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BBA Report

Size and stability of dipalmitoylphosphatidylcholine/cholesterol unilamellar vesicles are affected by interaction with proteins

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The effect of entrapping the enzyme ascorbate oxidase into dipalmitoylphosphatidylcholine/cholesterol vesicles, was studied by conventional transmission electron microscopy and freeze-fracture. The freeze-fracture technique has definitely demonstrated the unilamellar nature of empty and enzyme-loaded vesicles. Images of freeze-fractured and label-fractured liposomes also indicate that the observed reduction of vesicles volume could be related to the localization of ascorbate oxidase across the membrane. The membrane localization of ascorbate oxidase may explain the oxidation of externally added ascorbate by intact enzyme-loaded liposomes. Finally, the ageing of liposomes appears to be accelerated in the presence of proteins.

Unilamellar vesicles are being used as targetable vectors for several molecules such as drugs [1], enzymes [2] and genetic material [3].

However, little attention has been devoted in the past to the localization of the carried material within lipid vesicles. In fact, DNA and proteins may interact in several ways with lipids. In particular, proteins may be entrapped inside the vesicle, may interact with the inner and/or outer lipid leaflet or may span the lipid bilayers.

These different interactions are fundamental in the liposome-cell fusion process and eventually in the release of the carried material inside the target cells.

Previous work carried out in many laboratories showed that the survival time of liposomes and the ability of fusing with target cells are influenced by size [4], surface charge [5], lipid composition [6] and stability [7].

Our research deals with the entrapment of the plant enzyme ascorbate oxidase (EC 1.10.3.3.) into dipalmitoylphosphatidylcholine/cholesterol liposomes in order to deliver it to cells. In a recent study [8], we have reported the effect of loading liposomes with ascorbate oxidase on their physico-chemical properties and on the catalytic activity of the entrapped enzyme. In that paper we gave evidence that some enzyme was adsorbed on the membrane of liposomes. Since the presence of ascorbate oxidase outside the liposomes may prevent their use as carriers for this enzyme, we have further investigated by conventional transmission electron microscopy and freeze-fracture, the interaction of ascorbate oxidase with liposomes. The influence of the protein on the overall morphology and stability of the vesicles was also studied.

Information on the used chemicals, on the liposome preparation and on the enzyme activity determination is reported elsewhere [8].

Gel filtration of vesicles was performed as follows. A liposome suspension in PBS (4 ml), containing 50 μmoles of total lipids, was carefully layered onto a Sephadex G-25 (LKB, Bromma, Sweden) column (1.2 × 25 cm). The column was eluted, at room temperature, using PBS at a flow rate of 6 ml/h and 1 ml volume fractions were collected. The fractions were analyzed for the turbidity

Abbreviations: AAO, ascorbic acid oxidase; BSA, bovine serum albumin; CHO, cholesterol; DPPC, 1-α-dipalmitoylphosphatidylcholine; PBS, phosphate-buffered saline; TEM, transmission electron microscopy.

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due to the presence of liposomes by measuring the absorption at 640 nm, pooled and centrifuged to obtain a lipid concentration of about 10 μmoles/ml. Th e fractions and the unfractionated vesicle suspension were used for the size determination. The mean value of liposome diameter for the pre-column fraction, for the fraction fourth and washing of the column is $\bar{x} = 0.039 \pm 0.038$; 0.017 ± 0.009; 0.176 ± 0.093 μm, respectively. Standard deviation values are reported. As already reported [8], the presence of ascorbate oxidase significantly reduced the volume of liposomes.

Freeze-fracture electron microscopy was used to examine the morphology of the liposome preparations. Liposome samples were quenched from room temperature by plunging into partially solidified Freon 22. Vesicles were frozen in Freon 22, freeze-fractured in a freeze-fracture device ($-105°$C, 10^{-6} mmHg) and replicated by evaporation from a platinum/carbon gun. The replicas were cleaned in concentrated bleach for about 30 min and then extensively washed with distilled water. The replicas were examined using a Philips EM-400 transmission electron microscope at an accelerating voltage of 80 kV.

The procedure for the immunolocalization of ascorbate oxidase on liposome surface is reported elsewhere [8]. Vesicles were frozen and freeze-fractured as above. The replicas were not digested but were floated into distilled water [9]. In this way unfractured liposomes float away and vesicles attached to P faces (inner faces) remain on the replica. The exoplasmic halves of vesicles remain attached to their replicas.

