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Light-induced fusion of liposomes with release of trapped marker dye is sensitised by photochromic phospholipid

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Liposomes have been prepared from dipalmitoylphosphatidylcholine containing small amounts of a synthetic photochromic phospholipid, 'Bis-Azo PC'. In the dark, these are stable at room temperature, and contents do not significantly leak over weeks. Photoisomerisation results in immediate release of trapped marker, and in liposome fusion to form larger structures. Fusion has been detected using a fluorescence polarisation assay, and confirmed by electron microscopy. In mixtures, fusion occurs between 'photochromic' liposomes and those of pure lipid. Bis-Azo PC contains two photochromic acyl chains; analogues bearing a single photochromic chain appear to have little effect on bilayer permeability after isomerisation. Photo-induced leakage and liposome fusion suggest possible applications for localised drug delivery as an adjunct to phototherapy. The ability to non-invasively trigger fusion processes should be useful in fundamental studies of membrane interactions. We believe this to be the first report of photo-induced fusion to date.

Introduction

Recognition of the central role of photoisomerisation in visual processes has stimulated considerable interest in photochromism as a control process in a more general sense [1–3]. To date, relatively little work has considered modulation of lipid bilayer cooperativity using photochromic compounds. Most previous work of this sort has concentrated on *cis-trans* isomerisation in single chain azobenzene-containing amphiphiles [4]; *trans* isomers are essentially linear, while the *cis* forms have 'kinked' chains. In the cited study and others, interpretation was complicated by concentration-dependent effects on bilayer properties of the photochromic detergent. Recently, we introduced a photochromic phospholipid, Azo-PC, designed to avoid some uncertainties in previous work [5]. The phospholipid gave broadly similar results to those previously obtained by others using detergents [4]. Photoisomerisation caused no loss of bilayer integrity, but enhanced water permeability and proton permeability were observed. Although clear effects of isomerisation on phase equilibria were seen, experiments showed no obviously enhanced release of trapped marker dye on photolysis.

In this paper we report on a new photochromic phospholipid, Bis-Azo PC (Fig. 1). In size, Bis-Azo PC resembles dipalmitoylphosphatidylcholine (DPPC), and we have previously shown that the

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Abbreviations: Bis-Azo PC, 1,2-bis(4-(4-*n*-butylphenylazo)-phenylbutyroyl)-L-α-phosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; LUV, large unilamellar vesicles; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine.

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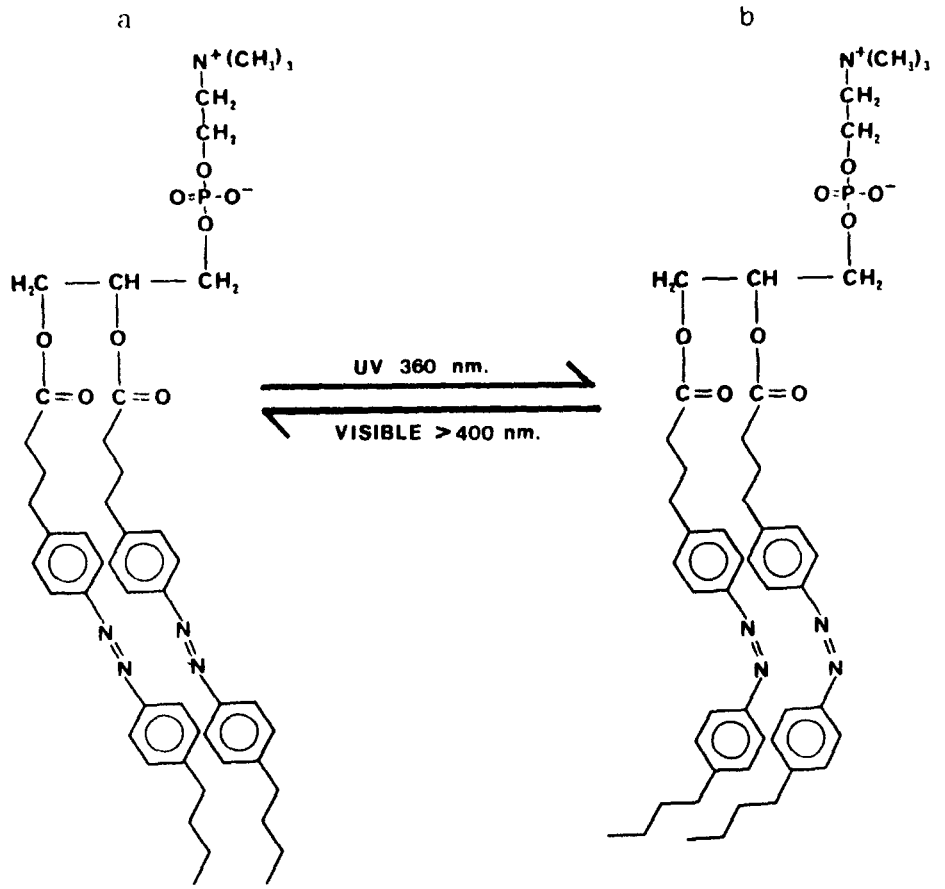

