Biochemical and Biophysical Research Communications **276,** 169–173 (2000)
doi:10.1006/bbrc.2000.3456, available online at http://www.idealibrary.com on IDEAL®

Wavelength-Programmed Solute Release from Photosensitive Liposomes

Roger H. Bisby,[1] Carole Mead, and Christopher G. Morgan
Division of Biological Sciences, School of Environmental and Life Sciences, University of Salford, Salford M5 4WT, United Kingdom

Received August 10, 2000

Liposomes of dipalmitoylphosphatidylcholine containing a photochromic lipid "Bis-Azo PC" release entrapped solutes on exposure to UV light. We have now demonstrated that on addition of cholesterol (up to 25 mol%) to the liposomal membrane the liposomes also release their contents in response to visible light in the region of 470 nm, to which liposomes lacking steroid are insensitive. In a mixed population of liposomes prepared with and without cholesterol, this enables wavelength-dependent release of entrapped solutes on sequential exposure to visible and UV light. Furthermore, the cholesterol-containing liposomes allow stepped partial release of entrapped solute following multiple periods of short visible illumination. It is suggested that the cholesterol-containing liposomes may be potentially useful for drug delivery and for "caging" of reagents.

Key Words: **azobenzene; caged reagents; cholesterol; liposome; photoisomerization.**

Liposomes have been investigated extensively both as models for biological membrane structure and function and as potential vehicles for targeted delivery and controlled release of drugs. In the latter area, much effort has been directed towards the development of liposomes that can be destabilized particularly in response to light (1). The most common approaches involve light-sensitized production of reactive species such as singlet oxygen (2), photopolymerization of membrane components (3) and photoisomerization of agents within the bilayer (4). Photoisomerization is a reversible process in some cases, with the isomerizable species reaching a photostationary-state equilibrium dependent on the wavelengths of illumination. Liposome properties might thus be controlled according to this photostationary state composition. In earlier work (5–7) photosensitive liposomes were described based on a host matrix of gel-phase phospholipid incorporating a synthetic phospholipid with acyl chains bearing azobenzene moieties. The lipid "Bis-Azo PC" (Fig. 1) is a compact molecule that exists as the stable E-isomer that is sterically compatible with a close-packed stable lipid bilayer. Liposomes of gel-phase lipid containing up to 10% (mol:mol) of this photosensitive lipid are stable in the dark and retain trapped solutes for months at room temperature. On photoisomerization with ultraviolet light a photostationary state dominated by the more bulky Z-isomer of the azobenzene moieties is formed. Solute release rates after UV exposure are markedly sensitive to temperature, and millisecond release kinetics can be achieved (8, 9) especially when the bilayer membrane contains cholesterol which enhances the release rate of trapped marker dye (10). Liposomes containing Bis-Azo PC in the absence of cholesterol show a threshold of photoisomerization below which leakage of trapped marker dye is not seen. Below about 3% of Bis-Azo PC (mol:mol), no leakage of trapped marker is seen on UV photolysis. The threshold effect has been studied by pulsed laser experiments in our earlier work (8). It was shown that samples containing Bis-Azo PC could be exposed to several low-intensity UV pulses without significant leakage of contents, but that rapid leakage ensued after a sufficient number of such exposures or above a threshold intensity. Inclusion of cholesterol has the effect of reducing the extent of photoisomerization necessary for leakage to occur, effectively increasing the sensitivity to light for liposomes having a given concentration of azobenzene derivative.

The influence of cholesterol on the properties of photosensitive liposomes suggested a possible means to control their permeability selectively. Control of the extent of photoisomerization can be achieved by limiting exposure to light, or more conveniently by manipulation of the photostationary state composition of the isomerizable species. The photostationary state composition of azobenzene derivatives depends on the wave-

[1] To whom correspondence should be addressed at Division of Biological Sciences, Peel Building, University of Salford, Salford M5 4WT, UK. Fax: +(44) 161 295 5210. E-mail: r.h.bisby@salford.ac.uk.

