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# PHOTOPOLYMERIZED PHOSPHOLIPID VESICLES

## STABILITY AND RETENTION OF HYDROPHILIC AND HYDROPHOBIC MARKER SUBSTANCES

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**Multilamellar vesicles were formed from photopolymerizable analogs of phosphatidylcholine. The polymerized vesicles differed markedly from conventional vesicles in terms of their stability to mechanical and chemical perturbations. Thus, polymerized vesicles, but not conventional ones, retained their overall physical integrity subsequent to ultrasonication or to exposure to organic solvents or detergents. Treatment of photopolymerized vesicles with detergents such as sodium dodecyl sulfate caused the release of entrapped hydrophilic solutes; however, lipophilic solutes were retained by the polymerized vesicles under circumstances in which conventional vesicles were completely solubilized. Thus, photopolymerized phospholipid membranes represent hybrid entities which seem to blend some of the characteristics of conventional lipid bilayers with properties more typical of polymer membranes.**

## Introduction

Phospholipid vesicles or liposomes have been extensively investigated as models for biological membranes [1]. Liposomes have also become an important tool for biotechnology. Thus, a number of groups have used liposomes as a system for the controlled delivery of drugs [2,3], while others have utilized lipid vesicles in technologies for photochemical solar energy conversion [4]. An inherent property of liposomes composed of natural phospholipids is their instability in the face of chemical and physical perturbations; this instability may limit their utilization in some circumstances. Recently, several groups have prepared synthetic surfactants [5] or phospholipid analogs [6–10] which can form bilayer membrane vesicles and then be induced to undergo polymerization. These polymerized bilayers represent interesting hybrid entities which may possess desirable attributes of both natural bilayer membranes and artificial polymer membranes. In this report we investigate the stability characteristics of multilamellar vesicles prepared from photopolymerizable analogs of phosphatidylcholine. We examine the retention of overall vesicle structure using light-scattering observations; we also examine the ability of the vesicles to retain both hydrophilic and lipophilic radioactive marker substances. Vesicles in either polymerized or nonpolymerized form are exposed to mechanical perturbations such as ultrasonication, or to chemical perturbations such as treatment with organic solvent, detergents or serum proteins. These investigations demonstrate that some types of photopolymerized vesicle

Abbreviations: DPL, 1,2-bis(methacryloyloxy)dodecanoyl-L-α-phosphatidylcholine; MPL, 1-palmitoyl-2-(methacryloyloxy)dodecanoyl-L-α-phosphatidylcholine.

are considerably more stable than conventional ones, especially with respect to the retention of lipophilic marker substances.

## Methods

*Preparation of vesicles*

The photopolymerizable phosphatidylcholine derivatives 1,2-bis(methacryloyloxy)dodecanoyl-L-α-phosphatidylcholine (designated dipolymerizable lipid or DPL) and 1-palmitoyl-2-(methacryloyloxy)dodecanoyl-L-α-phosphatidylcholine (designated monopolymerizable lipid or MPL) were synthesized as previously described [6]. The material was stored dry at −20°C, under $N_2$, in the dark until needed. Multilamellar liposomes were prepared by dissolving the lipids in chloroform/methanol, drying the lipids on to the wall of a glass tube by vacuum evaporation, adding $H_2O$ and then allowing liposomes to form during gentle vortexing while maintaining the temperature at 50°C. In some cases, water-soluble marker substances such as [$^3$H]sucrose were encapsulated by adding them to the water phase during liposome preparation; in other cases, lipophilic marker substances were incorporated in the vesicles by mixing them with the lipid prior to solvent evaporation. Small unilamellar vesicles were prepared by extensive sonication of larger vesicles prior to polymerization. These procedures are essentially identical to those used to form vesicles from conventional phospholipids [11,12] and no particular problems were encountered with the photopolymerizable analogs. Polymerization was accomplished by exposing samples in a quartz tube to intense ultraviolet radiation in a Rayonet Photochemical Reactor; illumination for 30–45 min resulted in complete polymerization. The polymerization of these lipids has been documented previously by infrared and NMR analysis [6]. In these studies, we evaluated the polymerization reaction by thin-layer chromatography; the lipid monomers migrated with an $R_F$ similar to dipalmitoylphosphatidylcholine, while polymerized lipids remained entirely at the origin. Free and liposomal marker substances were separated by column chromatography on Sephadex G-50.