Small aliquots of liposomes were spread on Formvar carbon-coated grids, treated with 1% bacitracin solution according to the method of Gregory and Pirie [10]. Samples were negatively stained using 2% phosphotungstic acid solution (pH 7.0) and observed under a Philips EM-400 transmission electron microscope at an accelerating voltage of 60 kV.

Micrographs were taken at random of representative areas of each liposome preparation at the same magnification (22000 ×). Liposome diameters were measured from video images of the micrographs using a Quanti-

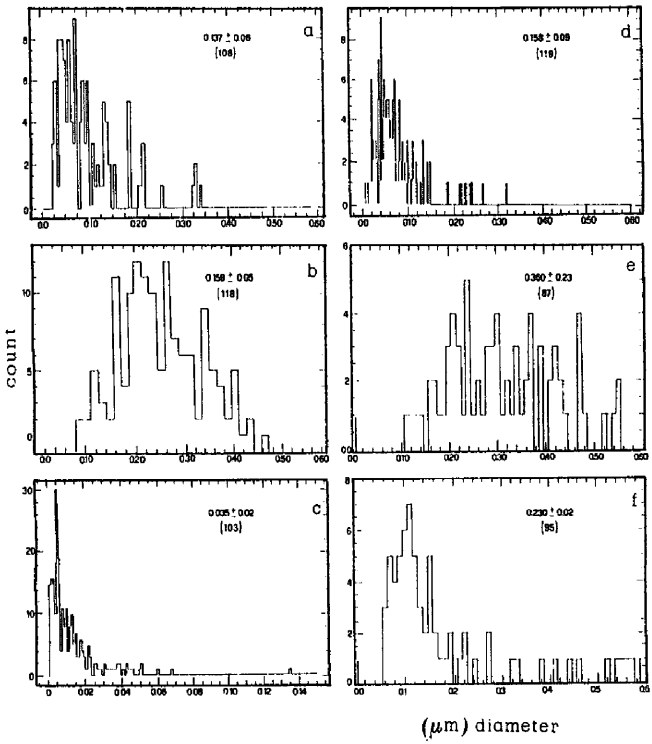

Fig. 1. Vesicle size distribution from micrographs of negatively stained DPPC/CHO liposomes. Electron microscopy for liposome diameter measurements was performed just after vesicle preparation and washings (0 days) (a, b, c) or after 15 days (d, e, f) liposomes storage at 4°C (15 days). Empty vesicles (a, d); albumin-loaded vesicles (b, e); ascorbate oxidase-loaded vesicles (c, f). The values of the mean diameter and of the standard deviation are reported in each diagram. The number of measured vesicles for each sample is reported between round brackets in each diagram.

met 970-Images analysis system (Cambridge Instruments) equipped with the standard software system QUIPS version V07.00. About 100 vesicles were measured for each preparation to calculate their mean diameter and the standard deviation. Data were analyzed by Student's *t*-test for differences. The diameter of the disc multiplied by 0.70 was taken as the diameter of the equivalent sphere [11]. Samples were observed the same