0006-291X/00 $35.00

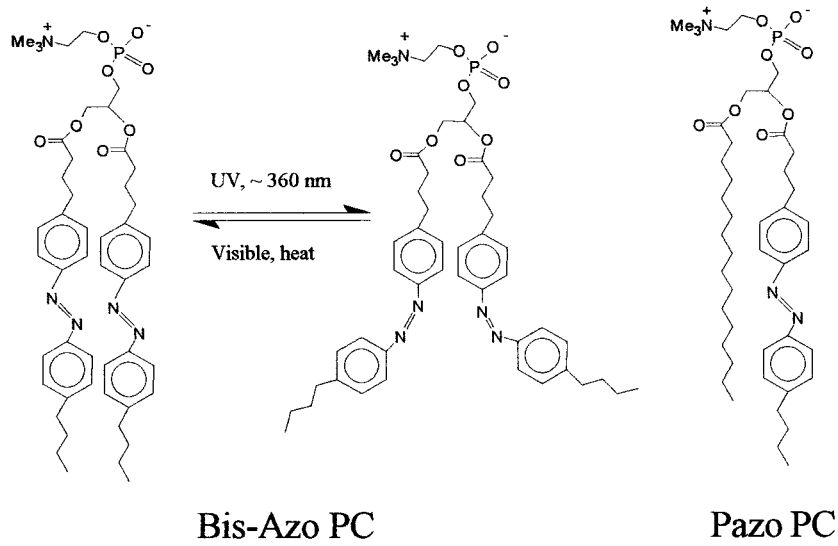

FIG. 1. Structures of the photochromic lipids, Bis-Azo PC and Pazo PC described in this work. The $E \rightarrow Z$ isomerization of Bis-Azo PC on illumination with UV light (~360 nm) is illustrated.

length of light used for isomerization (11) and the absorption spectrum of Bis-Azo PC in liposomes has been previously reported (6). Excitation at around 360 nm produces a photostationary state composition where the Z-isomer of azobenzene predominates. This absorbs more strongly around 420 nm than does the E-isomer, so that illumination with blue light causes partial reversion to the E-isomer. Coillumination by blue light and UV light can thus be used to determine the photostationary state composition of azobenzene derivatives according to the relative intensities of the two sources. Alternatively, an intermediate wavelength can be chosen to produce the desired photostationary state mixture of isomers.

In view of the observation that cholesterol reduces the threshold for UV-induced solute release, it was reasoned that solutes might be released from cholesterol-containing liposomes by light of a wavelength that has little or no effect on liposomes in absence of cholesterol.

MATERIALS AND METHODS

Bis-Azo PC was prepared as previously described (5). All other chemicals used were purchased from Sigma/Aldrich and used without further purification. Liposomes containing trapped marker dyes were prepared by extrusion under nitrogen pressure followed by gel-filtration as described previously (9). A blue LED (Nichia, 470-nm peak) was focused to give a fluence of approximately 20 mW cm^{-2} and was employed both to provide photolysis at 470 nm and to excite calcein fluorescence. Sulforhodamine-B fluorescence was excited by a filtered quartz-halogen source which was modulated with a square wave at 4 kHz using a liquid-crystal shutter (Meadowlark Optics Ltd, U.S.A.) and detected selectively using an SR830DSP lock-in amplifier from Stanford Instruments. UV excitation from a Spectron SL401 laser was provided as a 8 ns, 15 mJ pulse at 355 nm as described previously (9). Other experimental conditions are given in the figure legends.