Fig. 1. The structure of Bis-Azo PC (bis-(4-*n*-butylphenylazo-4′-phenylbutyroyl)-L-α-phosphatidylcholine) in (a) the *trans* form, and (b) the *cis* form.

molecule forms dispersions having a highly cooperative phase transition at a similar temperature to that of DPPC (though with very marked hysteresis between heating and cooling cycles). On photoisomerisation, Bis-Azo PC increases markedly in effective molecular area [6] and the interchain interactions are expected to be highly disordering to any bilayer containing the molecule.

Materials and Methods

Phospholipids were from Sigma Ltd. and were used without further purification, as was 1,6-diphenyl-1,3,5-hexatriene (DPH). 1,2-Bis(4-(4-*n*-butylphenylazo)phenylbutyroyl)-L-α-phosphatidylcholine (Bis-Azo PC) was synthesized as previously described [6].

Small unilamellar vesicles (SUV) were prepared by injection of ethanolic solutions containing phospholipid and Bis-Azo PC at appropriate concentrations into vortexing buffer [7].

Large unilamellar vesicles (LUV) of β-γ-dipalmitoyl-L-α-phosphatidylcholine (DPPC) containing Bis-Azo PC at appropriate concentrations were prepared by a modified deoxycholate removal method as previously described [8]. Calcein was included in the lipid/deoxycholate dispersion at a concentration of 30 mM and trapped within the LUV upon their formation. Untrapped material was removed by elution of vesicle fractions on a Sephadex G50 (medium) column (Pharmacia Ltd.) with eluting buffer containing EDTA for osmotic balance and as a chelator of metal impurities. Leakage of calcein was moni-

tored fluorimetrically as previously described [8,9]. Fluorescence polarisation measurements were made using a 'T-format' apparatus similar to that previously described [9]. The phase behaviour of phospholipid dispersions incorporating Bis-Azo PC was monitored as a function of temperature by measurement of fluorescence depolarisation of added DPH [10].

Photoisomerisation of lipid dispersions containing Bis-Azo PC in the *trans* photostationary state was performed by irradiating samples with the focused output of a high pressure mercury arc lamp as previously described [6]. The *cis* photostationary state is achieved within minutes by such exposure [5]. After photoisomerisation samples were kept in the dark to avoid reversion of the *cis*-Bis-Azo PC to the *trans* form. Reisomerisation of irradiated samples to the *trans* photostationary state was accomplished by exposure of *cis* samples to unfiltered visible light from a tungsten lamp [5]. Photoisomerisation of samples presented in Fig. 4 was accomplished using long wavelength light from a hand-held ultraviolet lamp. This served a dual purpose in that it also allowed visualisation of the extent of calcein leakage from vesicles.

Where the phase behaviour of Bis-Azo PC-labelled vesicles was studied by fluorescence depolarisation, the probe DPH was added to lipid dispersions after photoisomerisation to the *cis* photostationary state in order to avoid photobleaching of the probe by the ultraviolet output from the mercury arc lamp. DPH rapidly binds to vesicles, as indicated by the rapid increase in fluorescence of a sample to which the probe has been added.

Electron microscopy was performed using a Corinth 500 electron microscope. The samples were negatively stained with uranyl acetate on Formvar-coated grids previously treated with bacitracin.