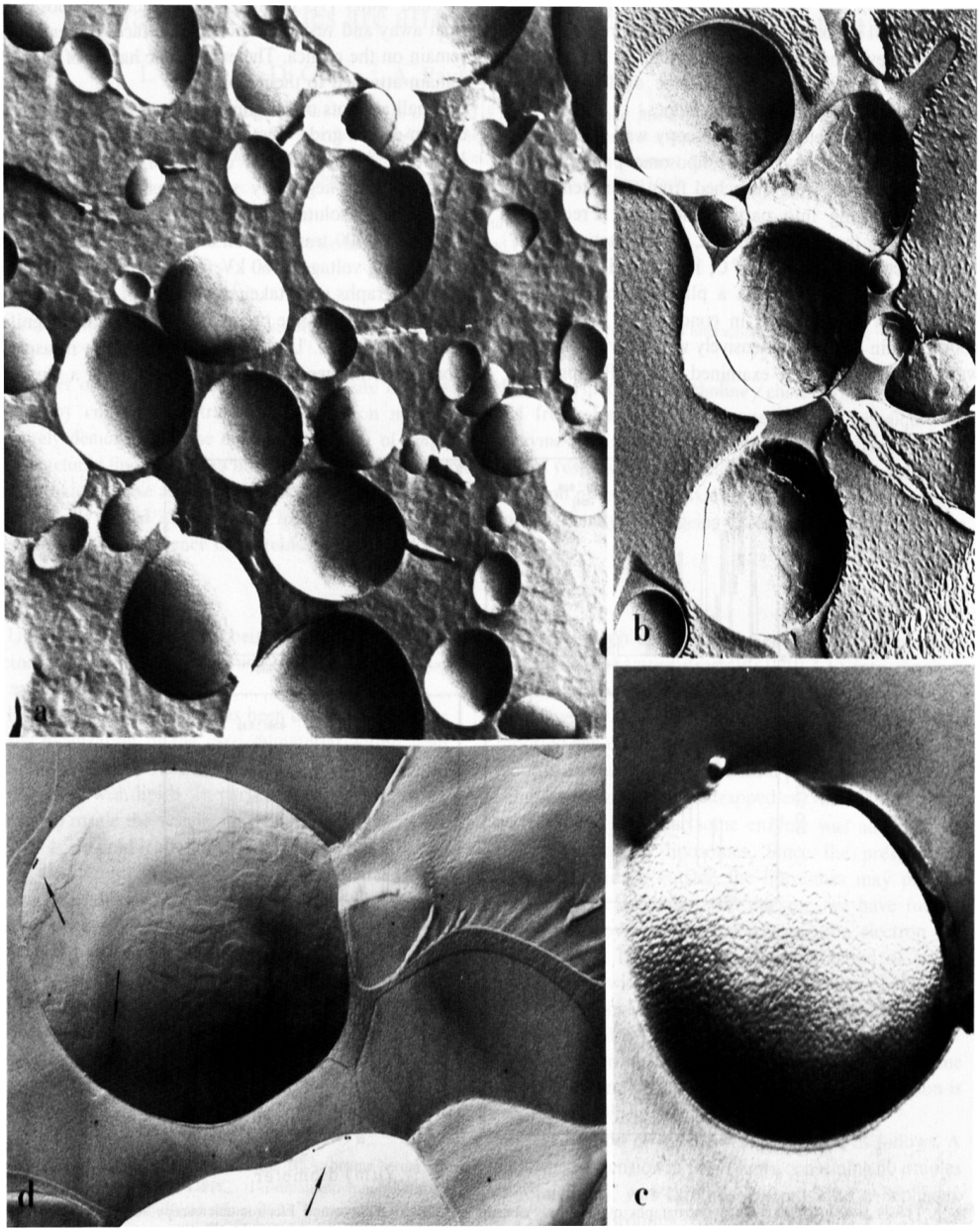

Fig. 2. Electron micrographs of freeze-fractured liposomes and label fracture of ascorbate oxidase loaded liposomes. Empty vesicles (a); ascorbate oxidase-loaded vesicles (b, c); ascorbate oxidase liposomes in the presence of rabbit specific antiserum (d). The protein is revealed by Protein A-Au 5 complexes (arrows).

day of preparation and after 15 days of storage at 4°C in PBS. The histograms were obtained with a sampling window given $d \pm 0.05$ μm, where d represents the liposome diameter.

Both empty and protein-loaded liposomes prepared by the detergent dialysis method were almost 100% unilamellar and distributed mostly around 0.137 μm. The homogeneity of the preparations could be furtherly improved by gel filtration through Sephadex G-25.

The effect of proteins on liposomes size and stability, was further studied to test whether the effect of ascorbate oxidase was specific. Fig. 1 shows the size distribution measured by TEM, of empty liposomes (a) and of liposomes loaded with BSA (b) or ascorbate oxidase (c). While ascorbate oxidase significantly decreased the size distribution profile of the vesicles, BSA slightly affected it.

Empty liposomes withstood much better the storage at 4°C. In fact their diameter was not significantly changed after 15 days of storage at 4°C (Fig. 1d) while the protein-containing liposomes and in particular those containing BSA (Fig. 1e) appeared to shift toward larger, less homogeneous forms.