RESULTS AND DISCUSSION

Figure 2 shows leakage of the impermeable fluorescent marker calcein from liposomes prepared from a mixture of 1,2-(dihexadecanoyl)-*sn*-glycerophosphocholine (dipalmitoyl-L-α-phosphatidylcholine, DPPC) and varying levels of cholesterol, containing a fixed level of 6% (mol:mol relative to total lipid) Bis-Azo PC. The samples were exposed to blue light from a light-emitting diode (LED) with emission centered at 470 nm, which also served to excite fluorescence from the calcein. Liposomes containing up to about 5% cholesterol (mol:mol relative to

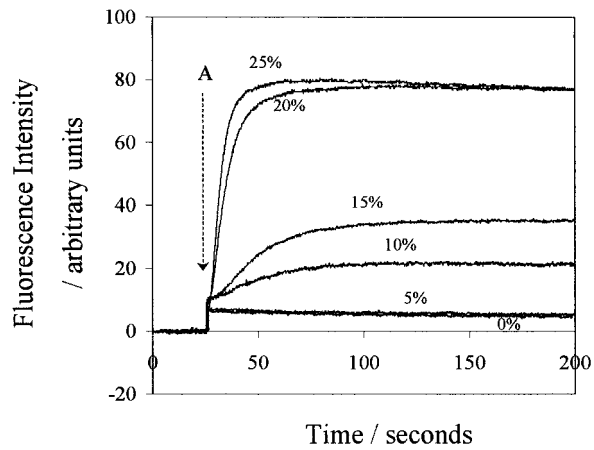

FIG. 2. Increase in fluorescence intensity at 510 nm (arbitrary units) indicating leakage at 25°C of entrapped calcein dye from liposomes containing increasing amounts of cholesterol. Exposure to blue light centered at 470 nm, which was also used to excite fluorescence, began at the point A. The liposomes were composed of DPPC with 6 mole% Bis-Azo PC and the indicated amounts of cholesterol between 0 and 25 mole%. The overall lipid concentration was 0.1 mg cm^{-3}.

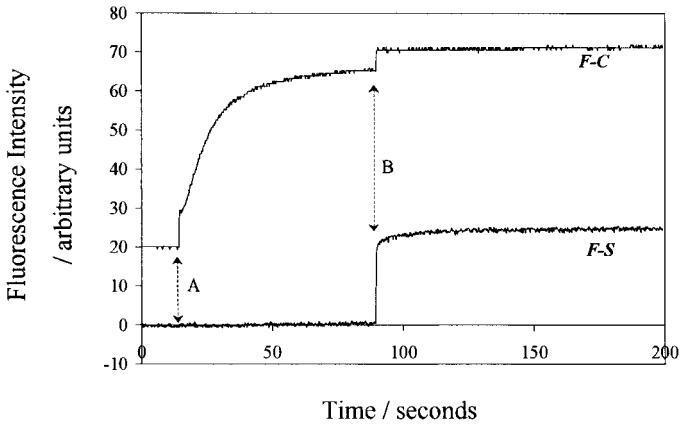

FIG. 3. Selective release by UV and visible light of entrapped dyes from a mixture of liposomes with and without cholesterol. One set of liposomes of DPPC with 6 mole% Bis-Azo PC (no cholesterol) contained entrapped sulforhodamine-B. The second population of liposomes of DPPC with 6 mole% Bis-Azo PC and 20 mol% cholesterol contained entrapped calcein. A mixture of equal amounts of the liposomes was exposed at 25°C to blue (470 nm) light from an LED source at the point marked A. After a short delay, the sample was exposed to a single UV laser pulse (355 nm, 15 mJ) at the point marked B. The experimental method used to discriminate between calcein and sulforhodamine-B fluorescence is described in Fig. 4. The total lipid concentration in the sample was 1 mg cm^{-3}.

total lipid) are not sensitive to light of this wavelength, and no release of trapped dye is seen, even on prolonged exposure to light. Very rapid release of trapped marker is however seen at higher cholesterol levels.

