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Triggered release of hydrophilic agents from plasmalogen liposomes using visible light or acid

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Triggered release from liposomes composed of semi-synthetic 1-alk-1'-enyl-2-acyl-*sn*-glycero-3-phosphocholine (plasmalogen) lipids has been demonstrated using either aerobic visible illumination or low pH to induce leakage. The photodynamic release system consists of three functional components: (1) small (< 1000 Å) unilamellar plasmalogen vesicles (SUVs) containing encapsulated glucose, (2) oxygen and (3) zinc phthalocyanine (ZnPc) incorporated within the hydrophobic region of the SUV membrane. Irradiation ($\lambda > 640$ nm) at 37°C of air-saturated 1-alk-1'-enyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (PlasPPC)/1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (8:1, mol/mol) liposomes at physiologically relevant temperatures results in glucose release rates that are twice those of the corresponding dark control. Photolysis of argon-saturated PlasPPC/DPPC liposomes or of identical vesicles lacking either ZnPc or the plasmalogen vinyl ether bond exhibit glucose release curves which are indistinguishable from the dark control. Irradiation under identical conditions, but in the presence of 100 mM sodium azide, also results in no increased rate of glucose release above that of the dark control. TLC analysis indicates that oxidized lipid species are produced only in air-saturated, irradiated plasmalogen liposomes. The acid lability of the plasmalogen vinyl ether linkage has also been used to trigger release of entrapped calcein. At pH 4.2, the release rate at 37°C is increased 4-fold over rates observed at pH 8. TLC analysis indicates formation of a lysoplasmalogen product. Taken together, these results indicate that both photodynamic and acid triggering can be used to increase plasmalogen liposome permeability and suggest that these liposomes are potentially useful for drug delivery applications.

Introduction

Encapsulation of drugs in liposomes to produce site-specific delivery systems has been widely discussed [1–4]. Results of early in vitro studies have led to Phase I clinical trials of liposome-encapsulated doxorubicin, amphotericin B, vincristine, indomethacin, and several other drugs [5,6]. Doxorubicin test results have shown enhanced circulation times, target tissue accumulation, and therapeutic index when delivered in liposome form; however, much of the accumulated drug remains entrapped within intact vesicles near the tumor site [7–9]. A general mechanism for rapid and complete release at the target tissue, therefore, is needed for both hydrophobic (e.g., doxorubicin, amphotericin B [9]) and lyophobic (e.g., methotrexate-γ-aspartate, 5-fluoroorotate [5]) materials, to increase the effectiveness of this therapy.

Although a variety of mechanisms have been described which increase the leakage rate of compartmentalized substances from liposomes [10–13], few reports of drug release triggered by visible or UV light have appeared [14–20]. Since light intensity and modulation are externally controllable variables that can be manipulated as required by the targeting strategy, phototriggered drug release using fiber optic sources [21], in principle, can provide a level of pharmacological control that is not easily obtained with many biochemical targeting methods. Photochemically induced liposome leakage should cause release of the encapsulated material with a relatively high degree of site specificity and preclude the tissue necrosis that often attends bolus-type direct injections.

Our approach to photostimulated drug delivery is based on the well-known peroxidation of membrane

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Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-3-phosphocholine; DSC, differential scanning calorimetry; PC, phosphatidylcholine; PlasPPC, 1-alk-1'-enyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; SUV, small unilamellar vesicle; ZnPc, zinc phthalocyanine.

lipids by Type I and/or Type II photodynamic sensitization [22–27]. Lipid photooxidation via sensitization of intact biomembranes [25–34], PC liposomes [22,29, 33,35–37], and Chinese hamster ovary cells [38–39] is well documented. In these systems, photodynamic sensitization has led to oxidation of unsaturated alkyl chains, increased permeability of the membrane, and release of entrapped reagents.

Plasmalogen lipids form unilamellar vesicles having membrane size and dynamic properties similar to diacyl phosphoglycerides [40–45]. Photoinduced plasmalogen vesicle leakage, based upon cleavage of the labile *sn*-1 vinyl ether linkage, is potentially well suited as a targeting and triggering mechanism for therapeutic applications. The advantages of this system are severalfold:

(1) The photooxidative instability of the vinyl ether linkage may be exploited to trigger the delivery system. The close proximity of the vinyl ether cleavage to the PC headgroup of the plasmalogen is expected to result in a more rapid, efficient disruption of liposome integrity during formation of fusogenic lysolipid photoproducts than would be produced from oxidation of unsaturated sites buried deeper within the bilayer [46].

(2) The irradiation wavelength required for drug release is dictated by the absorption spectrum of the liposome-bound sensitizer. Photostimulated liposome disruption by wavelengths having relatively high tissue penetration and low competitive absorption by the therapeutic agent, made possible by an appropriate choice of sensitizer, leads to liposome oxidation at wavelengths which are non-destructive to the drug or neighboring tissues.

(3) The acid-sensitivity of the vinyl ether bond may also be exploited to make plasmalogen liposomes potential delivery agents to areas of decreased pH, i.e., the gastrointestinal tract, or as a triggered release system when combined with an enzyme-substrate complex which decreases the local pH [47]. This method complements other pH-sensitive liposomal delivery systems that release their contents within the acidic environment of cellular lysosomes and endosomes [48–52].

