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CONTROL OF PRO-OXIDANT ACTIVITY OF CUPRIC IONS BY ENTRAPMENT IN UNILAMELLAR LIPID VESICLES

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As a demonstration of a potential means of delivering and controlling the biochemical and biological activity of metal ions, cupric ions have been trapped in unilamellar phospholipid vesicles. The activity of these cupric ion-containing vesicles as catalysts of the autoxidation of ascorbate and epinephrine has been investigated. A marked increase in autoxidation rate was observed on release of the cupric ion on addition of detergent. When an azobenzene-containing photochromic lipid was incorporated in the bilayer membrane of the vesicles, the release of cupric ions could be initiated by irradiation with ultraviolet light. In the dark, these vesicles remained stable for at least several weeks. Photo-controlled release of liposomally-entrapped species might find application in areas similar to those where 'caged' reagents are presently used.

KEY WORDS: cupric ions, autoxidation, ascorbate, epinephrine, unilamellar vesicles, photochromism.

INTRODUCTION

Metal ions have varied and important functions in cell biology, and may also be components of chemical and biochemically based assay systems. New methods for the non-invasive control or release of metal ion activity might find applications in the initiation of an assay reaction or biochemical reaction or in investigations of the effects of metal ions on cellular processes. Transition metal ions, such as Cu(II) and Fe(II) in redox-active form, are catalysts of the Haber–Weiss reaction and contribute to the development of free radical induced cellular oxidative stress.[1] Transition metal ions are also employed as components of site-selective DNA cleavage reagents.[2–4]

Previous methodologies for the intracellular release of compounds include the use of "caged" compounds, such as photolabile derivatives of ATP and an acetylcholine analogue.[5] Such "caged" compounds can be released by flash illumination on a millisecond timescale. Light may also be used to affect various properties of polymers to which the photochromic azobenzene group has been covalently attached in order to provide photoresponsivity.[6] The azobenzene group undergoes reversible cis $\rightleftharpoons$ trans isomerization on illumination with light of a suitable wavelength, and properties of the molecule such as dipole moment, shape and size change significantly. Parameters such as viscosity, solubility, sample volume, enzymic activity and ion chelation may be reversibly affected.[6,7] It has also been shown that the permeability of

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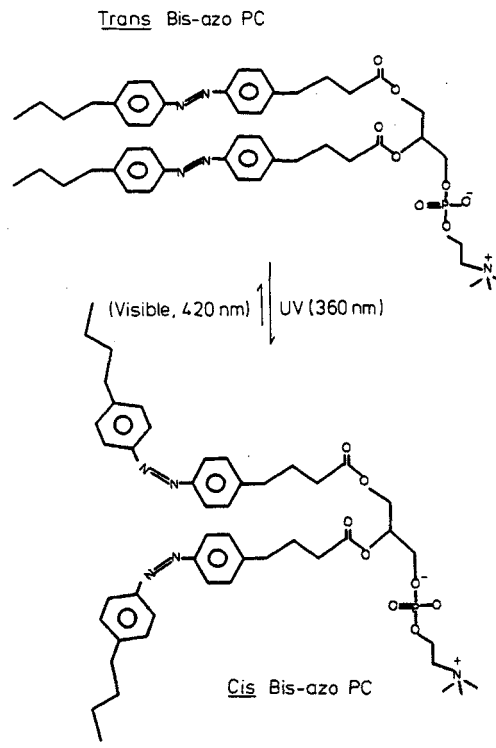

FIGURE 1 Structure of Bis-Azo PC [1,2-bis(4-(4-*n*-butylphenylazo)phenylbutyroyl)-phosphatidylcholine] and illustration of isomerization of the azobenzene groups by illumination.

membrane systems towards ions (Na^+ or K^+) may be photocontrolled either using an azobenzene-bridged crown ether[8] or by incorporation of an azobenzene-containing alkylammonium amphiphile into the membrane.[9,10] More recently a series of photochromic phospholipid compounds have been described which contain azobenzene groups in the fatty acyl chain(s).[10,11] Figure 1 shows the structure of one of these, Bis-Azo PC, and also illustrates the change in shape of this molecule on photoisomerisation. This structural change is sufficient to affect the physical properties of a bilayer into which the photochromic phospholipid is inserted.[13] Under appropriate conditions this can induce fusion and/or leakiness of the membrane vesicle.[14]

The present paper demonstrates the feasibility of using photochromic phospholipid vesicles as a means of containment of metal ions, and consequent restriction of their reactivity, until release is triggered by a brief exposure to ultra-violet irradiation.