Results

Fig. 2a, trace i, shows the temperature–polarisation profile measured immediately after vesicle preparation, and this profile was not significantly altered on repeated cycles of heating and cooling. Exposure of a sample of the lipid to 360 nm light from a medium pressure mercury lamp for several minutes caused a marked increase in vesicle turbidity. This might have been due to refractive index changes, vesicle aggregation or fusion. Control experiments using a lipid with a single

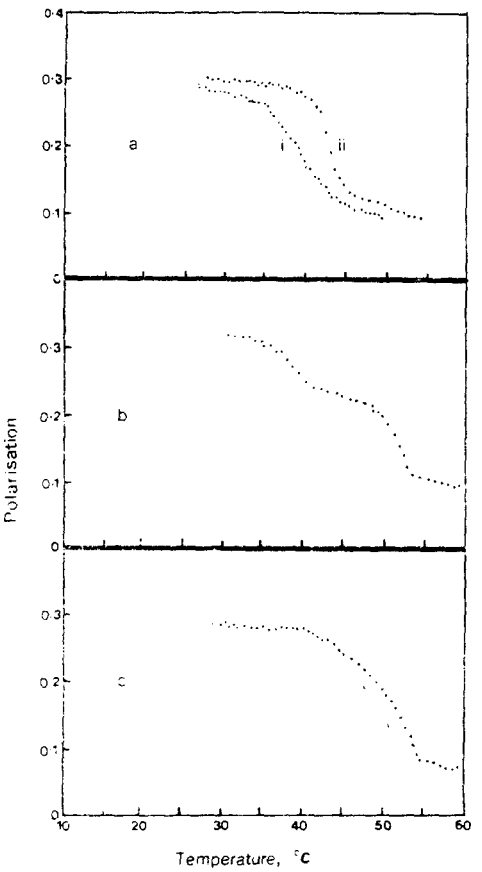

Fig. 2. Fluorescence polarisation vs. temperature profiles of lipid vesicles containing Bis-Azo PC, monitored by the fluorescent probe DPH. Vesicles were prepared by ethanol injection and were of the small unilamellar type (as in Ref. 7). Buffer was 50 mM phosphate, 0.02% NaN_3 (pH 7.4). (a) Trace i, shows the profile for vesicles of DPPC containing 6% (w/w) of Bis-Azo PC in the *trans* photostationary state; ii shows the profile for DPPC vesicles after photoisomerisation of the photochromic lipid to the *cis* photostationary state. The phase transition is considerably sharpened and shifted to higher temperature. Trace (b) shows a fluorescence polarisation vs. temperature profile for a mixture of vesicles of DPPC and vesicles of DSPC (each vesicle subset containing 6% (w/w) Bis-Azo PC) measured prior to photoisomerisation, showing transitions for each phospholipid component. Trace (c) shows the effect of photoisomerisation of a sample identical to that shown in profile (b). Such profiles are usually observed with vesicles formed from a mixture of DPPC and DSPC. The result indicates that photoisomerisation causes either lipid exchange or fusion.

azobenzene-containing chain showed that refractive index changes on isomerisation would decrease light scattering [5]. Photolysis of DPPC vesicles containing the single chain lipid results in photoisomerisation, but not in vesicle fusion. Light scattering from 'isomerised' vesicles is lower in this case than from unphotolysed vesicles, and recovers on reisomerisation to the *trans* photostationary state. Fig. 2a, trace ii, shows fluorescence polarisation vs. temperature for a lipid sample after photoisomerisation (DPH added after irradiation to avoid photobleaching). The transition is markedly sharpened and shifted to higher temperature. The profile is not significantly altered by visible light exposure, which rapidly reverses the ultraviolet-induced isomerisation. The effect of isomerisation on the phase behaviour is consistent with fusion of small unilamellar vesicles to give larger, more highly cooperative structures [11]; aggregation or lipid exchange will not give these changes. Confirmation of vesicle fusion was provided by the temperature profiles shown in Fig. 2b and 2c. Vesicles of distearoylphosphatidylcholine (DSPC) were prepared containing the same molar percentage of Bis-Azo PC as in the DPPC vesicles. A mixture of the two Bis-Azo-PC-labelled vesicle types was prepared and split into two portions, one of which was photoisomerised. After labelling with DPH, the phase transition profile was as shown in Fig. 2b, with individual phase transitions clearly visible. The isomerised sample was as shown in Fig. 2c, with a broad intermediate transition characteristic of vesicles prepared from a mixture of DPPC with DSPC [12]. If *cis*-isomerised vesicles not previously heated are exposed to visible light, rapid reversion to the *trans* photostationary state occurs. Such vesicles show broad 'mixed lipid' transitions. This suggests that the lipid mixing observed is not a consequence of the sample heating during measurement of fluorescence polarisation. Fluorescence measurements also show that DPPC vesicles containing Bis-Azo PC, on ultraviolet irradiation, can form mixed lipid structures with similar vesicles containing no Bis-Azo PC, so long as the unlabelled vesicles are not in large excess. These results (not shown) will

