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A RAPID METHOD FOR THE PREPARATION OF MICROVESICLES OF EGG YOLK LECITHIN

STEPHEN J. DEARDEN [a,*], THOMAS F. HUNTER [a] and JOHN PHILP [b]

[a] *School of Chemical Sciences, University of East Anglia, Norwich, NR4 7TJ and* [b] *Environmental Safety Division, Unilever Research, Colworth Laboratory, Sharnbrook (U.K.)*

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A new technique for the production of microvesicles of egg yolk lecithin is described. The method of preparation is significantly faster than contemporary methods, and is particularly useful for studying photodegradation in phospholipid vesicles.

Phospholipid liposomes and vesicles are particularly useful as physical models of biological membranes, and several methods for their preparation have been employed. These have varied from merely handshaking the lipid in an aqueous buffer to produce very large multilamellar liposomes [1], to injection of an ethanolic solution of lipid into an aqueous medium [2]. For mainly physical studies the most popular method of preparation is brief sonication followed by ultracentrifugation [3,4] or gel filtration chromatography [5–7], if liposomes of uniform size are required. A problem with the use of an ultracentrifuge is the wide disparity in conditions employed [8]. The largest multilamellae can be spun down in minutes at relatively low speeds [3], but for a homogeneous suspension of microvesicles 150000 $\times g$ for several hours is necessary. With gel filtration chromatography microvesicles can be collected after 5–8 h. During this time appreciable oxidation of unsaturated fatty acids in the bilayer chains can occur, unless oxygen is rigourously excluded from the apparatus. Furthermore, the final sample is considerably diluted.

As part of a study into the sensitized photooxidation of model biomembranes by singlet molecular oxygen (O_2, $^1\Delta_g$), and in the light of the above shortcomings of contemporary methods, it was considered necessary to have a quick and simple method for preparing vesicles with a limited and well-defined size distribution. We wish to report a rapid method for the preparation of microvesicles of egg yolk lecithin that has particular advantages when photodegradation studies are undertaken. The method involves membrane filtration using a millipore filtration cell. Olson et al. [9] have recently used a similar technique, sequentially extruding liposomes through a series of polycarbonate membranes of decreasing pore diameter using high (50 lb/inch^2) pressures, and they stressed the potential pharmacological applications of their method as a drug delivery system. Our technique employs very low applied pressures for filtration, and the rapidity of the method is emphasized in order to minimize ground state (thermal) oxidation of the lipid.

Millipore filtration of sonicated egg yolk lecithin through a membrane filter of mean pore diameter 0.22 μm produces a homogeneous dispersion of

* To whom correspondence should be sent. Present address: Department of Theoretical Chemistry, University of Bristol, Bristol BS8 1TS, U.K.

microvesicles of limited size distribution, that is very similar to microvesicles prepared by gel filtration chromatography (Fig. 1). Several membrane filters ranging from 0.05 μm to 0.5 μm pore diameter were tried, and the 0.22 μm type found to give the best results, causing no obvious rupturing of the vesicles on passage through the filter, whilst removing multilamellae that remained after sonication. Fig. 1A is a micrograph of egg yolk lecithin vesicles after passage through a column of Sepharose 4B. The time taken for gel filtration was about 8 h. Fig. 1B is a micrograph of an egg yolk lecithin sample, of the same initial concentration (0.5%), after passing five times through the 0.22 μm filter. This multiple filtration required 10–15 min.

Solubilization of photosensitizing dyes and fluorescent probes in lipid bilayers is an important requirement for photodegradation work. If an egg yolk lecithin solution is sonicated in the presence of Rose bengal so that the dye is present both inside and outside the vesicles, during gel filtration almost complete dye loss occurs, irrespective of the binding site. This is attributed to the very high water solubility of the dye, and its adsorption affinity for Sepharose 4B. However, passage of a similar sample through a 0.22 μm membrane filter allows the vesicles to retain the dye. The wavelength shift in the visible absorption spectrum of Rose bengal as a function of lipid concentration is presented in Table I. A 15 nm red-shift is observed on going from zero lipid concentration to 0.5%

