

# Fast solute release from photosensitive liposomes: an alternative to 'caged' reagents for use in biological systems

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**Abstract** The kinetics of release of soluble marker trapped in liposomes of gel phase phospholipid containing a photoisomerisable phospholipid analogue have been investigated. Marker release is triggered by UV laser flash photolysis at 355 nm. A markedly temperature-dependent release rate is seen, and above 25°C millisecond release kinetics can be achieved. These results suggest that such liposomes might find application as an alternative to conventional 'caged' reagents for photo-triggered reagent release in biological research.

**Key words:** Liposome; Photochromism; Photolysis; Laser; Solute release

## 1. Introduction

In biological research there are many applications of 'caged' reagents that are designed to be inert until activated by absorption of intense light of appropriate wavelength. Usually the reagent has a functional group essential to its biological action that is blocked by reaction with a photolabile species, and that can be regenerated when the blocking group is released on photolysis. 'Caged' derivatives of nucleotides, ion chelating agents, agonists and other molecules with biological activity are now available and widely used [1–3]. However there are several potential problems associated with covalently-caged compounds, including: (a) low quantum yield for solute release; (b) damage to other molecules by the short wavelength light required for cage photolysis; (c) a slow rate of uncaging; (d) the formation of by-products toxic to cellular systems; and (e) difficulties with preparative routes to synthesis of novel caged molecules. These and other considerations suggest that alternative strategies to achieve cellular delivery and rapid controlled release of reagents might be advantageous.

In previous papers [4–7] we have described a novel photosensitive liposome system based on a photochromic lipid molecule 'Bis-AzoPC' (1,2-bis(4-(4-*n*-butylphenylazo)phenylbutyryl)-phosphatidylcholine) (Fig. 1). This lipid exists in a photostationary state which is predominantly composed of the *trans*-isomer under visible light illumination, but which is converted to a *cis*-enriched photostationary state on exposure to near-ultraviolet light. Liposomes of dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine in the rigid 'gel' phase containing up to 5 mole% of Bis-AzoPC have retained trapped marker dye for up to several months at room temperature [4]. However, on exposure to ultraviolet light, conversion of Bis-

AzoPC to the *cis*-photostationary state disrupts the bilayer membrane leading to rapid leakage of contents [6].

Photosensitive liposomes might well offer a suitable means to 'cage' water-soluble reagents compatible with the bilayer structure and may provide solutions to the potential problems described above with covalently caged species. A wide variety of water-soluble materials including biologically important ions such as calcium and copper and drugs such as methotrexate [8] can easily be encapsulated under mild conditions without the need for specialised apparatus, and photo-induced release of such solutes from liposomes containing Bis-AzoPC has already been demonstrated [9,10]. In addition, liposomes might be appropriate as vehicles to introduce reagents into cells by endocytosis, minimising the reagent's exposure to degradative species until release is triggered.

One of the important advantages of some covalently caged molecules with biochemical and physiological activity is that release, on a time-scale of microseconds to seconds, may be triggered by a flashlamp or laser pulse. For those examples with the fastest rates of release, the time-scale is comparable with that obtained in stopped flow experiments. However, caged compounds are widely applied to situations, such as imaging of cells and tissues, in which stopped flow and related techniques are inappropriate. In these circumstances, photo-initiated release of solutes initially entrapped within a liposome system may be an attractive alternative to the use of covalently caged species. The purpose of the present experiments was to determine the kinetics of solute release from photosensitive liposomes of dipalmitoylphosphatidylcholine containing Bis-AzoPC. For this purpose calcein was used to represent a typical low molecular weight polar solute which may be encapsulated within a liposome. When entrapped at high concentrations within lipid vesicles the fluorescence of calcein is markedly self-quenched. Calcein release from within a vesicle may therefore be readily monitored by an increase in fluorescence as the dye is diluted into the bulk aqueous solution. We show that under suitable conditions, rapid release of calcein can be initiated by a single laser photolysis pulse.