We have developed a plasmalogen-based controlled release system wherein hydrophilic agents can be discharged from liposomal carriers via both triggering pathways – visible light or low pH (Fig. 1). Acid- and light-triggered liposome leakage rates, oxidation products, photooxidative stability of ZnPc, and other basic parameters required for the rational development of this delivery system are described.

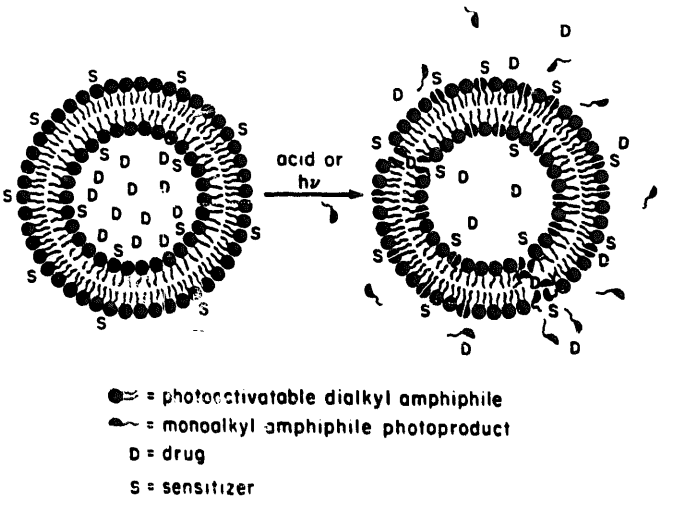


Fig. 1. Conceptual view of triggered liposomal drug delivery.

Materials and Methods

Chromatographically purified calcein was purchased from Molecular Probes. Palmitic anhydride (American Tokyo Kasei), *N,N,N',N'*-tetramethylphenylenediamine, and 98 + % ZnPc (Aldrich) were used as received. 4-(Dimethylamino)pyridine (DMAP, American Tokyo Kasei) was recrystallized from ether. NADP, ATP, hexokinase, and glucose-6-phosphate dehydrogenase were purchased from Sigma. DPPC (lyophilized powder) and bovine heart PC were obtained from Avanti Polar Lipids. DPPC and bovine heart PC showed single spots upon 1-dimensional silica TLC ($CHCl_3$/ MeOH/NH_4OH (65 : 35 : 6, v/v)). Lipids were stored under argon at −20°C. $CHCl_3$ and benzene were dried by distillation from P_2O_5 and CaH_2, respectively. Silica was pre-washed with $CHCl_3$/MeOH (1 : 1, v/v) and dried at 110°C before use.

Steady-state absorption spectra were recorded on a Hewlett-Packard 8452A UV-vis diode array spectrophotometer. Differential scanning calorimetry (DSC) was performed using a Perkin Elmer DSC 7/Intercooler capable of subambient operation. Sample and reference were heated at 2–5°C/min, typically from 5 to 75°C. Fluorescence measurements were conducted on a Perkin-Elmer MPF-66 fluorometer equipped with a thermostated sample compartment. ^{1}H-NMR spectra were recorded on a General Electric QE-300 spectrometer with tetramethylsilane as internal standard.

Preparation of 1-alk-1'-enyl-sn-glycero-3-phosphocholine

Lysoplasmenylcholine was prepared by alkaline deacylation of bovine heart phosphatidylcholine (0.05 M KOH in MeOH/benzene) and purified by flash chromatography on silica using sequential step gradients of $CHCl_3$/MeOH [53a]. Fractions containing pure lysoplasmalogen product were combined, concentrated, and lyophilized from benzene. Yields ranged from 50 to 60%, based on plasmalogen content of the natural lipid source. The purified lysoplasmenylcholine was a single spot by TLC in $CHCl_3$/MeOH/NH_4OH (65:35:7, v/v) and $CHCl_3$/MeOH/H_2O (65:35:5, v/v).

Preparation of 1-alk-1'-enyl-2-palmitoyl-sn-glycero-3-phosphocholine (PlasPPC)

Lysoplasmenylcholine was acylated by minor modification of the procedure described by Guivisdalsky et al. [54]. Lysoplasmenylcholine (213 mg; 0.44 mmol), palmitic anhydride (917 mg; 1.9 mmol), and DMAP (60 mg; 0.49 mmol) were combined with 10 ml of freshly distilled, ethanol-free $CHCl_3$ and stirred at room temperature under argon for 24 h. The reaction mixture was filtered through a pad of Celite, and the solvent removed under reduced pressure. The crude material was dissolved in 95:5 $CHCl_3$/MeOH and purified by silica gel flash chromatography using sequential step gradients of $CHCl_3$/MeOH (95:5, 90:10, 80:20, 60:40, and 40:60 (v/v)). Fractions containing pure PlasPPC were combined, concentrated, and lyophilized from benzene to yield PlasPPC (274 mg, 87%) that was one spot by TLC (R_f = 0.52 in $CHCl_3$/MeOH/NH_4OH, 65:35:6). ^{1}H-NMR ($CDCl_3$): 5.91 (d, J = 6.16 Hz, 1 H, -OCH=C-), 5.34–5.14 (m, 1.65 H, vinyl H and O-C=CH), 4.42–4.32 (m, 3 H, *sn*-2 CH and $POCH_2$), 4.02–3.78 (m, 4 H, *sn*-1,3 CH_2), 3.5 (s, 2 H, NCH_2), 3.38 (s, 9 H, $N(CH_3)_3$), 2.24 (t, J = 7.48 Hz, 2 H, $-OCOCH_2$), 2.02 (m, 2 H, allylic CH_2), 1.28 (m, 49–50 H), 0.9 (t, 6H, ω-CH_3).