Previous work has shown that liposomes of DPPC containing Bis-Azo PC at the concentration used in this experiment release trapped marker dye rapidly on exposure to near-UV light, whether or not cholesterol is present. Consequently, using the same photosensitive agent at the same concentration, it is possible to prepare two sets of liposomes, both of which leak contents on light exposure, but which have different wavelengths of activation. Figure 3 shows how a mixture of liposomes prepared with and without inclusion of cholesterol can be used to release two different marker species rapidly and sequentially in response to illumination with different wavelengths of light. Liposomes of DPPC containing 20% cholesterol (mol:mol relative to total lipid) were prepared with the trapped marker dye calcein, while DPPC liposomes without cholesterol were prepared similarly, but containing trapped sulforhodamine-B. Both sets of liposomes contained 6% Bis-Azo PC (mol:mol, relative to total lipid). At the point marked A in Fig. 3, a mixture of equal amounts of the liposomes was exposed at 25°C to blue (470 nm) light from an LED source which also excited fluorescence from calcein as it was released from the cholesterol-containing liposomes. The increase in fluorescence at 510 nm (curve marked $F-C$) indicates release of entrapped calcein from the DPPC-cholesterol liposomes. There was no increase in fluorescence from the sulforhodamine-B dye at 590 nm (curve $F-S$) in the second population of liposomes (without cholesterol) until the sample was exposed to a single UV laser pulse (355 nm, 15 mJ) at the point marked B. Sulforhodamine-B fluorescence was excited by green light at 550 nm that has little effect on the photostationary state of Bis-Azo PC. To perform this experiment, a lock-in detection scheme was used (Fig. 4). This was necessary to allow the emission of sulforhodamine-B (which was of relatively low intensity) to be distinguished from the long-wavelength "tail" of the calcein fluorescence.

In the course of these experiments another interesting difference was observed between photosensitive liposomes prepared with- and without cholesterol. In Fig. 5A the time course of calcein release from liposomes of DPPC containing Bis-Azo PC is shown after brief exposure to 365 nm UV light. After a rapid "burst" of release, the liposomes continue to release trapped marker slowly in the dark over a period of up to 2 h. A contrasting result is seen in Fig. 5B for liposomes containing 20% cholesterol (mol:mol, relative to total lipid) and the same level of Bis-Azo PC as used in Fig. 5A. Here the release of calcein is seen to occur only during the period of light exposure. Liposome contents can be released as a series of "pulses" by intermittent expo-

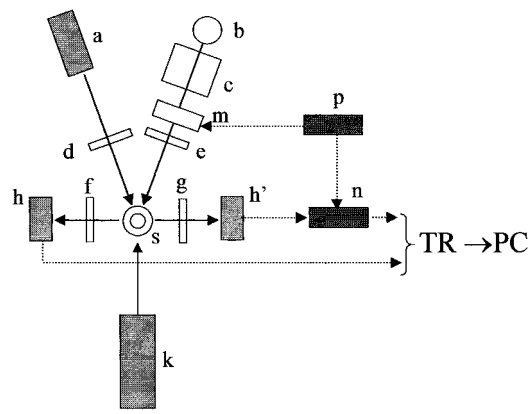

FIG. 4. Apparatus used for the simultaneous and separate measurement of fluorescence from calcein (λ_{max} 510 nm) and sulforhodamine-B (λ_{max} 590 nm). Calcein fluorescence from the sample in thermostated quartz tube (s) was excited by a blue LED (a) in conjunction with a 470 nm interference filter (d) which also constituted the blue light photolysis source. A photodiode (h) detected calcein fluorescence through a 510 nm interference filter (f). Sulforhodamine-B was excited using a quartz-halogen lamp (b), aqueous copper sulfate solution heat filter (c) and 550 nm interference filter (e). This beam was subject to square-wave modulation at 4 kHz using a liquid crystal light valve (m) driven by an oscillator (p). A second photodiode (h′) detected sulforhodamine-B fluorescence through a 590 nm interference filter (g). The sulforhodamine-B fluorescence signal modulated at 4 kHz is separated from the interfering unmodulated calcein fluorescence by use of the lock-in amplifier (n). The processed signals were recorded on a transient recorder (TR) linked to a PC. The sample was also irradiated with a UV pulse from the Nd:YAG laser (k).