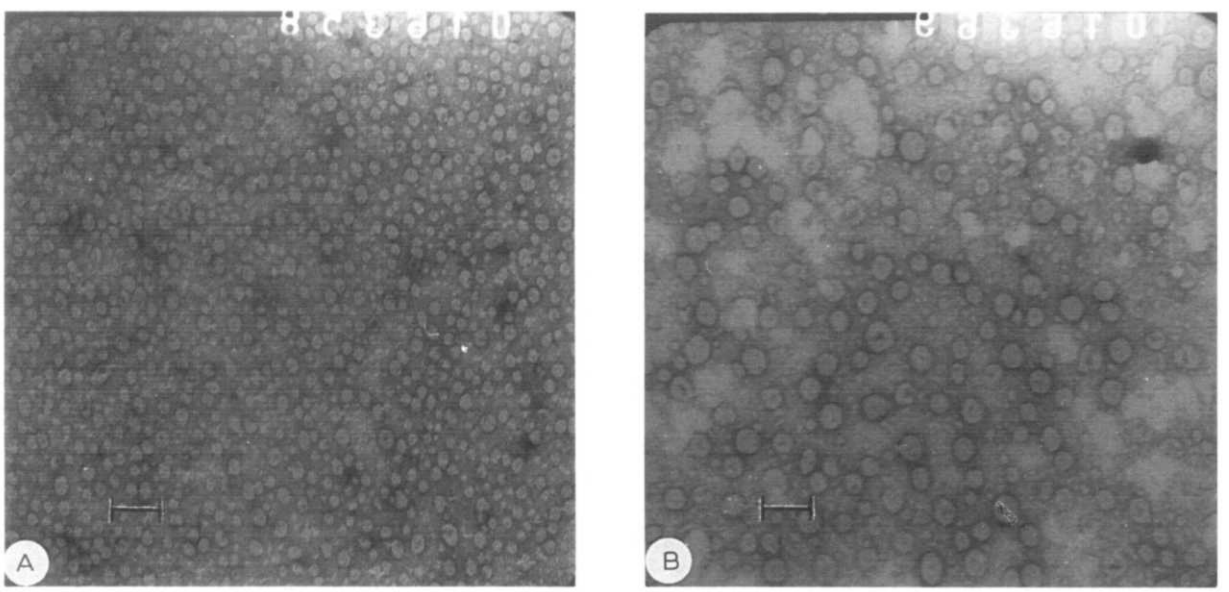

Fig. 3. Electron micrographs of vesicles of DPPC containing 6% (w/w) Bis-Azo PC. Samples were negatively stained with 1% uranyl acetate. Micrograph (a) shows vesicles prior to photoisomerisation. Magnification, ×60000; bar = 100 nm. Micrograph (b) shows vesicles after photoisomerisation to the *cis* photostationary state with long wavelength light from a hand-held ultraviolet lamp for 5 min. Vesicles were prepared by deoxycholate dilution as described in the text and negatively stained with 1% uranyl acetate solution. (Note that the presence of Bis-Azo PC depresses vesicle size relative to pure DPPC vesicles prepared under similar conditions). Magnification, ×60000; bar = 100 nm.

be discussed in detail in a later publication.