Photoinduced release

PlasPPC/DPPC (8:1 mol/mol) vesicles for photodynamic studies were prepared by combining dry DPPC with a $CHCl_3$ solution of PlasPPC and drying to a lipid film with an argon stream. The lipid was then dried under vacuum for 1–2 h to remove entrapped solvent. An ethanolic stock solution of ZnPc was prepared by dilution from a 5 mM pyridine solution of the sensitizer. 100–200 μl of the ethanolic ZnPc was added to 2.8 ml of 20 mM Tris (pH 8) containing 165 mg of glucose (0.3 M), and the solution combined with the lipid film. The suspension was repeatedly warmed to 45°C, vortexed, and put through five freeze-thaw cycles. Finally, the suspension was extruded through stacked 0.10 μm (five passes) and another five times through stacked 0.08 mm Nucleopore filters using a Lipex Biomembranes Extruder thermostated at 48°C. Unentrapped sensitizer and glucose were removed by passage down a small, buffer-equilibrated Sephadex G-25 column. UV-vis analysis (after solubilization with 10% Triton X-100) and phosphorus assay [55,56] indicated that the concentration of ZnPc and lipid in these vesicles was 0.05–0.1 μM and 4.8 mM, respectively.

Stirred, thermostated solutions of ZnPc liposomes were irradiated with a 1600 W high-pressure xenon lamp (Osram XBO-1600). The incident beam (80 mW/cm^2) was passed through a 10 cm cell through which flowed a cooled 0.02% Rhodamine B (aq) solution (λ_{irr} > 640 nm [57]). The temperature inside the photolysis cell was monitored periodically with a calibrated Fluke 51 K/J thermocouple and did not vary by more than 0.2°C over the course of the experiment. Light intensity at the irradiation cuvette surface was measured with a Coherent 210 power meter.

Contents leakage was monitored by withdrawing 25–50-μl aliquots of liposomal solution at various times during the course of the photolysis. Glucose leakage was analyzed spectrophotometrically using the glucose oxidase assay [56,58,59]. The total amount of entrapped glucose was determined following disruption with 10% Triton X-100.

Lipid peroxidation

Hydroperoxide formation was examined by thin-layer chromatography with slight modifications of the procedure reported by Girotti et al. [60]. All manipulations were conducted under dim room lights. Following extraction with 2:1 (v/v) $CHCl_3$/MeOH, the lipids were spotted on Baker silica 1B-F plates and developed in 75:25:4 (v/v) $CHCl_3$/MeOH/H_2O. The plates were then dried and sprayed with 1% *N,N,N',N'*-tetramethylphenylenediamine in 1:1 (v/v) $CHCl_3$/MeOH containing 1% acetic acid.

pH-induced release

Liposomes for pH-triggered release studies were prepared as described above. In a typical experiment, 4 mg of PlasPPC and 0.7 mg of DPPC were combined with 1 ml of $CHCl_3$, and dried under high vacuum for 1–2 h. Calcein (46.8 mg in 2.5 mls of 20 mM Tris) was added to the film. Following adjustment of the pH to 8 with 5 M NaOH, the vortexed suspension was put through five freeze-thaw cycles. Liposomes were extruded as before. Liposomes (50 μl) were collected from a G-25 column and diluted with 1.45 ml of 20 mM NaOAc/HOAc/30 mM NaCl, pH 4.2 buffer or with the same volume of 20 mM Tris/30 mM NaCl, pH 8 buffer. Release of calcein was monitored as described by Allen [61]. The percentage of release was calculated following disruption with 100 μl of 10% Triton X-100.

Results

Differential scanning calorimetry

Table I gives the main gel-to-liquid crystalline phase transition temperature, T_c, of DPPC and PlasPPC liposomes (< 1000 Å), as well as the T_c of liposomes containing the two lipids in varying molar ratios. PlasPPC and 8:1 PlasPPC/DPPC exhibit a single, broad transition with width at half-maximum of 5°C. Such broad, noncooperative transitions may be attributed to the dispersity of molecular species which comprise the semi-synthetic liposomes [62]. It has been shown by Creer and Gross [63] that in the absence of further chromatographic separation, preparation of PlasPPC by the method described above results in a

mixture of 1-alk-1'-enyl lipid chains of which 96% are saturated, with 68% being 16:0.

Previously reported DSC studies of phosphatidylethanolamine plasmalogens of varying chain length and unsaturation have yielded gel-to-lamellar T_c values which were 6–7°C lower than that of either the diether or diacyl counterparts [64]. Calorimetric measurements of PlasPPC revealed a transition temperature of 39.4°C, a depression of 3°C relative to that of DPPC (T_c = 41.4°C [65]). Han and Gross have observed a similar depression in transition temperature by fluorescence anisotropy measurements [66]. It is likely that the observed T_c depression is due at least in part to the vinyl ether linkage of the *sn*-1 chain that induces different packing and molecular conformations between plasmenylcholines and phosphatidylcholines as proposed by Gross, et al. [66,67] on the basis of proton NMR NOE's.