MATERIALS AND METHODS

DL-α-Dipalmitoylphosphatidylcholine (DPPC) was obtained from Sigma. Other chemicals were AnalaR grade wherever possible. 1,2-Bis(4-(4-*n*-butylphenylazo) phenylbutyroyl)-phosphatidylcholine (Bis-Azo PC) was synthesised and purified

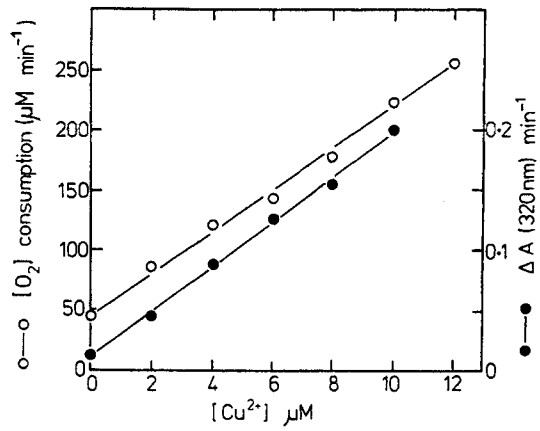

FIGURE 2 Effect of cupric ion concentration on A: the rate of oxygen consumption by solutions of ascorbate (0.5 mmol dm^{-3}) in phosphate buffer (0.2 mol dm^{-3}, pH 7.3); and B: the autoxidation rate of epinephrine (16 mmol dm^{-3}), measured by ΔA at 320 nm, in Tris buffer (0.05 mol dm^{-3}, pH 10.4).

as described previously.[12] Solutions were prepared with water from a Barnstead "Nanopure II" apparatus.

Ascorbate autoxidation was measured by determination of oxygen uptake in a pyrex oxygen electrode (Rank Brothers, Cambridge). Epinephrine autoxidation[15] was monitored at 320 nm in a Cecil model 272 spectrophotometer. Both assays were performed at 23°C. Unilamellar vesicles (ULV's) were prepared by repeated extrusion[16] at 60°C of an aqueous dispersion of phospholipid (5 mg/ml, DPPC or DPPC + 8 mol% Bis-Azo PC) containing $CuSO_4$ (0.8 mol dm^{-3}). Samples were extruded through polycarbonate filters (Nuclepore, pore size 100 nm) in a Lipex Thermobarrel Extruder (Lipex Biomembranes Inc, Vancouver). The ULV preparation was then separated from untrapped cupric ions by gel filtration on a column (1 × 12 cm) of Sephadex G-50. The vesicles appeared in the void volume of the eluate from this column. For ultraviolet irradiations the focused output of a high pressure mercury lamp was used after passing through a heat filter and a UV filter (60 nm bandwidth, centered at 360 nm).

RESULTS AND DISCUSSION

Cupric ions catalyse the autoxidation of both ascorbate and epinephrine.[18] Figure 2 shows the rate of oxidation of ascorbate (0.5 mmol dm^{-3}), measured from the rate of oxygen consumption in an oxygen electrode, as a function of cupric ion concentration in buffered solution at neutral pH (phosphate buffer, 0.2 mol dm^{-3}, pH 7.3). Also shown is the rate of oxidation of epinephrine (16 mmol dm^{-3}) at pH 10.4 in Tris buffer (0.05 mmol dm^{-3}), monitored by the formation of adrenochrome at 320 nm. Although both assays show detectable oxidation in the absence of added Cu^{2+}, presumably due to transition metal impurities in the aqueous buffer, they are sensitive to added Cu^{2+} at micromolar concentrations.

