Photosensitive liposomes as 'cages' for laser-triggered solute delivery: the effect of bilayer cholesterol on kinetics of solute release

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Received 13 September 1999; received in revised form 6 November 1999

Edited by Richard Cogdell

Abstract Liposomes containing acyl chains incorporating azobenzene chromophores have been investigated as potential 'caging' agents for fast solute release. On photolysis, trapped marker dye can be released from gel-phase liposomes within milliseconds. Solute release is markedly sensitive to the presence of cholesterol in the bilayer. Phospholipids bearing one saturated acyl chain and an azobenzene-substituted chain are ineffective as sensitisers unless cholesterol is present, while doubly substituted phospholipids sensitise release in its absence. Cholesterol markedly affects the temperature profile of solute release depending on the host phospholipid chain length. Solute release is not seen for lipid hosts with unsaturated acyl chains.

Key words: Liposome; Caged reagent; Azobenzene; Photosensitization; Drug delivery; Photodynamic therapy

1. Introduction

Liposomes have long been of interest as model systems to study membrane phenomena and have also been proposed and investigated as carriers for drug delivery in vivo. For drug delivery applications, the liposomes can entrap soluble materials within the aqueous interior or alternatively hydrophobic materials can be bound to the bilayer membrane. In the former case, the well known permeability increase at the phase transition temperature can provide a means to trigger release of trapped solute, for example in locally heated tissues [1]. In previous publications [2–5], others and we have shown that efficient solute release from liposomes can also be triggered by light if the liposomal membrane contains a photoisomerisable species. It was postulated that such triggered release might form an alternative or adjunct to 'photodynamic' drug treatments based on the generation of reactive species from excited sensitising agents. In recent publications [6,7], we have shown that liposomes containing a phospholipid (1,2-(4′-*n*-butylphenyl)azo-4′′(γ-phenylbutyroyl))-glycero-3-phosphocholine ('Bis-Azo PC'), substituted with azobenzene moieties in both acyl chains, can be photoisomerised by a fast laser pulse. Solute release can subsequently occur on the milliseconds timescale and it was suggested that photosensitised liposomes containing Bis-Azo PC might be useful as 'cages' to allow for the triggered release of soluble reagents for biological research. Our previous paper [7] investigated the release kinetics of a trapped marker dye, calcein, which is highly fluorescent when dilute, but is 'quenched' when trapped at a high concentration within a liposome. The cited work investigated the effect of phospholipid acyl chain length and temperature on release kinetics of calcein and demonstrated that liposome composition could be tailored to allow for rapid solute release across a range of temperatures.

In this paper, we investigate the effect of cholesterol and lipid chain composition on the rate and efficiency of laser-triggered calcein release from liposomes. The addition of cholesterol is shown to have a marked effect on kinetics of calcein release and in some circumstances can result in substantial enhancement of light sensitivity of the liposomes.

2. Materials and methods

Phospholipids, cholesterol, calcein and buffer media were purchased from Sigma and used without further purification. Calcein was brought into solution with sodium bicarbonate and adjusted to pH 7.4. (1-Hexadecanoyl-2-(4′-*n*-butylphenyl)azo-4′′(γ-phenylbutyroyl))-glycero-3-phosphocholine (Pazo PC) and Bis-Azo PC were prepared as described elsewhere [8,9]. Lipids were mixed in chloroform solution and evaporated under nitrogen to give a thin film. Liposomes were prepared by vortex mixing of lipid films hydrated by heating above the lipid phase transition with 45 mM calcein solution and subsequent repeated extrusion under nitrogen through 400 nm Unipore polycarbonate membranes using a 'Lipex' extruder as described [7]. The final lipid concentration after extrusion was 2 mg/ml. The extrusion apparatus was heated by circulating water to a temperature at least 5°C above the phase transition of the lipid sample. Liposomes were separated from untrapped dye by gel filtration at room temperature on Sephadex G-75 equilibrated with phosphate-buffered saline pH 7.4 containing 10 mg/dm^3 EDTA. The presence of the chelator was necessary to complex traces of heavy metal ions which otherwise give a time-dependent quenching of calcein when this is released from liposomes at a low bulk concentration. The samples were shielded from direct sunlight during chromatography. Liposomes were diluted with elution buffer to a final concentration of 0.2 mg/ml total lipid.

The apparatus and protocol for the measurement of calcein release were as described previously [7]. Liposome samples were placed in a thermostatted silica tube and were irradiated with a pulse of 355 nm laser radiation (8 ns duration, 15 mJ energy). Calcein fluorescence was excited with a filtered 470 nm light-emitting diode source and emission was monitored at right angles to the light source using a fast semiconductor diode with an interference filter to block exciting light. Samples were only exposed to the 470 nm source for brief periods during and after photolysis to minimise any effects of this radiation on the photostationary state of the azobenzene moieties.