T_c values for PlasPPC SUV preparations containing only lipophilic ZnPc (< 1 μM) as well as the same concentration of sensitizer and glucose entrapped within the aqueous core of the vesicles are also presented in Table I. Comparison of these data with those from unloaded PlasPPC liposomes indicates that molecular packing is not perturbed appreciably upon incorporation of either ZnPc or glucose within the liposome.

Photosensitized disruption of liposomes

PlasPPC/DPPC liposomes containing ZnPc show an absorption maximum at 674 nm superimposed on a highly scattering background (Fig. 2). The solubility constraints of ZnPc within PlasPPC liposomes prepared as described earlier are such that the optical density of ZnPc, which varies slightly in each experiment due to differing amounts of sensitizer incorporated in the liposome, is generally no greater than 0.1 absorbance units above the background light scattering of the 1000 Å liposomes. The 674 nm absorption maximum of ZnPc in the PlasPPC/DPPC vesicles represents a shift of 8 nm relative to that measured in ethanol. A similar red shift has been observed in benzene and mouse myeloma cell suspensions [68].

TABLE I

Calorimetric data for plasmalogen liposomes

Composition [a]	T_c (°C) [b]
PlasPPC	39.4
DPPC	41.5 [c]
PlasPPC/DPPC [d] (8:1)	38.5
PlasPPC/DPPC [e] (8:1)	38.9
PlasPPC/DPPC (1:1)	39.4, 43.4

[a] 10 mM lipid in 20 mM Tris buffer, pH 8; < 1000 Å diameter.
[b] ±1°C.
[c] cf. 41.4°C [65].
[d] Contains < 1 μM ZnPc + 0.3 M glucose.
[e] Contains 0.3 M glucose.

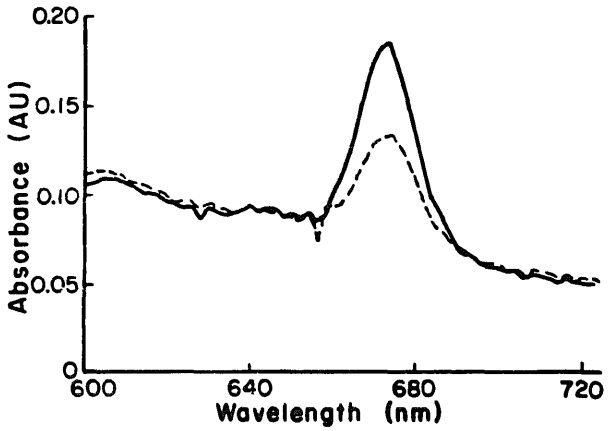


Fig. 2. Absorption spectrum of PlasPPC/DPPC liposomes containing < 1 mM ZnPc after 0 (———) and 45 (— — —) min of irradiation.

Dye photobleaching was monitored spectrophotometrically by scanning the absorption spectrum of either the photolysis cell directly or of Triton X-100 disrupted aliquots of the vesicle solution. Under the conditions of our experiments, ZnPc bleaching was found to be significant at 37°C, with 60–70% of the dye being bleached after 45 min of irradiation (Fig. 2).

Sensitized release rates of glucose from 8:1 PlasPPC/DPPC liposomes containing very low concentrations of ZnPc ([lipid]/[ZnPc] > 10^4) were measured initially at 15°C. This temperature was chosen since steady-state fluorescence studies of carboxyfluorescein leakage at 15°C (data not shown) indicated that the dark permeability of PlasPPC/DPPC liposomes is extremely slow when the liposomes are in the gel phase. Similar results for diacyl lipid vesicles have also been reported previously [69,70]. Although dark leakage of glucose at 15°C was less than 5 (±3)% after 60 min, photoinduced glucose release was also slow in the gel phase, with less than 15 (±3)% of the entrapped glucose released after 60 min of irradiation (Fig. 3).

Release rates were then measured at 37°C, where a more fluid bilayer predominates (Fig. 3). Since passive permeability of lipid bilayers is greatest at the bilayer T_c [71,72], we expect that any purely photoinduced effect will be more difficult to discern at 37°C. Nevertheless, the physiological relevance of 37°C makes the release kinetics at this temperature especially important for any potential drug delivery application. As expected, the passive leakage at 37°C is significantly higher than that in the gel phase, with 30 (±3)% of the glucose released in 60 min. The photoinduced glucose release rate, however, is twice that measured in the dark control, with 62 (±3)% of the glucose being released after 60 min of photolysis.