*Light scatter*

Effects of organic solvents or detergent on the overall physical integrity of polymerized or nonpolymerized vesicles was assesed by mixing equal volumes of a vesicle suspension and the agent of interest and then measuring light scatter at 400 nm in a spectrophotometer.

*Retention of marker substances*

The ability of vesicles to retain hydrophilic markers such as [$^3$H]sucrose or lipophilic markers such as [$^{14}$C]cholesterol oleate, [$^3$H]dipalmitoylphosphatidylcholine (DPPC) or [$^{14}$C]stearic acid was assesed as follows. Radiolabelled vesicles were exposed to various perturbations such as detergents, ultrasonication or protein containing solutions; the remaining intact vesicles were sedimented at 10000 × $g$ for 20 min and the pellet was rinsed, dissolved in Aquasol (New England Nuclear) and counted in a β-counter. Thus, several processes, including true release and solubilization, formation of very small nonsedimentable vesicles and exchange processes, could all conceivably contribute to the estimate of the amount of marker released; we have made no attempt to discriminate these various events in the present study.

*Material.*

Radioactive compounds including [$^3$H]sucrose (spec. act. = 10.8 Ci/mmol), [$^3$H]dextran ($M_r$ 10000, spec. act = 176 mCi/g), [$^{14}$C]cholesterol-oleate (spec. act. = 58 mCi/mmol), [$^3$H]DPPC (spec. act. = 37 Ci/mmol) and [$^{14}$C]stearic acid (spec. act. = 57.6 mCi/mmol) were obtained from Amersham. Lipids other than the photopolymerizable derivatives were from Avanti Polar Lipids. The detergents sodium dodecyl sulfate, deoxycholate, Brij 56, Triton X-100 and X-45 were obtained from Sigma. All solvents were reagent grade and were obtained from Fisher Scientific.

## Results

We encountered no difficulties in preparing photopolymerized multilamellar vesicles, using essentially conventional means [11,12]. A variety of water-soluble or lipophilic markers could readily be entrapped in the liposomes. Vesicles composed of MPL readily entrapped [$^3$H]sucrose with ef-

ficiencies comparable to those of conventional vesicles [12].

*Mechanical perturbations*

We have examined the ability of polymerized and nonpolymerized vesicles of DPL to retain lipophilic ([$^3$H]DPPC) and hydrophilic ([$^3$H]sucrose) markers when subjected to the intense hydrodynamic forces generated by a probe-type ultrasonic generator. Thus, as seen in Fig. 1, non-polymerized vesicles are rapidly destroyed by sonication; both radiolabelled markers are lost from the liposome pellet and indeed the liposomal suspension visible clears as the large MLVs are converted to smaller structures. The release of [$^3$H]sucrose probably represents simple efflux of soluble marker from disrupted liposomes; the loss of [$^3$H]DPPC is unlikely to represent a true solubilization, since this marker is water-insoluble; rather, it may represent the conversion of large vesicles to so-called 'limit vesicles' (small unilamellar vesicles) which do not pellet under the conditions of the experiment. By contrast, polymerized DPL vesicles do not clear appreciably upon sonication; they retain most of the [$^3$H]DPPC marker and a substantial amount of [$^3$H]sucrose. These results seem to indicate that the size profile of the original vesicle population is largely preserved and that a fraction of the population (or possibly a compartment within the population) retains its impermeability to sucrose, even upon extensive sonication.

*Chemical perturbations*

When conventional liposomes are diluted into solutions containing water-miscible organic solvents such as short-chain alcohols, the vesicles partially or completely dissolve (depending on the solvent concentration) and the suspension clears. Thus, by monitoring the light scatter of liposome suspensions one can obtain at least a semiquanti-