A typical oxygen uptake trace recorded on addition to an ascorbate solution of DPPC ULV's, prepared to contain entrapped Cu^{2+} as described in Materials and

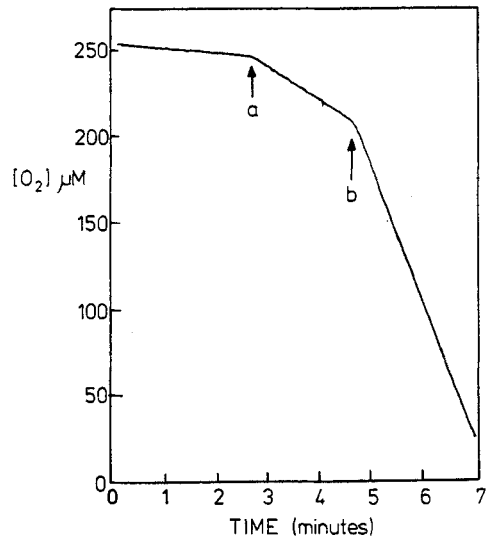

FIGURE 3 Oxygen consumption in an ascorbate solution (0.5 mmol dm^{-3}) in phosphate buffer (0.2 mol dm^{-3}, pH 7.3). At the point marked (a) was added an aliquot of DPPC unilamellar vesicles (100 μl), prepared to contain entrapped cupric ions as described in the text. At the point marked (b) Triton X-100 was added to a final concentration of 0.4 mmol dm^{-3}.

Methods, is shown in Figure 3. On addition of the vesicles the rate of oxidation increased slightly. A further pronounced increase in the rate of oxygen consumption and ascorbate oxidation was observed on addition of the detergent Triton X-100 (100 μl of a 20 mmol dm^{-3} aqueous solution). The increase in oxygen consumption on addition of the vesicles is due either to incomplete separation of untrapped cupric ions by the gel chromatography or adsorption of cupric ions to the external vesicle surface, since further measurements showed that leakage of Cu^{2+} from DPPC vesicles was insignificant when the vesicles were stored at room temperature for up to 3 weeks. Vesicles of DPPC are known to be impermeable to ionic solutes at temperatures below that of their thermotropic gel to liquid crystalline phase transition.[19] The addition of Triton X-100 is used to release trapped cupric ions from within the vesicles and make them available for catalysis of ascorbate oxidation. In a typical experiment, such as that shown in Figure 3, 3–4 μmol dm^{-3} of Cu^{2+} was released. The result of a similar experiment, but using the epinephrine oxidation assay, is shown in Figure 4. An appreciable increase in the rate of absorbance increase, indicative of substrate oxidation, is seen on release of trapped Cu^{2+} by addition of detergent. These results therefore show that using extrusion technology, measurable amounts of cupric ions may be trapped within vesicles of DPPC in the gel phase.

Vesicles composed of DPPC and the photochromic lipid Bis-Azo PC (8 mol%) were also prepared in a similar way with a view to trapping cupric ions within a photolabile vesicle. It has been demonstrated that vesicles formed with Bis-Azo PC release entrapped fluorescent markers[13] and calcium ions[20] on illumination. The results of adding cupric ion-containing DPPC/Bis-Azo PC vesicles to an ascorbate solution (0.5 mmol dm^{-3}, pH 7.3) are shown in Figure 5. As in the previous examples, the vesicle preparation contains some untrapped or adsorbed redox active cupric ions. In

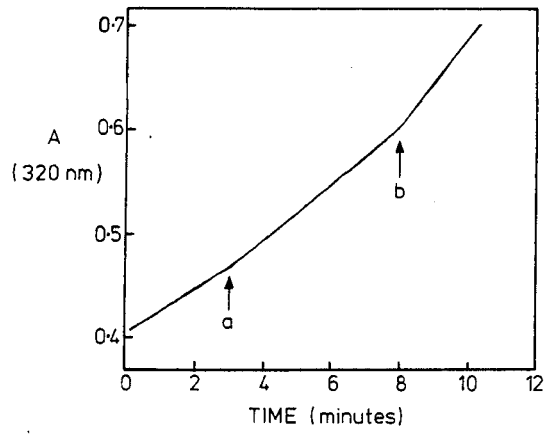

FIGURE 4 Rate of change of absorbance at 320 nm in a solution of epinephrine (16 mmol dm^{-3}) in Tris buffer (0.05 mol dm^{-3}, pH 10.4). An aliquot (100 μl) of DPPC unilamellar vesicles containing entrapped cupric ions, prepared as described in the text, was added at the point marked (a). At (b) Triton X-100 was added to a final concentration of 1 mmol dm^{-3}.