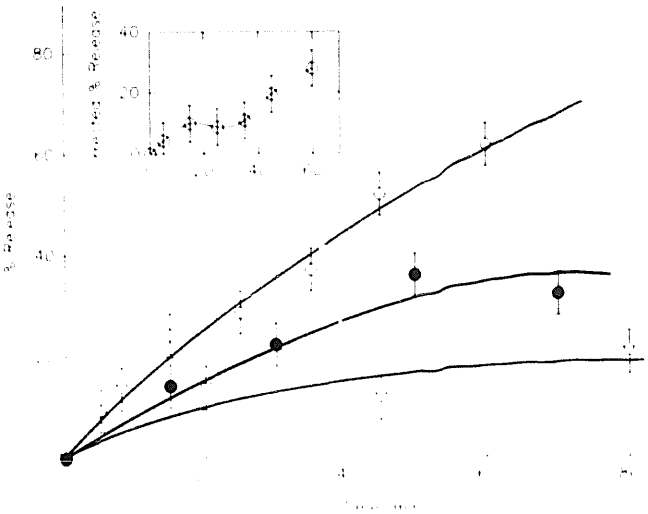


Fig. 3. ZnPc-sensitized release of glucose from PlasPPC/DPPC liposomes. ○, Irradiated, $\lambda > 640$ nm, 37°C; ●, dark control, 37°C; ▽, irradiated, $\lambda > 640$ nm, 15°C. Inset: Glucose release corrected for dark permeability, 37°C.

Photoinduced release rates at 37°C were also measured for several control experiments. Irradiation of identically prepared liposomes lacking either ZnPc or oxygen (argon purged) yielded release rates which were experimentally indistinguishable from the dark permeation profile shown in Fig. 3. These results strongly suggest that leakage from plasmalogen liposomes can be accelerated by at least a factor of two when irradiated in the presence of oxygen and submicromolar concentrations of ZnPc.

The involvement of the vinyl ether linkage during photoinduced release was probed by irradiating DPPC vesicles at 37°C and 41°C under identical conditions of ZnPc, glucose and oxygen used in the PlasPPC/DPPC liposome suspensions described above (Fig. 4). At 37°C, DPPC SUVs are in the gel phase (Table I) and 60 min of photolysis results in only 3 (±3)% release of encapsulated glucose. At 41°C, the light-induced and dark permeation release rates, while both much faster are identical within experimental error. The similarity of light and dark release rates in DPPC liposomes is in contrast to the photoinduced vs. passive leakage profiles of the PlasPPC/DPPC liposomes and suggests that the vinyl ether bond of the plasmalogen lipid is required for the photorelease evidenced in Fig. 3.

Lipid hydroperoxides were detected only in irradiated samples when analyzed by the method described by Girotti [60]. Such TLC methods, while lacking the sensitivity of GC or HPLC techniques, have been used extensively for rapid detection of lipid hydroperoxides [60,72]. Samples of dark, sensitizer-free, and argon-purged control liposomes revealed no new lipophilic products using a variety of solvent systems, whereas a small amount of a new product with R_f slightly lower than that of PlasPPC (0.30 cf. 0.32 in 65:35:4 $CHCl_3$/MeOH/H_2O) was observed in the irradiated samples. This spot stained blue with *N,N,N',N'*-tetramethylphenylenediamine spray reagent, suggesting that the new product is a hydroperoxide of PlasPPC.

Possible involvement of singlet oxygen ($^1O_2$) in this system was studied by irradiating an identical liposome preparation containing 100 mM sodium azide, a known $^1O_2$ quencher [73]. The glucose release profile at 37°C is shown in Fig. 5. No protection against vesicle release was afforded by azide under these experimental conditions as determined from a comparison of this data with those shown in Fig. 3.

Acid-triggered release of calcein

The increase of fluorescence emission intensity upon release of entrapped calcein has been used extensively

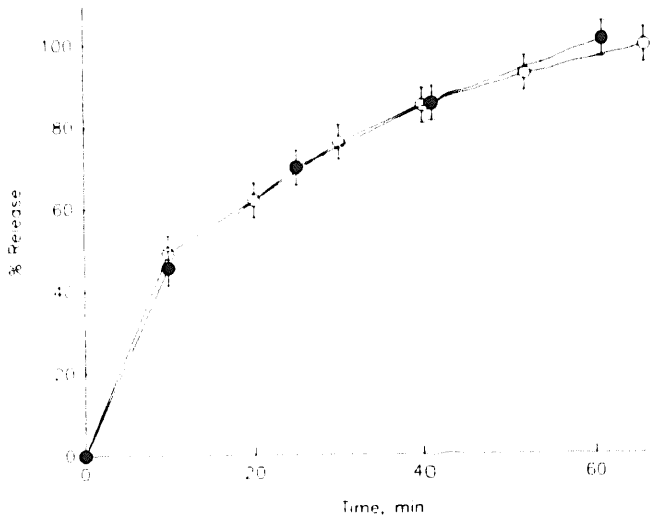


Fig. 4. Release of glucose from DPPC/ZnPc liposomes at 41°C. ○, Irradiated, $\lambda > 640$ nm; ●, dark control.