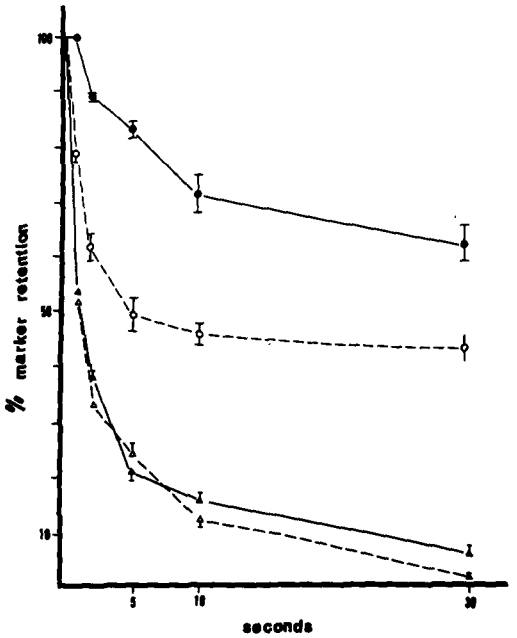


Fig. 1. Release of markers from vesicles by sonication. Polymerized (circles) or non-polymerized (triangles) vesicles of DPL containing either [$^3$H]sucrose (open symbols) or [$^3$H]DPPC (filled symbols) were briefly sonicated with a Heat Systems W375 probe-type instrument at full power. The residual liposomes were pelleted by centrifugation at 10000 × *g* for 20 min and the remaining radioactivity in the pellet was determined. Results represent the means and standard errors for triplicate determinations. The lipid concentration was 1 mg/ml. Ordinate, % marker retention; abcissa, time of sonication.

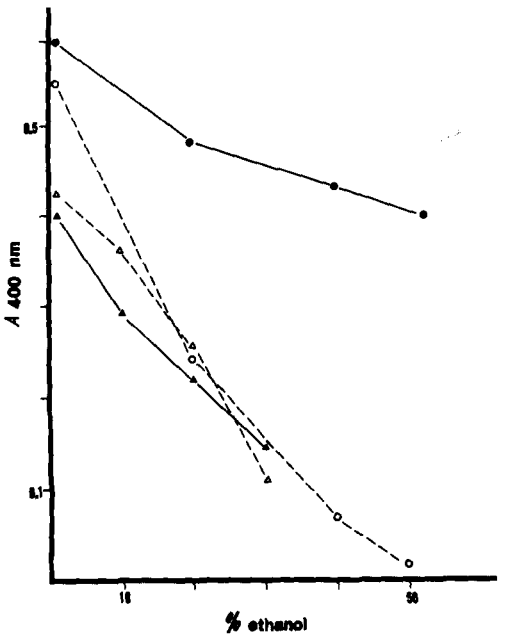


Fig. 2. Solubilization of vesicles by ethanol. Polymerized (filled symbols) or non-polymerized (open symbols) vesicles of DPL (circles) or MPL (triangles) were mixed with an equal volume of an ethanol/water solution. After 15 min at room temperature the $A_{400}$ was determined. The lipid concentration was 1 mg/ml.

tative evaluation of the structural integrity of these particles. In our studies we have used this approach to compare conventional liposomes and polymerized and non-polymerized vesicles composed of DPL or MPL. As seen in Fig. 2, the light scattering of liposomal suspensions of DPPC, and non-polymerized DPL or MPL all markedly decreased as the solvent (ethanol) concentration was raised. Somewhat surprisingly, polymerized vesicles of MPL behaved in a similar fashion; although the the lipid was clearly polymerized (measured by TLC), apparently the lipid polymers (which are not crosslinked with this derivative) remained soluble in ethanol. By contrast, suspensions of polymerized DPL remained quite turbid; indeed, one can even resuspend polymerized DPL vesicles in chloroform/methanol without evidence of solubilization (suspension remains turbid). Thus the highly cross-linked structure formed by DPL resists solubilization by organic solvents.

We have also investigated, in a preliminary way, the stability of DPL vesicles in serum. As seen in Table I, both polymerized and non-polymerized DPL multilamellar vesicles are fairly stable to release of [$^3$H]DPPC or [$^3$H]sucrose in the presence of 10% serum. Conventional multilamellar vesicles display a similar degree of stability in serum, whereas small unilamellar vesicles are much less stable [13]; we have not investigated the stability of DPL small unilamellar vesicles in the present studies.

One of the most striking differences we have noted between conventional and photopolymerized liposomes concerns their relative stabilities in the presence of detergents. Treatment of vesicles composed of conventional phospholipids or of non-polymerized MPL or DPL with ionic or non-ionic detergents leads to a reduction in the turbidity of the vesicle suspension and a complete release of either hydrophilic or hydrophobic marker substances. However, treatment of polymerized DPL vesicles with detergents gives quite a different picture. For example, as shown in Fig. 3a, treatment of polymerized DPL vesicles with SDS does not result in a reduction in turbidity, even at extremely high detergent concentrations. Thus, these vesicles retain their overall structure in detergent solution; one can even visualize typical vesicle profiles, by electron microscopy, in polymerized DPL samples treated with SDS and then subject to gel filtration to remove detergent (data not shown).