this case, untrapped cupric ions were titrated into a less redox active form with EDTA until the rate of oxygen uptake became negligible. The sample was then illuminated with ultra-violet light. As can be seen in Figure 5, following a short delay ($\sim$ 30 s) after the start of illumination the rate of oxygen uptake increased markedly. This may be

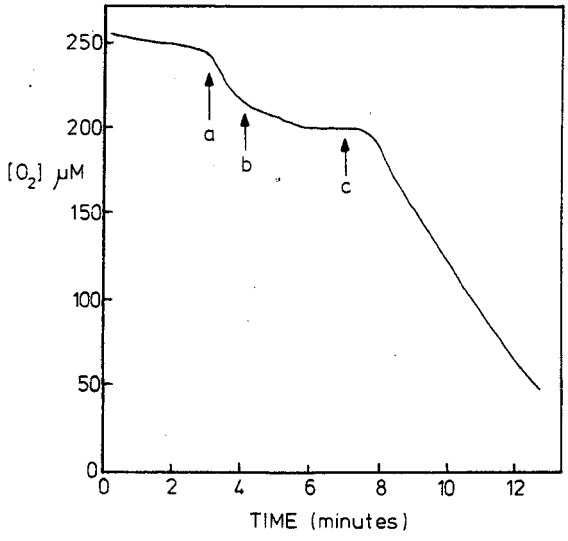

FIGURE 5 Oxygen uptake recorded in a solution of sodium ascorbate (0.5 mmol dm^{-3}) buffered to pH 7.3 with sodium phosphate (0.2 mol dm^{-3}). At the point marked (a) a 100 μl aliquot of Bis-Azo PC/DPPC (8 : 92 molar ratio) unilamellar vesicles prepared to contain trapped cupric ions was added. At point (b) EDTA (final concentration 75 μmol dm^{-3}) was added to chelate untrapped cupric ions, before the solution was irradiated with ultraviolet (360 nm) light at the point marked (c).

ascribed to the photo-initiated release of cupric ions from the Bis-Azo PC/DPPC vesicles. A control experiment showed that UV illumination had no effect on the rate of ascorbate oxidation.

These experiments act as a demonstration that metal ions such as Cu^{2+} can be trapped in photochromic phospholipid vesicles and released on brief illumination with near ultra-violet (~ 360 nm) light. In the experiments performed here, Cu^{2+} concentrations of several micromolar were released into solution. It is quite feasible that this could be substantially increased by both increasing the lipid concentration in the initial dispersion to give a higher Cu^{2+} trapping efficiency and by the use of membrane filters with a wider pore diameter to give larger vesicles with considerably greater trapped volumes.[16] More effective separation of untrapped metal ions from vesicles containing trapped metals is also required and could be achieved by ion-exchange chromatography.

The potential uses of vesicle-entrapped metal ions remain to be explored but may include the intracellular delivery of redox-active metal ions either by membrane fusion or by phagocytosis. This may be used to activate DNA cleavage agents. Another possibility is that they may be used to enhance the effects of redox-active drugs in the chemotherapy of parasitic diseases such as Leishmaniasis, the causative organisms of which are susceptible to oxidative stress and reside in phagocytic cells to where liposomes may be targetted.[21]