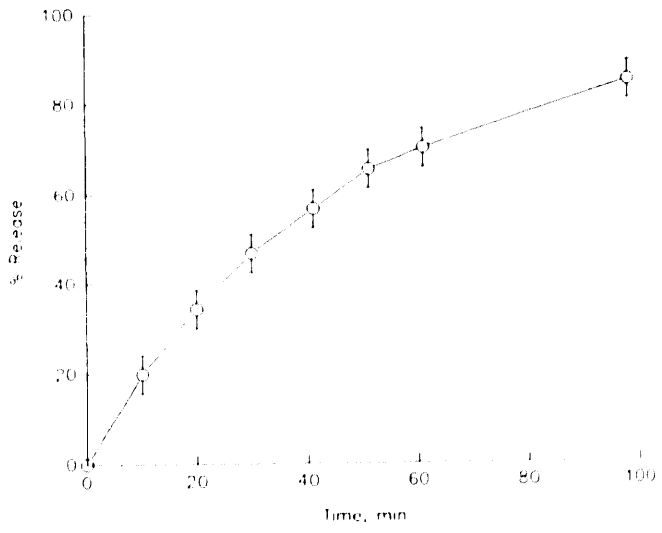


Fig. 5. Release of glucose from PlasPPC/DPPC liposomes at 37°C containing 100 mM sodium azide, irradiated, $\lambda > 640$ nm.

to monitor liposome destabilization under a variety of environmental stimuli [61,74–76]. Fig. 6a shows the time course of calcein release in the gel phase (15°C) at pH 8.0 and 4.2 from PlasPPC/DPPC liposomes. The analogous plot at 37°C is given in Fig. 6b. While release rates of calcein at 15°C are independent of pH, a substantially increased rate is observed at 37°C. Thus, after 3 h at 37°C and pH 4.2, 37 (±5)% of the calcein has been released whereas only 7 (±5)% has leaked out of the liposomal core at pH 8.

TLC product analysis confirmed that acid hydrolysis of the vinyl ether linkage is responsible for the leakage of calcein at low pH. Extraction of lipids into 2:1 $CHCl_3$/MeOH followed by TLC in 65:35:6 $CHCl_3$/MeOH/NH_4OH revealed the presence of a new lipid product that was not present in PlasPPC stock or in lipids recovered from the pH 8 liposomes. The R_f of the new product was identical to that of authentic

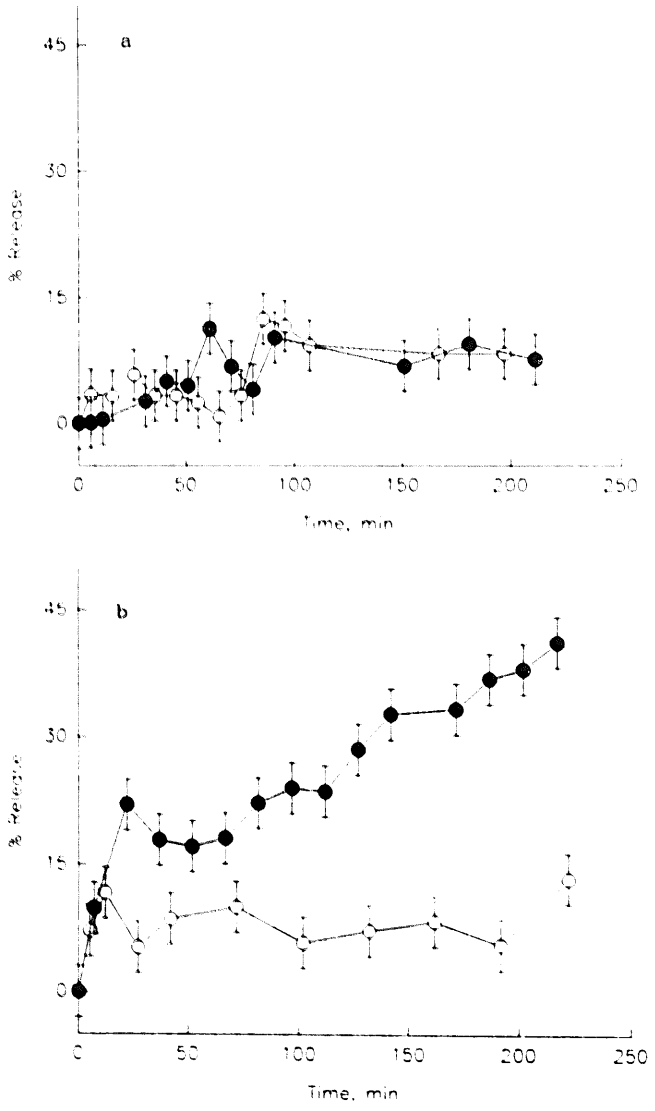


Fig. 6. Acid-triggered release of calcein from PlasPPC/DPPC liposomes at (a) 15°C and (b) 37°C. ○, pH 8; ●, pH 4.2.

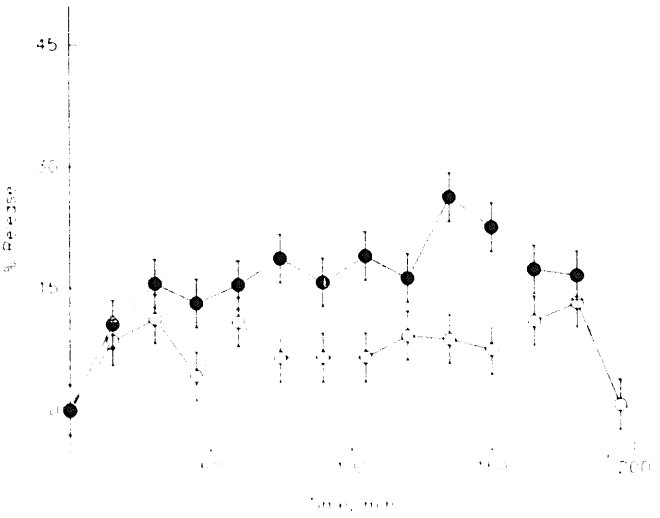


Fig. 7. Calcein release from DPPC liposomes at 41°C. ●, pH 4.2; ○, pH 8.

lysoplasmalogen prepared from $HgCl_2$ hydrolysis of PlasPPC [53b].