The permeability of both polymerized and non-polymerized DPL vesicles to hydrophilic markers such as [$^3$H]sucrose is markedly increased by exposure to low concentrations of detergent (Fig. 3b). However, the extremely lipophilic marker [$^{14}$C]cholesterol oleate is completely retained by polymerized DPL vesicles at concentrations of detergent which would lead to its complete solubilization from conventional or non-polymerized vesicles. Other lipophilic markers such as [$^3$H]DPPC or [$^{14}$C]stearic acid are partially retained by polymerized vesicles in the presence of detergent. To a first approximation, the ability of the detergent to extract marker substances from the polymerized vesicles seems to be inversely re-

TABLE I

VESICLE STABILITY IN SERUM

Multilamellar vesicles of DPL in polymerized (P DPL) or nonpolymerized (N DPL) form were incubated with 10% calf serum at 37°C for the indicated times and the release of entrapped marker was determined as described in Methods.

| Incubation (h) | Marker retention (%) | | | |
|---|---|---|---|---|
| | P DPL | | N DPL | |
| | [$^3$H] sucrose | [$^3$H] DPPC | [$^3$H] Sucrose | [$^3$H] DPPC |
| 0 | 100 | 100 | 100 | 100 |
| 1 | 95 | 100 | 79±1 | 100 |
| 3 | 81±2 | – | 65±7 | – |
| 5 | 75±6 | 92±5 | 59±1 | 87±5 |
| 15 | – | 85±3 | – | 80±2 |
| 24 | 65±2 | 72±3 | 44±3 | 70±7 |

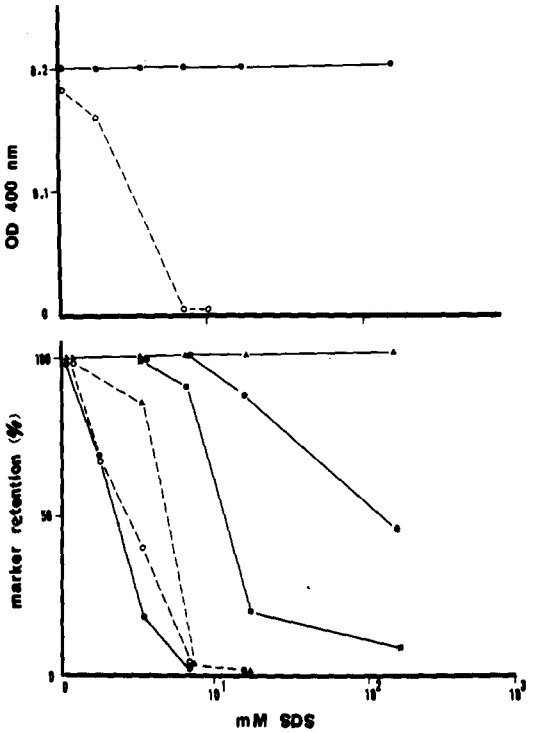


Fig. 3. Detergent effects on vesicle integrity and marker release. Multilamellar vesicles were prepared from DPL; in some cases the vesicles contained [$^3$H]sucrose as a marker for the aqueous phase or [$^{14}$C]cholesterol oleate, [$^3$H]DPPC or [$^{14}$C]stearic acid markers for the lipid phase. Polymerized and non-polymerized samples of DPL vesicles (1 mg/ml) were treated with various concentrations of SDS for 60 min at room temperature. At this time, the absorbance at 400 nm was measured, or the samples were centrifuged at 10000 × *g* for 20 min and the amount of radioactivity remaining in the liposomal pellet was measured. The results shown are the means of triplicate determinations. (a) Turbidity: polymerized DPL, ●———●; non-polymerized DPL, O- - -O. (b) Retention of marker: (P, polymerized; N, non-polymerized). N DPL sucrose, O- - - O; P DPL sucrose, ●———●; P DPL stearic acid, ■———■; P DPL DPPC, ◒———◒; P DPL cholesterol oleate, ▲———▲; N DPL stearic, DPPC, cholesterol oleate, △- - -△.

lated to the hydrophobicity of the marker. Thus, detergents such as SDS can apparently cause defects in the membranes of polymerized vesicles which lead to the leakage of encapsulated water-soluble markers. The size of these defects ('pores') must be substantial, since preliminary experiments indicate that [$^3$H]dextran ($M_r$ 10000) leaks as readily as sucrose. By contrast, the detergent does not readily extract lipophilic markers which must tend to bind to the polymer matrix.