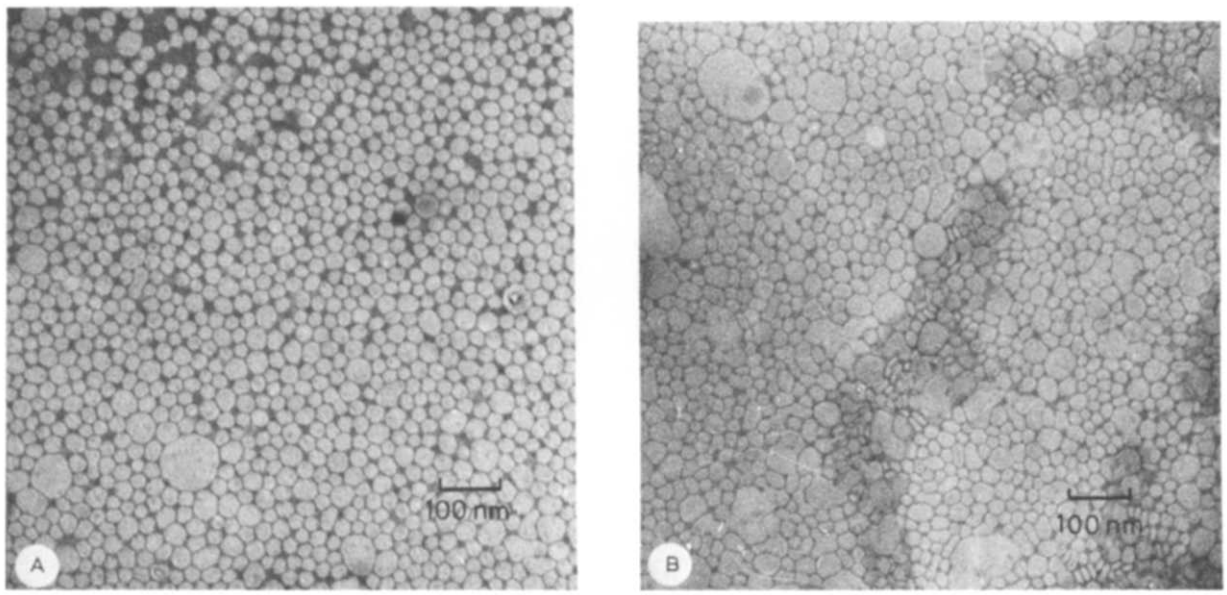

Fig. 1. Negatively stained microvesicles. (A) Representative field of egg yolk lecithin microvesicles prepared by gel filtration chromatography. (B) Representative field of egg yolk lecithin microvesicles after filtering five times through a 0.22 μm membrane filter. Electron microscopy of the microvesicles was carried out using a JEOL JEM 7 instrument operating at 80 kV. Samples were prepared by placing one drop of sample solution on a carbon-coated copper grid, adding one drop of 1% potassium phosphotungstate solution (pH 7.0), and immediately blotting the grid with filter paper. The micrographs were taken at an instrumental magnification of 37500×, photographic enlargement 1.95×. Samples of egg yolk lecithin vesicles (Lipid Products Ltd., U.K.) were prepared in the following way: 25 mg of lipid were dissolved in chloroform/methanol (1:1, v/v). The solvent was evaporated to dryness under nitrogen, the lipid remaining was held under vacuum for 5 min, and then dispersed in 5 cm^3 of Tris buffer (0.1 M NaCl, 0.01 M Tris, 10^{-3} M EDTA, $3 \cdot 10^{-3}$ M NaN_3). The dispersion was sonicated for 15 min, in an ice bath under an atmosphere of O_2-free nitrogen, using a Kerry Vibrason 150, with a probe amplitude of 5 μm. The resulting liposomes were then centrifuged at 360× g for about 10 min to remove any suspended titanium particles originating from the probe tip. Separate aliquots were taken for gel filtration and membrane filtration. Gel filtration chromatography was carried out at room temperature using a Pharmacia column (40 cm × 1.6 cm^2) containing 70 cm^3 of Sepharose 4B. Membrane filtration was performed using a Millipore 25 mm stirred cell containing a 25 mm membrane filter (type GSWP) of 0.22 μm pore diameter. A 10 cm^3 aliquot of solution was filtered under an applied nitrogen gas pressure of 2 lb/$inch^2$. The flow rate through the cell was approx. 5 $cm^3 \cdot min^{-1}$.