Empty and ascorbate oxidase-loaded liposomes were processed for freeze-fracture microscopy. Fig. 2 (a, b and c) clearly shows that both samples contain unilamellar liposomes only. The ascorbate oxidase seems mostly embedded in the lipid bilayer (Fig. 2c). Moreover, the ascorbate oxidase is also localized over the exoplasmic face of freeze-fractured vesicles as observed by label-fracture (Fig. 2d).

The size distribution of the vesicles was furtherly checked throughout the freeze-fracture pictures. The results are shown in Fig. 3 for empty (A) and ascorbate oxidase-loaded (B) liposomes. After taking into account the systematic difference in diameter extimation, the data of TEM and freeze-fracture for empty and protein-carrying liposomes are in close agreement each other. In particular the figure shows that the presence of ascorbate oxidase causes a shrinking of the vesicles.

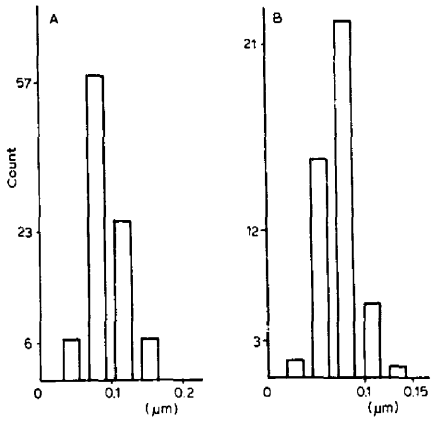

Fig. 3. Vesicle size distribution from micrographs of freeze-fractured liposomes. The size distribution profiles were determined from micrographs of freeze-fractured empty (A) and ascorbate oxidase-loaded (B) liposomes, just after vesicle preparations. About 100 vesicles for each sample were measured.

The aim of this investigation was the characterization of vesicles obtained by the detergent dialysis method. The interaction of DPPC-cholesterol vesicles with a copper-containing oxidase, ascorbate oxidase, was also studied to check whether ascorbate oxidase-loaded liposomes might be a good model for a two-compartment bioreactor and a convenient tool to vehiculate the enzyme to target cells. Previous results [12] have suggested that the liposome preparations obtained were rather homogeneous. The presence of ascorbate oxidase induced a drastic reduction of vesicle volume. Preliminary immunostaining data showed that some ascorbate oxidase was sitting in the lipid bilayer.

In this paper, thanks to the freeze-fracture technique [11,13], the unilamellar nature of the empty and protein loaded vesicles was definitely assessed (Fig. 2). The size distribution of vesicles was found fairly homogeneous with this technique and significantly lower in the presence of ascorbate oxidase (Fig. 3). The observed reduction of vesicle volume could be related to the localization of ascorbate oxidase across the membrane, clearly shown by direct observation of freeze-fracture pattern and by labelled-fracture (Figure 2; c, d).

These results have bearing both on the structure of ascorbate oxidase-loaded liposomes and on the physiology of this enzyme. The membrane localization of ascorbate oxidase explains why intact enzyme-loaded liposomes may oxidize externally added ascorbate [8], which at the pH studied should not permeate the lipid membrane [14].

The increase of enzyme activity after disrupting the vesicle integrity with detergents [8] might be due to a partial masking of ascorbate oxidase active site in intact liposomes.

The presence of ascorbate oxidase also in the internal aqueous space of liposomes cannot be, however, ruled out. These enzyme molecules might be those responsible for the criptic activity.

The relative lipophily of ascorbate oxidase may explain the ubiquitous localization of this enzyme in the plant cell and the possibility for it to cross the cellular membrane towards the extracellular fluids [15].

The vesicles volume shrinking ability of ascorbate oxidase appears to correlate with its localization BSA, a very hydrophilic protein, does not affect significantly the vesicle diameter. Instead both proteins and particularly BSA, have a dramatic effect on vesicle ageing and stability. The ageing seems to be accelerated by the coalescence of vesicles into larger aggregates through the fusion of their membranes.

The fusion of membranes is not a simple process, as membranes do not spontaneously coalesce. Several proteins, along with Ca^{2+}, have the ability of inducing the membranes to adhere tightly together. These proteins might also participate in membrane fusion [16].

Further work is needed to characterize which mechanisms are responsible for the decay of our proteoliposomes. This issue is particularly important because of the possible pharmacological use of liposomes as protein vectors.

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