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Abbreviations: DPPC, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (dipalmitoyl-L-α-phosphatidylcholine); DSPC, 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine (distearoyl-L-α-phosphatidylcholine); Pazo PC, (1-hexadecanoyl-2-(4′-*n*-butylphenyl)azo-4′′(γ-phenylbutyroyl))-glycero-3-phosphocholine; Bis-Azo PC, (1,2-(4′-*n*-butylphenyl)azo-4′′(γ-phenylbutyroyl))-glycero-3-phosphocholine

0014-5793/99/$20.00
PII: S0014-5793(99)01612-9

3. Results

Previous work [7] demonstrated that good liposome stability and efficient photo-induced release was obtained for a composition containing 6% (mol:mol) of Bis-Azo PC in the lipid host. This concentration of sensitising lipid was therefore used for the present experiments. Under the conditions of the experiment, it is calculated that each azobenzene chromophore in the sample absorbs at least one photon from a single laser pulse. This indicates that the photostationary equilibrium concentrations of the *cis*- and *trans*-isomers should be achieved after exposure to a single laser pulse. In Fig. 1, the time course of calcein release is shown for a host lipid of 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine (dipalmitoyl-L-α-phosphatidylcholine) (DPPC) containing varying levels of cholesterol. The data were measured at 15.7 ± 0.5°C. There are two noteworthy features. The release kinetics show a slight lag phase, after which the data can be fitted to a double exponential curve and the overall rate of dye release is markedly increased in the presence of cholesterol in the bilayer. The apparent lag phase is reproducible but is noticeable only at relatively low temperature. The origin of this effect is not known at present, but might well be associated with lateral redistribution or phase separation of photoisomerised lipid within the host membrane. Previous results using DPPC as host lipid suggest that no solute leakage is seen unless the concentration of the photoisomerised *cis*-form of Bis-Azo PC reaches a critical level in the bilayer [6] and a co-operative effect is therefore suspected. Attempts to treat the data of Fig. 1 by first order sequential kinetic schemes failed to give good fits and therefore, the data were characterised simply by measurement of the time required for 50% of maximal light-induced leakage (t_{50}). This treatment allows for trends to be discerned as the cholesterol level increases and is a reasonable approach in view of the decreasing influence of the slight lag phase as temperature increases. Fig. 2 shows data plotted using the reciprocal of this parameter as a pseudo 'rate constant' to investigate the effects of temperature and cholesterol concentration on rate of calcein release. It is clear that leakage rates increase with temperature at all concentrations of cholesterol, but that above 15% cholesterol (mol:mol relative to total lipid), leakage rates decrease once more. This is illustrated most clearly by the inset to Fig. 2, which shows the rate of calcein leakage as a function of cholesterol concentration at representative fixed temperatures. Data were not measured beyond these temperature ranges because the liposomes begin to release calcein spontaneously at higher temperature. Data were also measured for liposomes of 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine (distearoyl-L-α-phosphatidylcholine) (DSPC) at a fixed concentration of Bis-Azo PC of 6% (mol:mol) as a function of temperature and cholesterol concentration. Results are shown in Fig. 3. It is worth noting that the results for DSPC alone are somewhat variable and apparent discontinuities are sometimes seen in Arrhenius plots of leakage rate as a function of temperature. This suggests that the system might not be at equilibrium and can be interpreted in terms of possible time and temperature-dependent lateral redistribution of Bis-Azo PC within the bilayer. Cholesterol appears to have little effect on the rate of calcein leakage from liposomes of DSPC at temperatures below about 25°C. At higher temperatures, the presence of cholesterol has a very marked effect on the leakage kinetics, as demonstrated in

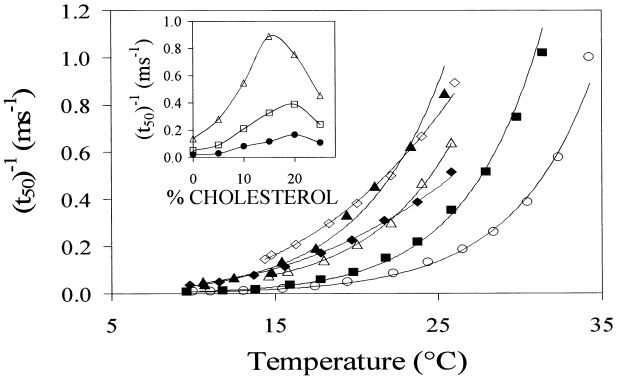

Fig. 2. Effect of temperature on the reciprocal of the time to 50% release of calcein ($(t_{50})^{-1}$) in liposomes of DPPC and 6 mol% Bis-Azo PC containing 0 mol% (○), 5 mol% (■), 10 mol% (△), 15 mol% (▲), 20 mol% (◇) and 25 mol% (◆) cholesterol. Inset: data from the main figure showing values of $(t_{50})^{-1}$ versus mol% cholesterol at 15°C (●), 20°C (□) and 25°C (△).