Further confirmation that acid-induced permeation changes are due to hydrolysis of the plasmalogen vinyl ether bond was obtained by monitoring calcein release from DPPC liposomes as a control. DPPC exhibited pH-independent release rates at 37°C and 41°C (Fig. 7) in contrast to the plasmalogen results (Fig. 6b). The lack of acid sensitivity of DPPC SUVs along with TLC product analysis from PlasPPC liposome decomposition indicates that acid-catalysed hydrolysis of the plasmalogen vinyl ether linkage does trigger contents release from plasmalogen liposomes.

Discussion

Photosensitized release

Photoinduced oxidation of lipids in natural and artificial membranes is known to result in chain scission and lipid decomposition [77]. Hydration of the bilayer, enhanced transbilayer movement of phospholipids, increased fluidity and permeability to solutes, cytotoxicity and cell death may occur as a result of lipid photooxidation [78,79].

In general, two types of photodynamic processes are known. In the Type I process, the electronically excited sensitizer reacts directly with the lipid, forming radicals which then react further with molecular oxygen or other cellular components. In the Type II process, the excited sensitizer reacts directly with molecular oxygen via energy transfer producing singlet oxygen ($^1O_2$) [73].

While the photodynamic reactions of diacyl phospholipids have been studied extensively [24], relatively little is known about the photodynamic decomposition of plasmalogen lipids. Recently, Morand et al. [38,39] showed that Chinese hamster ovary cells and

aqueous dispersions of plasmenylcholine are subject to photooxidation when irradiated in the presence of 12-(1'-pyrene)dodecanoic acid or merocyanine 540. Their work suggests that the predominant photooxidative mechanism in both the liposomal and cellular systems is a Type II ($^1O_2$) pathway, although contributions from Type I could not be discounted.

Scherrer and Gross [80] have demonstrated that $CHCl_3$/MeOH solutions of plasmenylcholine are also photooxidized when irradiated in the presence of Methylene blue. Product analysis was consistent with reaction of $^1O_2$ at the vinyl ether linkage of the plasmenylcholine. Dispersions of radiolabeled plasmenylcholine and Methylene blue were also irradiated. The results indicate that under these conditions, reaction of $^1O_2$ with the vinyl ether linkage of plasmalogen lipids occurs within the bilayer and results in cleavage of the *sn*-1 chain.

ZnPc is known to oxidize unsaturated phospholipids in the presence of visible light and oxygen [33,81–83]. In most cases, ZnPc-sensitized peroxidation of lipid chains in phosphatidylcholine bilayers has been ascribed to reaction of alkenes with $^1O_2$ via a Type II process [83]; however, the intermediacy of both Type I and II processes have been demonstrated, with the relative contribution of each being dependent upon a host of experimental parameters, including the polarity of the sensitizer microenvironment, lipid/oxygen ratios in the immediate environment of the sensitizer, and the absolute light intensity [27,84].

The conditions of the experiments described here do not preclude either mechanistic pathway. While the high quantum yield of $^1O_2$ from ZnPc in diacyl PCs [83] and the comparatively high solubility of O_2 in the lipid bilayer relative to that in the aqueous core [85] would both favor a Type II process, the relatively large photon fluxes combined with high local concentrations of lipid relative to ZnPc in the hydrophobic bilayer interior where ZnPc is preferentially localized [83] tend to favor a Type I mechanism. The observation that azide does not decrease the photostimulated leakage of glucose is consistent with a Type I mechanism in which $^1O_2$ is not an intermediate. However, since azide is preferentially solubilized within the aqueous phase while ZnPc is sequestered within the hydrophobic environment of the bilayer, it is likely that azide has little or no access to any $^1O_2$ that may be generated within the bilayer. Thus, a Type II process with $^1O_2$ intermediacy may still be operative in this liposome environment even though azide does not reduce the observed photostimulated leakage rate.

