious to the liposomes, but may lead to adverse biological effects if such liposomes are used therapeutically [27,28].

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