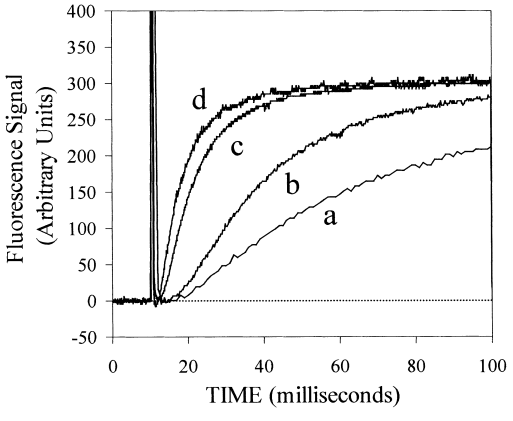

Fig. 1. Increase in fluorescence intensity due to calcein release, following exposure to a single 355 nm laser pulse, from liposomes of DPPC at 15.7 ± 0.5°C containing 6 mol% Bis-Azo PC and (a) 0 mol%, (b) 5 mol%, (c) 10 mol% and (d) 15 mol % cholesterol. The spike at 10 ms is due to excitation of fluorescence by the laser pulse.

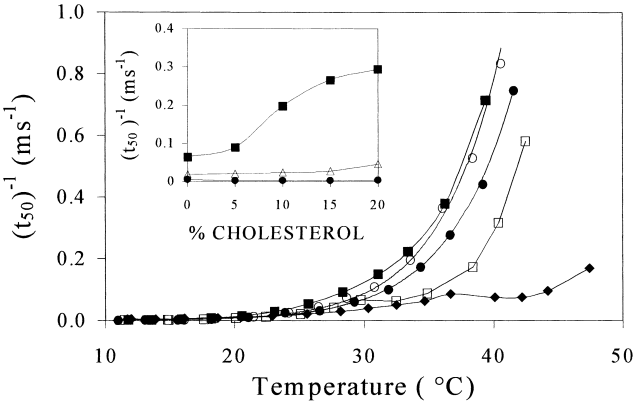

Fig. 3. Effect of temperature on the reciprocal of the time to 50% release of calcein ($(t_{50})^{-1}$) in liposomes of DSPC and 6 mol% Bis-Azo PC containing 0 mol% (◆), 5 mol% (□), 10 mol% (●), 15 mol% (○) and 20 mol% (■) cholesterol. Inset: data from the main figure showing values of $(t_{50})^{-1}$ versus mol% cholesterol at 15°C (●), 25°C (△) and 35°C (■).

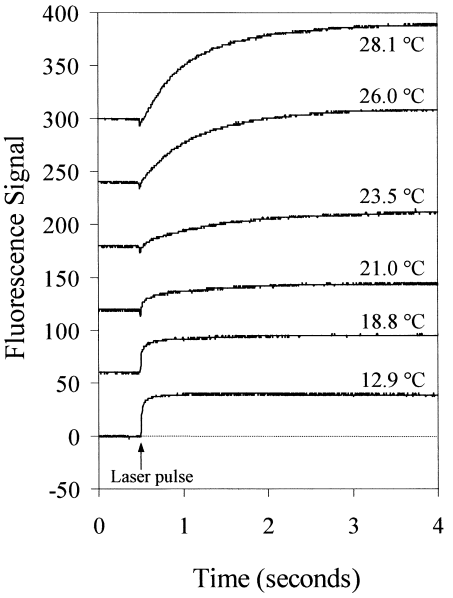

Fig. 4. Kinetics of calcein release from DPPC liposomes containing Pazo PC (12 mol%) and cholesterol (20 mol%). The curves measured at the indicated temperatures have been displaced on the vertical scale for comparison purposes.

the inset to Fig. 3. With the laser power used in these experiments (~15 mJ/pulse), between 60 and 80% of the entrapped calcein was released from the liposomes after a single pulse, when compared with the total release obtained on addition of detergent (Triton X-100).

In marked contrast to the results for saturated chain lipids DPPC and DSPC, liposomes prepared with Bis-Azo PC and 1,2-di-(9-octadecenoyl)-*sn*-glycerophosphocholine (dioleoyl-L-α-phosphatidylcholine) (both acyl chains unsaturated) or 1-hexadecanoyl-2-(9-octadecenoyl)-*sn*-glycero-3-phosphocholine (1-palmitoyl-2-oleoyl-L-α-phosphatidylcholine) (one unsaturated acyl chain) trapped calcein efficiently, but showed no significant leakage on prolonged photolysis with an UV handlamp or with the pulsed laser. The presence of up to 30% cholesterol (mol:mol relative to total lipid) did not induce any significant leakage of solute on photolysis in the temperature range used for the DPPC experiments.