The failure of SDS to extract the lipophilic

TABLE II

EXTRACTION OF LIPOPHILIC MARKERS FROM POLYMERIZED VESICLES BY SOLVENTS

Pellets of polymerized (P DPL) or nonpolymerized (N DPL) vesicles containing the indicated radioactive marker were extracted with 5 ml chloroform/methanol (1:1.) The samples were then repelleted by centrifugation at 10000 × *g* for 20 min and the amount of radioactivity extracted into the supernatant was determined. Results (%) are means of triplicate determinations

| Marker | P DPL | N DPL |
|---|---|---|
| [$^{14}$C]Cholesterol oleate | 84 | 98 |
| [$^3$H]DPPC | 82 | 92 |
| [$^{14}$C]Stearic acid | 88 | 92 |

markers from polymerized DPL vesicles is not due to the formation of covalent bonds between the marker and the lipid during the course of the polymerization reaction. Thus, as seen in Table II, almost all of the [$^{14}$C]cholesterol oleate, [$^3$H]DPPC or [$^{14}$C]stearic acid can be extracted from the polymerized liposomes by organic solvents. [$^3$H]DPPC incorporated into either polymerized or non-polymerized DPL vesicles retains the $R_f$ typical of unmodified phosphatidylcholine upon thin-layer chromatography.

It should be noted that some detergents are much more effective than SDS in extracting lipophilic markers from photopolymerized liposomes. The extraction takes place, however, in the absence of detectable changes in turbity; this is in sharp contrast to the case of non-polymerized vesicles, where extraction and dissolution of the vesicles go hand in hand. As seen in Table III, Triton X-100 and Triton X-45 extract [$^3$H]DPPC as readily from polymerized vesicles as from non-polymerized ones. By contrast, Brij 56, SDS and deoxycholate are considerably more active on non-polymerized vesicles than on polymerized ones. No apparent relationship between the detergent's chemical structure, ionic nature or CMC and its ability to extract polymerized vesicles was noted in this limited series of studies.

## Discussion

Our studies have demonstrated that photopolymerized DPL vesicles display substantially more

TABLE III

RELEASE OF [$^3$H]DPPC FROM POLYMERIZED OR NON-POLYMERIZED DPL VESICLES BY DETERGENTS

Polymerized (P DPL) or non-polymerized (N DPL) multilamellar vesicles containing [$^3$H]DPPC were incubated with 10 ml of various concentrations of detergents. After 1 h at room temperature the residual vesicles were pelleted by centrifugation and the amount of radioactivity in the pellet determined. Curves of % [$^3$H]DPPC released versus detergent concentration were constructed for each detergent. Concentrations required for 20% and 50% release of marker were estimated by linear interpolation. Critical micelle concentrations (CMC) were from Refs. 4,14.

| Detergent | CMC (mM) | Concentration required for release (mM) | | | |
|---|---|---|---|---|---|
| | | 20% release | | 50% release | |
| | | P DPL | N DPL | P DPL | N DPL |
| Deoxycholate | 5 | 12 | 6 | 20 | 8 |
| SDS | 8 | 25 | 4 | 130 | 5 |
| Triton X-100 | 0.24 | 0.25 | 0.25 | 0.35 | 0.35 |
| Triton X-45 | 0.11 | 0.5 | 0.6 | – | – |
| Brij 56 | 0.002 | 1 | 0.1 | – | – |

stability than conventional vesicles or photopolymerized vesicles prepared from MPL. This includes enhanced physical and chemical stability with overall retention of size and structure during hydrodynamic shear (sonication) or exposure to organic solvents. It also includes the interesting property of being able to retain hydrophobic marker substances in the presence of high concentrations of certain detergents. On the other hand, polymerized DPL vesicles do not retain water-soluble markers upon exposure to detergents any better than do conventional liposomes. The substantially different behavior of photopolymerized liposomes composed of MPL versus DPL may stem from the fact that the latter compound is bifunctional and can form cross-linked polymers in the bilayer, while the former compound in monofunctional and can form only a linear polymer [6].

At this point, it seems premature to speculate as to how the properties of polymerized DPL or similar compounds might contribute to their eventual practical application. However, it seems clear that the advent of polymerizable lipids and the consequent ability to generate extremely thin yet quite stable membrane barriers may find utilization in a number of interesting ways in biological research and applied biology.

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