## 2. Materials and methods

Both calcein and 1- $\alpha$ -dipalmitoyl phosphatidylcholine (DPPC) were obtained from Sigma. Bis-AzoPC was synthesised as described previously [4]. Calcein was encapsulated at a concentration of 30 mM in liposomes of a mixture of DPPC and Bis-AzoPC in a 90:10 molar ratio. Under these conditions the dye fluorescence is substantially quenched by self-association. A calcein solution (30 mM) was dissolved by careful addition of sodium hydroxide solution to a suspension of calcein in water and sodium chloride added to a final concentration of 47 mM to osmotically match the calcein solution to standard phosphate buffered saline. Vesicles were formed by sonication at 50°C of a dispersion of lipid (DPPC + Bis-AzoPC, total lipid concentration 5 mg/ml) in this

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solution of calcein. To increase the mean liposome size and enhance trapping efficiency [8] the resulting dispersion was frozen in liquid nitrogen and thawed in water at 50°C. This process was repeated six times. The predominantly unilamellar dispersion of liposomes was separated from untrapped calcein by gel chromatography on Sephadex G-75 eluting with standard phosphate-buffered saline prepared by dissolving Dulbecco A tablets (Oxoid) in distilled water containing EDTA (1 mM). The EDTA was required to chelate any contaminating heavy metal ions which would otherwise quench the fluorescence of calcein. Samples were used within 3 h of chromatographic separation, on which time-scale no significant calcein leakage was evident.

Dye release was initiated by laser flash photolysis using either a single pulse or a train of pulses of 355 nm radiation from a dye laser (Lambda-Physik FL3002) pumped by a XeCl excimer laser (Lumonics HE460). The direct beam of the dye laser output (7 mJ in a 10 ns pulse) was used to give an energy density at the sample estimated to be ca.  $2 \text{ mJ} \cdot \text{mm}^{-2}$ . Alternatively the dye laser beam was diffused by a liquid-filled light guide to give an energy density of ca.  $0.1 \text{ mJ} \cdot \text{mm}^{-2}$ . Liposomes were irradiated in a thermostatted 1 cm pathlength quartz cuvette after

dilution to  $A_{355\text{nm}} < 0.1$ , to ensure uniform laser illumination across the lightpath. Fluorescence from released calcein was excited by a quartz-halogen lamp filtered with a 470 nm interference filter. Fluorescence was detected at right angles through a monochromator (Applied Photophysics) set to 520 nm (10 nm bandwidth) using a photomultiplier (Hamamatsu 1P21) linked to a digital oscilloscope and microcomputer.

### 3. Results and discussion

Initial experiments were undertaken with the low power density, diffused laser beam. In this case no release of calcein, as evidenced by an increase in fluorescence intensity, was observed when the sample was exposed to a single laser pulse. Further experiments involved the use of low laser power density and a train of closely spaced laser pulses. Fig. 2A shows the result of an experiment at 20°C involving a train of six pulses, each separated by 33 ms. In this experiment there was little dye