Experiments were conducted with a photochromic lipid, Pazo PC (which has a single photoisomerisable acyl chain and a palmitoyl acyl chain) in DPPC as the host lipid. In these experiments, 12% Pazo PC (mol:mol) was used so that the average concentration of photoisomerisable acyl chain was the same as that with the Bis-Azo PC experiments discussed above. It was found that photoisomerisation of Pazo PC caused essentially no leakage of calcein except at temperatures where the sample was beginning to show spontaneous thermally induced solute leakage. Only at temperatures above approximately 30°C, there is significant leakage induced by the laser pulse. At 32.8°C for example, laser photolysis caused leakage with an apparently exponential profile and a rate constant of 0.62 s^{-1}, of the order of 1000-fold slower than the rate of leakage seen for 6% Bis-Azo PC in DPPC. However, slow thermally induced leakage is also observed at this temperature. The addition of 20% (mol:mol) cholesterol, however, had a dramatic effect on calcein leakage in this system. The effect of cholesterol is shown in Fig. 4, where traces are offset on the vertical axis for clarity. Below room temperature, the cholesterol induces a rapid partial loss of solute on laser photolysis. The data can be fitted to an exponential process with a rate constant of the order of 100 s^{-1}. This is of the same order as the rapid release of calcein seen with 6% Bis-Azo PC:20% cholesterol in DPPC at the same temperature. As temperature is increased, the extent of release during this fast phase decreases and a slower but more extensive solute leakage is seen.

4. Discussion

The data presented demonstrate that cholesterol has a clear and marked effect on kinetics of solute release from liposomes sensitised with the photochromic phospholipids Bis-Azo PC and Pazo PC. These results point towards possible mechanisms for the photo-induced solute leakage. The results also suggest which liposomal compositions are likely to prove most useful in practical application for controlled release of solutes.

Only liposomes prepared from a saturated host lipid appear to become permeable to calcein in the presence of the photoisomerised sensitiser. The requirement for a saturated host lipid suggests that the rigidity of the bilayer must play a part in the leakage process. An alternative effect might be related to lateral phase separation causing the sensitising lipid to cluster within the bilayer, possibly forming a transient discontinuity in acyl chain packing. It seems likely that both of these effects operate to some extent. A rigid bilayer might not be able to accommodate the excess volume introduced by photoisomerisation of an azobenzene moiety, while the greater free volume of the acyl chains in a fluid lipid might be expected to buffer the effect of the photoisomerisation. The lack of sensitising efficiency of Pazo PC, even within a rigid host in the absence of cholesterol, suggests that some degree of interaction between adjacent azobenzene-substituted chains is required to cause the bilayer to become permeable to solutes. Previous work [6] has shown that Bis-Azo PC in DPPC does not sensitise leakage of solute unless the extent of photoisomerisation is above a threshold level and this suggests that a lateral clustering of sensitising lipid is involved in the leakage process. This model is reinforced by the effect of cholesterol. The very marked effect of cholesterol on liposomes containing Pazo PC and the 'burst' release kinetics at low temperature argue strongly for a lateral phase separation of sensitiser in this system. Cholesterol is well known to act as a 'buffer' of membrane order. In gel-phase lipids, it is thought to disorder the rigid phase while above the phase transition, the effect is to increase the order of the 'fluid' phase. It is likely that cholesterol has effects both to influence lateral phase separation and also to modulate the overall rigidity of the bilayer at a given temperature, and both of these will influence rates of solute release from the sensitised liposomes.

From a practical viewpoint, the photosensitive liposomes are likely to find application as 'cages' for release of trapped reagents and might also be of value in drug delivery. For both of these applications, rapid and efficient release of trapped solutes is required. Liposomes used for drug delivery purposes frequently include cholesterol to modulate bilayer stability. In some cases, liposomes will bind cholesterol when incubated with plasma, so that the effect of this component on liposomal properties cannot be ignored. The present work has demonstrated that suitable liposome compositions can be formulated

to give rapid and efficient solute release at a chosen temperature and that release rates that are competitive with more conventional 'caging' methods can be achieved. On storage at room temperature in the dark, liposomes of DPPC containing Bis-Azo PC (6 mol%) and cholesterol (up to 15 mol%) show no significant leakage of calcein for at least 8 weeks. However, liposomes containing higher concentrations of Bis-Azo PC (e.g. 10 mol%) are stable overnight, but release their contents on long term storage. Future work will investigate the influence of molecular size of solute on release kinetics from liposomes.

Acknowledgements: The authors wish to acknowledge the assistance of Mr. J.G. Murray and Mr. E.J. Wall in construction of electronics for the project. This work was funded by BBSRC Grant 106/B09058 to C.G.M. and R.H.B., and this funding is gratefully acknowledged.

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