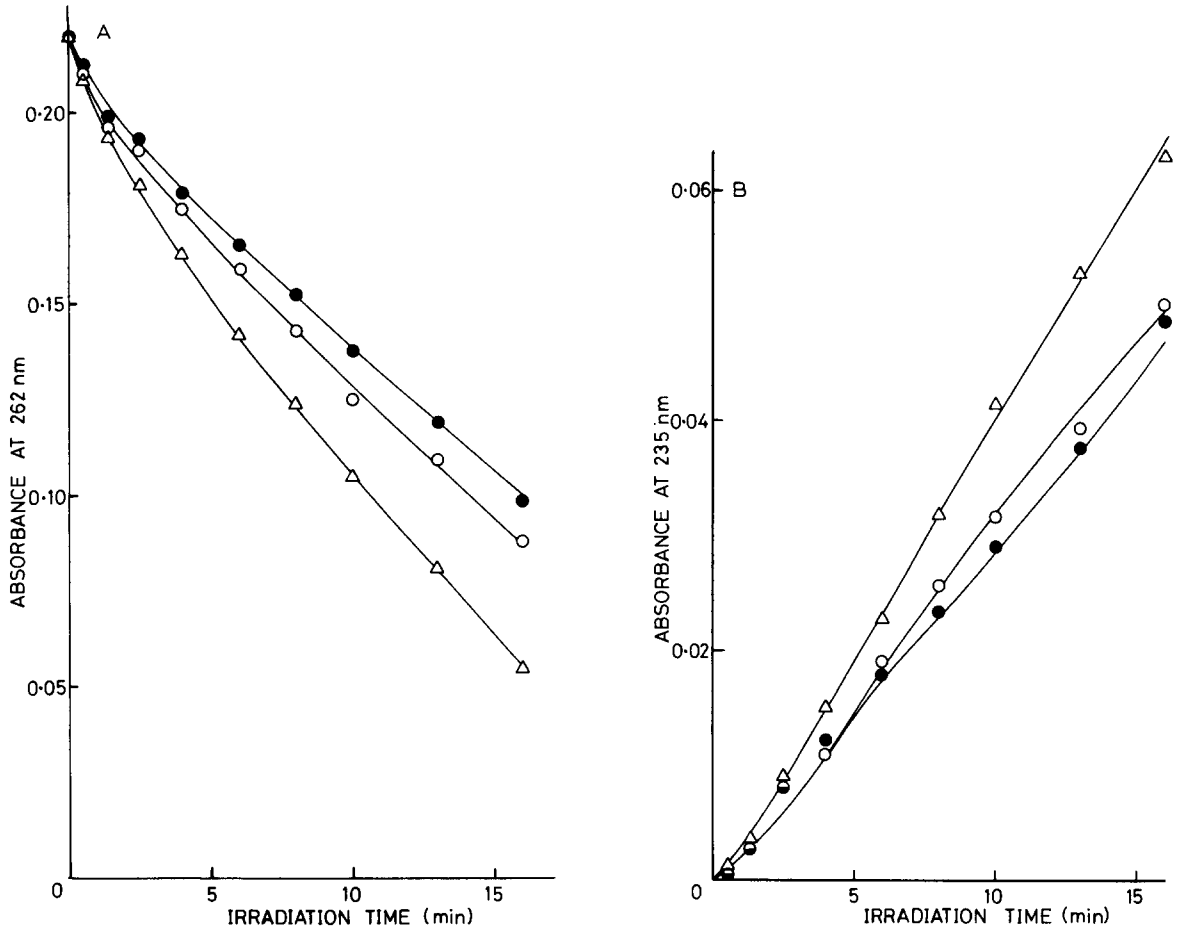

Fig. 2. Effect of age of vesicle preparation on photooxidation kinetics. (A) On the rate of photooxidation of 9,10-dimethylanthracene. △, Fresh vesicle solution; ○, 5-h old; ●, 30-h old. Sample irradiation was carried out using filtered light (Chance-Pilkington type OX1 UV band-pass) from a 250 W medium pressure mercury lamp (Hanovia), limiting light absorption to the first electronic band of 9,10-dimethylanthracene at 404 nm. The principal excitation wavelength was the 365 nm line. (B) On the rate of formation of lipid hydroperoxide. △, Fresh vesicle solution; ○, 5-h old; ●, 30-h old. Irradiation conditions identical to those in (A). In these experiments the 9,10-dimethylanthracene (DMA) molecule acts as its own sensitizer, and its photooxidation in phospholipid vesicles proceeds by the following reaction sequence:

$^1DMA \xrightarrow{h\nu} {}^1DMA^*$	light absorption
$^1DMA^* \rightarrow {}^3DMA^*$	intersystem crossing
$^3DMA^* + {}^3O_2 \rightarrow {}^1O_2^*({}^1\Delta_g) + {}^1DMA$	singlet oxygen production by triplet-triplet annihilation
$^1O_2^* + {}^1DMA \rightarrow {}^1DMA\text{-}O_2$	endoperoxide formation
$^1O_2^* + Lipid \rightarrow LOOH$	lipid hydroperoxide formation

where * represents electronic excitation. The photooxidation of 9,10-dimethylanthracene and unsaturated fatty acids were followed by difference absorption spectrophotometry (Cary 118c), details of which are to be published separately. Absorbance changes were monitored at 262 nm for 9,10-dimethylanthracene (the second electronic band), and at 235 nm for lipid hydroperoxide. The growth of an absorption band in this region indicates an increase in diene conjugation [12] and represents a relatively early stage in lipid peroxidation. The lipid concentration was $6.67 \cdot 10^{-4}$ M and the initial DMA concentration $2.3 \cdot 10^{-6}$ M.

TABLE I

WAVELENGTH VARIATION OF THE FIRST ELECTRONIC TRANSITION OF ROSE BENGAL AS A FUNCTION OF EGG YOLK LECITHIN CONCENTRATION

Rose bengal concentration 10^{-5} M.

Lipid (% v/v)	Wavelength (nm)
0	538
0.0005	538
0.001	539
0.005	549
0.01	552
0.05	553
0.5	553

lipid. With 0.005% an intermediate absorption maximum is observed, probably where bound and free aqueous dye occur in roughly the same concentrations. Furthermore, passage of Rose bengal plus sonicated 0.5% lipid through the much smaller 0.05 μm filter produces no dye in the filtrate, indicating retention of the vesicles by the filter in which a high proportion of Rose bengal is located.

It is well known that polyunsaturated lipids are susceptible to oxidation [10–12]. Both ground state oxidation by a free-radical mechanism and photosensitized oxidation by a singlet oxygen mechanism bring about migration of the C=C bond to give a conjugated diene, followed by hydroperoxide formation. Ring formation and subsequent cleavage of the fatty acid chain to give malonaldehyde and short chain aldehydes and ketones, complete the process. Our work is primarily concerned with the oxidation of lipid vesicles by the photosensitized production of singlet oxygen [13]. The rate of photooxidation of the fluorescent probe 9,10-dimethylanthracene intercalated within the vesicle bilayer is critically dependent of the age of the preparation. Figs. 2A and 2B report the effect of age of vesicle preparation on the rate of photooxidation of 9,10-dimethylanthracene, and on the rate of formation of lipid hydroperoxide, respectively. Significant changes in the kinetics occur after only several hours. By ageing it is meant that a 0.5% sonicated egg yolk lecithin solution was left exposed to the air, whilst stored in the dark at 4°C. Therefore it is most unlikely that separate photodegradation experiments on vesicles prepared by gel filtration and by membrane filtration will show similar photooxidative rates. The significantly faster millipore method will be more suitable for studies of photochemical reactions in vesicles.

In conclusion, the filtration method of preparing microvesicles should prove useful when minimal degradation of unsaturated fatty acid is the main concern. The method should lend itself to the preparation of other types of microvesicles e.g. dipalmitoylphosphatidylcholine (with a phase transition temperature of 41°C), since thermostatic control of the filtration cell and eluant is easily established. The method offers the advantages of rapid preparation and the possibility of retaining probe molecules within the vesicles, two desirable features in any photodegradation experiment with model biomembranes.

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