References

1. B. Halliwell and J.M.C. Gutteridge (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemical Journal*, **219**, 1–14.
2. A.M. Pyle, E.C. Long and J.K. Barton (1989) Shape-selective targetting of DNA by (Phenanthrenequinone diimine) rhodium III photocleaving agents. *Journal of the American Chemical Society*, **111**, 4520–4522.
3. D.W. Celander and T.R. Cech (1990) Iron(II)-ethylenediaminetetraacetic acid catalysed cleavage of RNA and DNA oligonucleotides: similar reactivity towards single and double stranded forms. *Biochemistry*, **29**, 1355–1361.
4. R.V. Prigodich and C.T. Martin (1990) Reaction of single stranded DNA with hydroxyl radical generated by Iron(II)-ethylenediaminetetraacetic acid. *Biochemistry*, **29**, 8017–8019.
5. J.A. McCray and D.R. Trentham (1989) Properties and uses of photoreactive caged compounds. *Reviews of Biophysical Chemistry*, **18**, 239–270.
6. G.S. Kumar and D.C. Neckars (1989) Photochemistry of azobenzene-containing polymers. *Chemical Reviews*, **89**, 1915–1925.
7. I. Willner, S. Rubin and A. Riklin (1991) Photoregulation of papain activity through anchoring photochromic azo groups to the enzyme backbone. *Journal of the American Chemical Society*, **113**, 3321–3325.
8. A. Kumano, O. Niwa, T. Kajiyama, M. Takayanagi, K. Kano and S. Shinkai (1983) Photoinduced ion permeation through ternary composite membrane composed of polymer/liquid crystal/azobenzene-bridged crown ether. *Chemistry Letters*, 1327–1329.
9. K. Kano, Y. Tanake, T. Ogaw, M. Shimomura, Y. Okahata and T. Kunitake (1980) Photoresponsive membranes: regulation of membrane properties by photoreversible cis-trans isomerizaiton of azobenzenes. *Chemistry Letters*, 421–424.
10. Y. Okahata, H.-J. Lim and S. Hachiya (1983) Permeability control of NaCl for a nylon membrane capsule coated with a synthetic bilayer membrane containing an azo-chromophore. *Die Makromolekulare Chemie, Rapid Communications*, **4**, 303–306.
11. C.G. Morgan, E.W. Thomas, Y.P. Yianni and S.S. Sandhu (1985) Incorporation of a novel photochromic phospholipid molecule into vesicles of dipalmitoylphosphatidylcholine. *Biochimica et Biophysica Acta*, **820**, 107–114.
12. S.S. Sandhu, Y.P. Yianni, C.G. Morgan, D.M. Taylor and B. Zaba (1986) The formation and Langmuir-Blodgett deposition of monolayers of novel photochromic azobenzene-containing phospholipid molecules. *Biochimica et Biophysica Acta*, **860**, 253–262.

13. C.G. Morgan, E.W. Thomas, S.S. Sandhu, Y.P. Yianni and A.C. Mitchell (1987) Light induced fusion of liposomes with release of trapped marker dye is sensitised by photochromic phospholipid. *Biochimica et Biophysica Acta,* **903**, 504–509.
14. C.G. Morgan, S.S. Sandhu, Y.P. Yianni and N.J.F. Dodd (1987) The phase behaviour of Bis-Azo PC: photoregulation of bilayer dynamics via lipid photochromism. *Biochimica et Biophysica Acta,* **903**, 495–503.
15. M. Sun and S. Zigman (1978) An improved spectrophotometric assay for superoxide dismutase based on epinephrine autoxidation. *Analytical Biochemistry,* **90**, 81–89.
16. L.D. Mayer, M.J. Hope and P.R. Cullis (1986) Vesicles of variable size produced by a rapid extrusion technique. *Biochimica et Biophysica Acta,* **858**, 161–168.
17. M.J. Hope, M.B. Bally, L.D. Mayer, A.S. Janoff and P.R. Cullis (1986) Generation of multilamellar and unilamellar phospholipid vesicles. *Chemistry and Physics of Lipids,* **40**, 89–107.
18. D.M. Miller, G.R. Buettner and S.D. Aust (1990) Transition metals as catalysts of "autoxidation" reactions. *Free Radicals in Biology and Medicine,* **8**, 95–108.
19. M.K. Jain and R.C. Wagner (1980) *Introduction to Biological Membranes.* Wiley-Interscience, New York, Chapter 6.
20. C.G. Morgan and R. Arain (1991) *Proceedings of the Society for Photoinstrumentation Engineering,* "Medtech '91", In Press.
21. S.L. Croft (1988) Recent developments in the chemotherapy of leishmaniasis. *Trends in Pharmaceutical Sciences* **9**, 376–381.