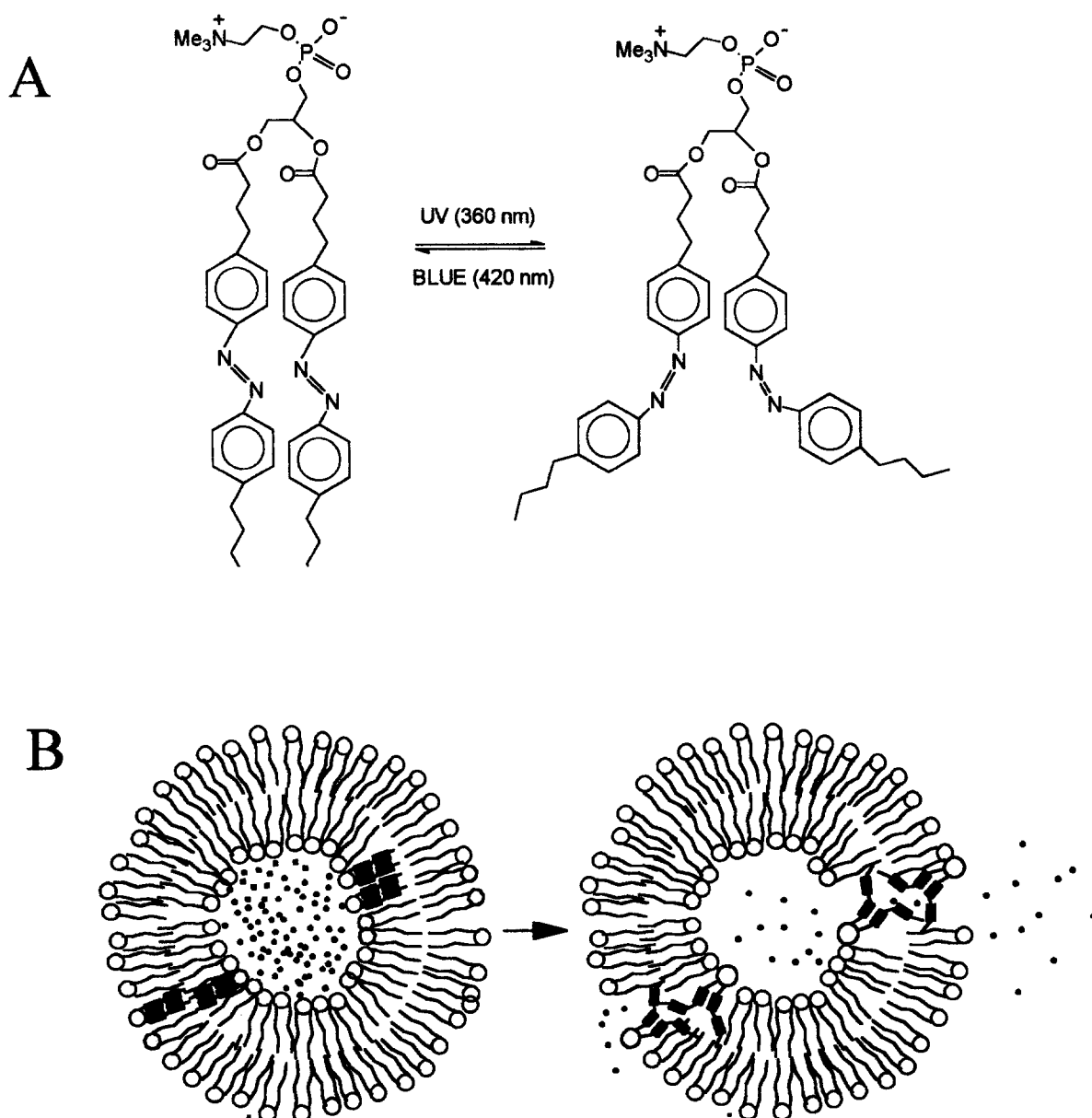


Fig. 1. (A) Structure of Bis-AzoPC and change in structure on *cis-trans* isomerisation. (B) A diagrammatic representation of the disruption of a liposome and leakage of its contents following exposure to ultraviolet light and conversion of Bis-AzoPC to the *cis*-enriched photostationary state.

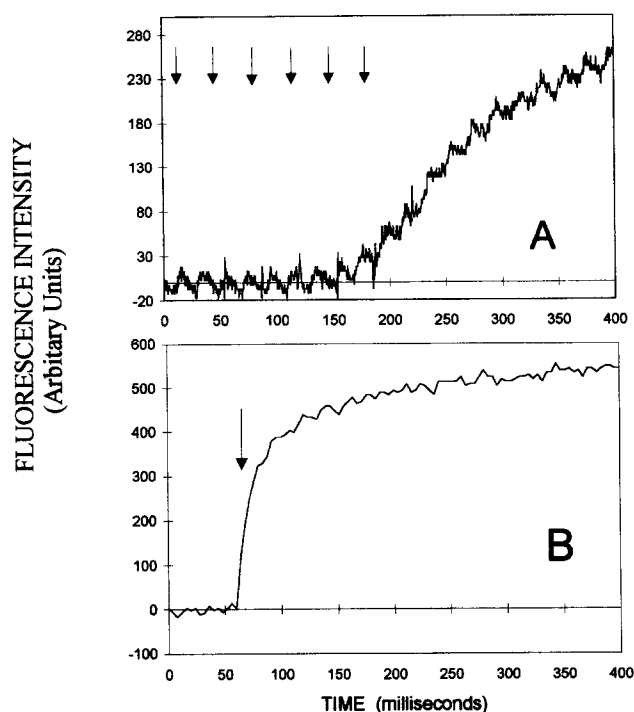


Fig. 2. Increase in fluorescence intensity due to release of calcein from DPPC/Bis-AzoPC liposomes at 20°C after flash photolysis at 355 nm. (A) On exposure to six laser pulses of low-power density. (B) On exposure to a single higher power density laser pulse. The position of the laser pulse(s) is indicated by the vertical arrow(s).