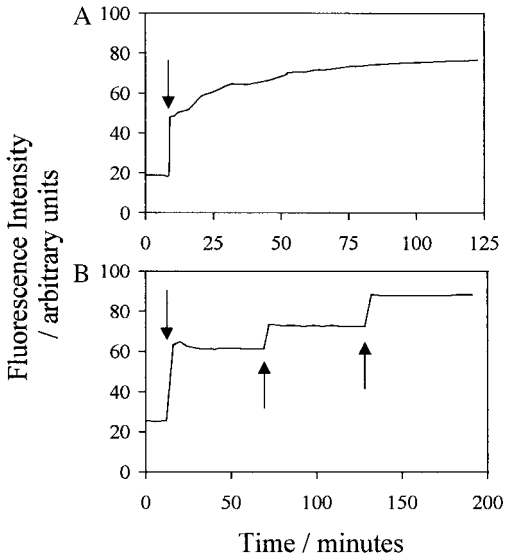

FIG. 5. Increase in calcein fluorescence on release from DPPC liposomes containing 6 mole% Bis-AzoPC at 22°C. Illumination with 365 nm light from a UVG-58 (UVP Ltd) handlamp (incident light intensity ~4 mW cm^{-2} is indicated by arrows). (A) Liposomes in the absence of cholesterol were exposed to a single brief (25 s) period of illumination; (B) liposomes of DPPC containing both 6 mole% Bis-AzoPC and 20 mole% cholesterol were illuminated with repeated very brief (~1 s) exposures. For these experiments the liposomes were diluted to a lipid concentration of 10^{-2} mg cm^{-3}.

sure to UV light for brief periods as shown in Fig. 5B. These results were achieved with very low exposure to UV light. Leakage during exposure to light is likely to be due to bilayer disorder resulting from the continuous cycling of azobenzene units between isomeric forms. Such cycling continues, even when the photostationary state composition has been reached. After illumination ceases the photostationary state composition provides a residual level of disorder that has effects on permeability dependent on membrane composition. In gel-phase lipids where diffusion is relatively slow, isomer distribution might not be at equilibrium after photolysis, and this might also have time-dependent effects on membrane permeability.

It is suggested that, at least in part, the effects of cholesterol in sensitizing the liposomes to blue light arise from cholesterol-induced phase separations in the bilayer. Recent work has suggested that lipid domain formation is promoted by cholesterol in bilayer membranes of saturated gel-phase lipids (12), such as were used in our experiments. Further support for this proposal has been obtained from our previous experiments (10) involving photoisomerization of lipids containing a single azobenzene moiety ("Pazo PC," see Fig. 1). In the absence of cholesterol, isomerization of Pazo PC has little effect on liposome permeability, even at high concentration of the photochromic species. Liposomes containing cholesterol, however, release trapped marker rapidly on isomerization of single-chain azobenzene derivatives. This suggests that the Pazo PC-rich domains might be formed on addition of cholesterol giving rise to sites with a lower threshold for light-induced solute leakage. The possible role of phase-separation in the leakage process needs further investigation and calorimetric measurements, NMR data and freeze-fracture electron microscopy would be useful in this respect.

The results presented demonstrate that a controlled release of solutes in a programmed fashion is easily achieved using photosensitive liposomes. These findings could have practical application both in scientific areas, such as sequential release of "caged" reagents, and in technological areas such as photo-stimulated drug delivery. Although blue light and UV light do not penetrate deeply into tissues, liposomes such as described might find use as adjuncts to photodynamic therapy in accessible areas such as skin, eyes and mucous membranes. It is noteworthy that photostimulated release from liposomes can be achieved with very low exposure to light, for example in seconds or less using the light intensity and wavelengths associated with cosmetic "sun-tanning" apparatus. Photo-release induced by blue light might be particularly useful for ocular drug delivery, since the relatively low illumination intensity is unlikely to be hazardous.