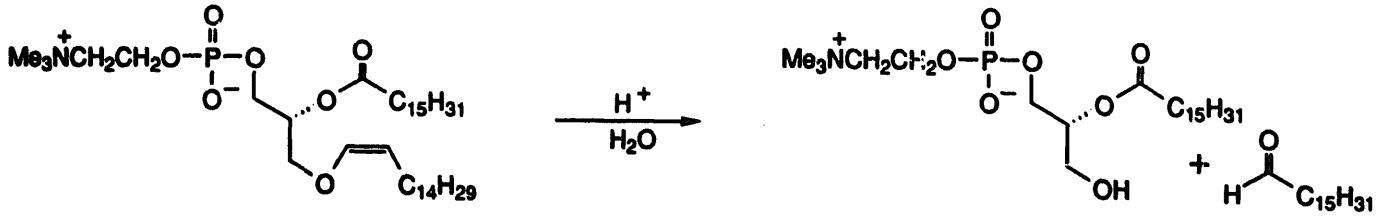
TLC data show that irradiation produces a new product that stains positive for hydroperoxide as would be expected for a Type I process. This result contrasts with unirradiated liposomes and liposomes irradiated in the absence of ZnPc or oxygen which do not produce any detectable amount of hydroperoxide. Oxidation of the vinyl ether bond and its importance in triggering liposomal leakage of hydroperoxide product is further indicated by the absence of hydroperoxide formation during the photolysis of similarly loaded, pure DPPC vesicles. HPLC analysis is currently underway to establish the identity of the oxidized species as well as the mechanistic details of the photooxidation pathway.

The data in Fig. 3 show both the release of glucose at 37°C from the PlasPPC/DPPC liposome core following irradiation at $\lambda > 640$ nm in the presence of oxygen and the passive (dark) permeation control. The photostimulated glucose release rate, corrected for dark permeation, is shown in Fig. 3, inset. Qualitatively, the plot shows an apparent induction period such that at early times (< 10 min) the leakage from irradiated liposomes is within experimental error of that observed in the dark control. Following this induction period, the magnitude of the corrected release increases with time, until approx. 60 min when the photolysed sample has released 28 (±5)% more glucose than the dark control. Given the precision of the data, such a difference greatly exceeds experimental error yet defies further quantitative analysis. We can infer from these results, however, that increased liposome permeability is induced by the combined effects of light and oxygen.

The data presented above suggest that plasmalogen liposomes, in combination with the photodynamic release mechanism, offer significant potential as a drug carrier/controlled release system. Many obstacles remain before plasmalogen liposomes become a practical delivery system, particularly the high passive glucose leakage rates observed at 37°C, although leakage rates probed by monitoring glucose release are typically quite high. Glucose permeation has been measured in a number of other membrane model systems and has been found to be consistently more rapid than that of other smaller, ionic markers [40]. Indeed, when plasmalogen vesicle permeation is followed by monitoring the dequenching of calcein fluorescence, dark leakage rates are found to be less than 10% over 5 h at 38°C (data not shown).

Further reduction of the passive permeation of glucose from PlasPPC liposomes can most likely be achieved by a variety of techniques. These include the addition of cholesterol or another sterol [85,86] and/or preparation of vesicles from lipids with increased C-2 chain length or with mixed chain lengths. Recently, we have measured the passive permeation from PlasPPC/cholestanol (30 mol%) liposomes [87]. Passive leakage rates are greatly reduced in this case, since only 20% of the entrapped glucose was released after 65 min at 37°C (cf. Fig. 3).

In addition to decreased dark permeation rates, a more rapid and efficient release of contents must be



Scheme 1.

developed which does not result in sensitizer decomposition. We have recently prepared several phthalocyanine derivatives which are more soluble within the bilayer than ZnPc [88]. Experiments are now in progress using these as sensitizers for plasmalogen vesicle photooxidation.

Acid-triggered release

Acid hydrolysis of the *sn*-1 vinyl ether linkage of plasmalogens produces fatty aldehydes and lysolipids [40], as shown in Scheme I above. The data in Figs. 6a and b demonstrate that calcein leakage at 37°C is accelerated by a pH drop from 8 to 4.2, consistent with a destabilization of the vesicle at low pH. No analagous increase in vesicle permeability was noted with identically prepared DPPC liposomes. TLC product analysis of the PlasPPC liposomes subjected to decreased pH indicated the formation of a new product with the same R_f as authentic lysoplasmalogen. As noted for the photosensitized release, no increased leakage was observed at pH 4.2 when the solution temperature was below T_c.

Several other liposome systems [50,52,90] have been reported that have faster pH release kinetics. While significant modifications of liposome structure and composition may be necessary to improve release kinetics before the present approach can assume any practical utility, these initial results do demonstrate that the plasmalogen vinyl ether may be sufficiently acid labile to serve as a basis for a slow-releasing, pH-sensitive delivery system.

The combined results from photodynamic and pH sensitive release experiments reported herein represent our initial attempts to develop a delivery system based on naturally occurring plasmalogen lipids. While many problems remain to be solved, we believe that plasmalogen liposomes offer great promise for externally triggerable drug delivery applications.

Conclusion

Stimulated release of glucose from plasmalogen liposomes triggered by visible light has been demonstrated. The photosystem is based upon the naturally occurring 1-alk-1'-enyl-2-acyl-*sn*-glycero-3-phosphocholines which in the presence of light, O_2, and sensitizer are known to photooxidize via radical oxygen and/or $^1O_2$ intermediates. Glucose has been shown to be actively released from these liposomes at modest rates and hydroperoxide products have been detected by TLC. We have also demonstrated that decreasing the pH from 8 to 4.2 results in increased permeation of calcein from plasmalogen liposomes as a result of hydrolysis of the vinyl ether linkage. Experiments designed to decrease dark permeation and establish optimal conditions for rapid and complete light- and acid-induced release using different sensitizers and lipid compositions are in progress.

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