released until after the fifth laser pulse had irradiated the sample, after which the release proceeded relatively slowly over several hundred milliseconds. Increasing the spacing between pulses to several seconds gave similar results, as did experiments at 35°C except that the fraction of calcein ultimately released was greater at the higher temperature. These results indicate that a threshold level of photoisomerisation of Bis-AzoPC was required to initiate leakage.

In contrast it was found that in higher power density experiments, using the direct dye laser beam, rapid release could be initiated by a single laser pulse. The result at 20°C is shown in Fig. 2B on the same time-scale as in the lower power density experiments. The rate of calcein release from the liposomes induced by a single laser pulse appeared biphasic, and was fitted well to a double exponential process. Most of the build-up in fluorescence intensity occurred in the rapid phase with a time constant of  $10 \pm 0.5$  ms. The slower phase had a time constant of  $80 \pm 10$  ms. These were not thermal effects of the laser pulse, since control experiments using liposomes not containing Bis-AzoPC did not release calcein under any conditions tested, despite the fact that their dye contents would strongly absorb the incident radiation. The use of the higher power density laser beam therefore induces solute release from within the Bis-AzoPC/DPPC liposomes at 20°C on a time-scale comparable with that of the usual stopped-flow technique.

The rate of calcein release in response to a single high intensity laser pulse is markedly temperature dependent. Fig. 3 shows the results of experiments performed between 5 and 35°C (below the phase transition temperature of DPPC

liposomes). The time constant for the predominant fast component for dye release varied from about 5 ms at 25°C to greater than 100 ms at 5°C. The release rate at temperatures between 25 and 35°C could not be accurately measured because of saturation of the detector during and shortly after the laser pulse in the present experimental apparatus, as indicated by a transient coincident with the laser pulse as seen in Fig. 3. Fitting the rate data obtained at 5°C intervals between 5 and 25°C to the Arrhenius equation gave an activation energy of  $101 \pm 13$  kJ·mol<sup>-1</sup>. Using this value and assuming that the system obeys the Arrhenius law up to the transition temperature, a rate of release of  $0.81$  ms<sup>-1</sup> ( $\tau = 1.2$  ms) at 35°C is calculated.

The marked temperature dependence suggests that the physical state of the lipid bilayer might well be important in the dye release process. As is well known, liposomes of pure DPPC undergo a gel:liquid-crystalline phase transition at around 37–41°C, with a transition profile and midpoint temperature determined by a variety of factors such as liposome size, lipid purity and thermal history of the sample [11]. Our previous experiments have established that liposomes of DPPC containing *trans* Bis-AzoPC retain a similar phase transition to those of DPPC alone, and dispersions of *trans* Bis-AzoPC also show a similar co-operative phase transition at approximately 41°C, though with marked hysteresis between heating and cooling cycles [7]. The phase transition of Bis-AzoPC is abolished by isomerisation to the *cis* photostationary state. Interpretation of the effect of isomerisation of Bis-AzoPC in mixtures with DPPC is complicated by the fact that liposomes fuse together after photolysis [6], and this causes a time-dependent change in thermal properties. The fusion process is likely to proceed through initiation of locally disordered regions in the bilayer membrane where the relatively bulky *cis* isomer of Bis-AzoPC interferes with bilayer packing. The relatively slow release of calcein at low temperature also argues for a release mechanism involving dislocations or channels in the ordered bilayer structure whose dimensions are similar to those of the trapped marker. The effective size of such defects would be markedly affected by temperature in the neighbourhood of a lipid phase transition, and a very steep temperature dependence of solute leakage rate would be predicted on this basis. The behaviour in a lipid bilayer host of azobenzene-containing lipids similar to those used in this study has been investigated by Song et al. [12] who find spectroscopic evidence of aggregate formation in