We believe that this is the first attempt to use control of photostationary state composition of a sensitizing species to achieve selective solute delivery, although Mueller *et al.* (13) have very recently demonstrated visible light-induced destabilization of liposomes sensitized with a cyanine dye.

ACKNOWLEDGMENT

This work was supported by BBSRC Project Grant 106/B09058 to C.G.M. and R.H.B. and this funding is gratefully acknowledged.

REFERENCES

1. Gerasimov, O. V., Boomer, J. A., Qualls, M. M., and Thompson, D. H. (1999) Cytosolic drug delivery using pH- and light-sensitive liposomes. *Adv. Drug Deliv. Rev.* **38,** 317–338.
2. Thompson, D. H. Gerasimov, O. V. Wheeler, J. J., and Anderson, V. C. (1996) Triggerable plasmalogen liposomes: Improvement of system efficiency. *Biochim. Biophys. Acta* **1279,** 25–34.
3. Bondurant, B., and O'Brien, D. F. (1998) Photoinduced destabilization of sterically stabilized liposomes. *J. Am. Chem. Soc.* **120,** 13541–13542.
4. Pidgeon, C., and Hunt, C. A. (1983) Light sensitive liposomes. *Photochem. Photobiol.* **37,** 491–494.
5. Morgan, C. G., Thomas, E. W., Sandhu, S. S., and Yianni, Y. P. (1985) Incorporation of a novel photochromic phospholipid molecule into vesicles of dipalmitoylphosphatidylcholine. *Biochim. Biophys. Acta* **820,** 107–114.
6. Morgan, C. G, Sandhu, S. S., Yianni, Y. P., and Dodd, N. J. F. (1987) The phase behaviour of dispersions of Bis-Azo PC—Photoregulation of bilayer dynamics via lipid photochromism. *Biochim. Biophys. Acta* **903,** 495–503.
7. Morgan, C. G., Thomas, E. W., Sandhu, S. S., Yianni, Y. P., and

Mitchell, A. C. (1987) Light-induced fusion of liposomes with release of trapped marker dye is sensitized by photochromic phospholipid. *Biochim. Biophys. Acta* **903,** 504–509.

8. Morgan, C. G., Bisby, R. H., Johnson, S. A., and Mitchell, A. C. (1995) Fast solute release from photosensitive liposomes—An alternative to caged reagents for use in biological systems. *FEBS Lett.* **375,** 113–116.
9. Bisby, R. H., Mead, C., Mitchell, A. C., and Morgan, C. G. (1999) Fast laser-induced solute release from liposomes sensitized with photochromic lipid: Effects of temperature, lipid host, and sensitizer concentration. *Biochem. Biophys. Res. Commun.* **262,** 406–410, doi:10.1006/bbrc.1999.1206.
10. Bisby, R. H., Mead, C., and Morgan, C. G. (1999) Photosensitive liposo 'cages' for laser-triggered solute delivery: The effect of bilayer cholesterol on kinetics of solute release. *FEBS Lett.* **463,** 165–168.
11. Ross, D. L., and Blanc, J. (1971) Photochromism by *cis-trans* isomerization. *In* Techniques of Chemistry: Photochromism (Brown, G. H., Ed.), Vol. III, pp. 471–556, Wiley Interscience, New York.
12. Xu, X., and London, E. (2000) The effect of sterol structure on membrane lipid domains reveals how cholesterol can induce lipid domain formation. *Biochemistry* **39,** 843–849.
13. Mueller, A., Bondurant, B., and O'Brien, D. F. (2000) Visible-light-stimulated destabilization of PEG-liposomes. *Macromolecules* **33,** 4799–4804.