Further evidence that vesicle interaction is a result of fusion to form larger structures, rather than mere lipid exchange, and that such fusion proceeds at room temperature, is provided by electron microscopy. Electron micrographs of DPPC vesicles containing Bis-Azo PC are shown in Fig. 3a. After isomerisation, vesicular structures are replaced by a fusion product which shows larger, apparently unilamellar structures (Fig. 3b). These are early fusion products. Very much larger structures appear on standing, and precipitation eventually occurs.

We note in passing that incorporation of Bis-Azo PC into DPPC vesicles appears to reduce the average vesicle size relative to vesicles of pure DPPC. Photoisomerisation also causes total loss of trapped marker from vesicles. Leakage of vesicle contents was studied using the impermeable fluorescent dye, calcein, which is self-quenched when trapped in vesicles [13]. LUV containing trapped calcein were prepared from a mixture of DPPC with 6% (w/w) Bis-Azo PC as previously described for DPPC [8]. Leakage of calcein results in intense fluorescence. On exposure of calcein-loaded LUV to 360 nm light, rapid increase in fluorescence occurred, and total release of contents was essentially instantaneous with an intense mercury arc source. Fig. 4 shows the slower release caused by an ultraviolet handlamp. Photo-induced release of marker dye has previously been demonstrated for vesicles of pure retinoyl phospholipids [14], though sensitised release from DPPC vesicles accompanied by vesicle fusion has never before been reported to our knowledge.

Photo-induced fusion should provide a useful tool for scientific study of primary processes in vesicle and cell fusion. Preaggregation of vesicles (e.g., with low concentrations of poly(ethylene glycol) [15]), would allow time-resolved studies of membrane perturbations, free of complications of diffusional encounters. Photo-induced release of vesicle contents might also find a role for flash synchronisation of cellular processes. A very interesting possibility is the application of photosensitive liposomes for localised drug delivery, and as an adjunct to phototherapy using sensitisers [16]. Although Bis-Azo PC is sensitive to ultraviolet light, which has a limited penetration in tissue,

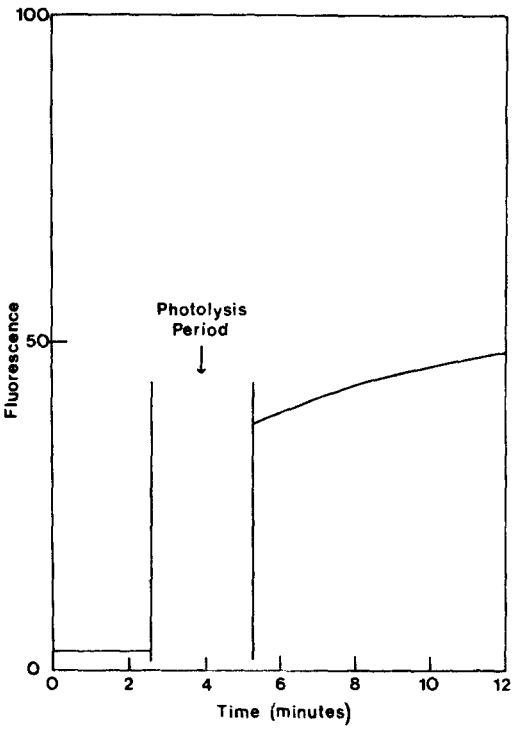

Fig. 4. Photo-induced leakage of entrapped calcein from vesicles of DPPC. Buffer was as in previous experiments, but with EDTA present to osmotically balance entrapped calcein (as in Ref. 8). Vesicle concentration was 0.2 mg lipid/ml. Full scale (100% leakage) was determined using Triton X-100 to totally lyse the vesicles. Vesicles were exposed to an ultraviolet handlamp (approx. 360 nm) for photolysis.

topical application might be possible. For example, drug-loaded liposomes have been located in lung tissue by intravenous injection and inhalation of aerosols [17] and localised release of soluble drugs might be contemplated. We note however, that Bis-Azo PC might be toxic and/or carcinogenic in animals, and therefore suggest that synthesis of analogues should be investigated. There seems no reason in principle why long-wavelength sensitive analogues of Bis-Azo PC should not be synthesised, and work is proceeding to this end.

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