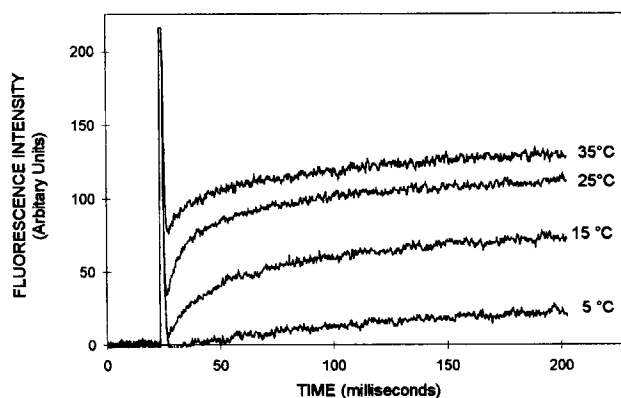


Fig. 3. Release of calcein from DPPC/Bis-AzoPC liposomes, after exposure to a single high-power density laser pulse at 355 nm, at the indicated sample temperatures.

some circumstances. It is well known that solute release from liposomes of pure gel-phase lipids is markedly increased at the midpoint of a phase transition [13] where regions of ordered and disordered lipid coexist, and indeed this has been proposed as a method to control localised delivery of soluble drugs trapped within liposomes [14]. Millisecond release of liposome contents is unlikely to be caused by an overall transition in the membrane to the fluid, liquid crystalline state, since these transitions in pure DPPC bilayers occur on a time-scale of tens of milliseconds [15].

The present results show that the DPPC-Bis-AzoPC liposome system investigated here could be readily applied to effect rapid release of a variety of relatively low molecular weight polar solutes. Further investigation of the effects of acyl chain length and lipid composition may provide even more effective systems. In addition it would be of considerable interest to determine the effect of molecular size of the solute on release kinetics. The release can be triggered by long wavelength UV light from a flashlamp or dye laser, and the 337 nm output of a low-cost nitrogen laser is also suitable. It is likely that such liposomes will find applications as 'cages' for reagents which are difficult to modify using conventional chemical techniques, and in particular the release of metal ions or metal ion chelators appears promising in view of our earlier work [9,10].

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## References

- [1] Guernsey, A.M. and Lester, H.A. (1987) *Physiol. Rev.* 67, 583–617.
- [2] McCray, J.A. and Trentham, D.R. (1989) *Rev. Biophys. Chem.* 18, 239–270.
- [3] dams, S.R. and Tsien, R.Y. (1993) *Annu. Rev. Physiol.* 55, 755–784.
- [4] Morgan, C.G., Thomas, E.W., Yianni, Y.P. and Sandhu, S.S. (1985) *Biochim. Biophys. Acta* 820, 107–114.
- [5] Sandhu, S.S., Yianni, Y.P., Morgan, C.G., Taylor, D.M. and Zaba, B. (1986) *Biochim. Biophys. Acta* 860, 253–262.
- [6] Morgan, C.G., Thomas, E.W., Sandhu, S.S., Yianni, Y.P. and Mitchell, A.C. (1987) *Biochim. Biophys. Acta* 903, 504–509.
- [7] Morgan, C.G., Sandhu, S.S., Yianni, Y.P. and Dodd, N.J.F. (1987) *Biochim. Biophys. Acta* 903, 495–503.
- [8] New, R.R.C. (1990) *Liposomes: a Practical Approach*, IRL Press, Oxford.
- [9] Morgan, C.G., Mitchell, A.C. and Chowdhary, R.K. (1991) *Proc. SPIE* 1525, 391–396.
- [10] Bisby, R.H., Morgan, C.G. and Munro, L.H. (1992) *Free Rad. Res. Commun.* 16, 65–71.
- [11] Taylor, K.M.G. and Morris, R.M. (1995) *Thermochim. Acta* 248, 289–301.
- [12] Song, X., Perlstein, J. and Whitten, D.G. (1995) *J. Am. Chem. Soc.* 117, 7816–7817.
- [13] Jansen, M. and Blume, A. (1995) *Biophys. J.* 68, 997–1008.
- [14] Ueno, M., Yoshida, M. and Horikoshi, I. (1991) *Bull. Chem. Soc. Jpn.* 64, 1588–1593.
- [15] van Osdel, W.W., Johnson, M.L., Ye, Q. and Biltonen, R.L. (1991) *Biophys. J.* 59, 775–785.