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PREPARATION OF UNILAMELLAR LIPID VESICLES AT 37°C BY VAPORIZATION METHODS

DAVID S. CAFISO *, HOWARD R. PETTY ** and HARDEN M. McCONNELL ***

John Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, CA 94305 (U.S.A.)

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We have developed a method utilizing low boiling solvents to prepare large, unilamellar vesicles at physiologic temperatures. Solutions of ethyl methyl ether or dichlorofluoromethane (Freon-21) at 4°C containing solubilized lipids were injected into a column of a aqueous buffer at 37°C. Vesicles prepared in this manner have been examined by freeze-fracture, negative stain electron microscopy, and fluorescence microscopy. The principal advantages of this technique are: (1) heat labile substances may be more readily entrapped in the internal vesicle volume without thermal denaturation, and (2) the range of lipids which are soluble in dichlorofluoromethane is greater than that of many other solvents, e.g. diethyl ether.

Lipid vesicles have proven to be valuable models for the study of biological membranes. A wide range of problems from the physical chemistry of membranes to the reconstitution of biological function have been approached using this system. Since it is easy to entrap small molecules as well as proteins within the internal aqueous space of lipid vesicles, the possibility of using vesicles as a method for drug delivery has been a topic of interest. The ether vaporization method of Deamer and Bangham [1] is one of the most useful techniques for preparing relatively uniform, unilamellar lipid vesicles with a high trapping efficiency. Additional extensive studies of the general method have been carried out by Schieren et al. [2]. The latter authors employed a low boiling fraction of petroleum ether as lipid solvent. Both of these methods have the disadvantage that the aqueous injection buffer employed was 60–65°C, a temperature which destroys many labile biological molecules. In the present communication we describe the use of ethyl methyl ether and dichlorofluoromethane as lipid injection solvents, which permit the formation of vesicles when injected into aqueous buffers maintained at 37°C. The dichlorofluoromethane also appears to be a good solvent for a large variety of lipids.

We have utilized ethyl methyl ether and dichlorofluoromethane ('Freon-21'), which have boiling points of 10.8°C and 9°C, respectively, to prepare vesicles at 37°C. Dichlorofluoromethane was obtained from Matheson gas (Newark, CA), and Dupont, Linde Division (New York, NY). Ethyl methyl ether was synthesized according to the standard Williamson synthesis for ethers (see Streitweiser and Heathcock [3]) and purified twice by distillation from room temperature.

Lipids were obtained from Calbiochem or Sigma

* Present address: Department of Chemistry, University of Virginia, Charlottesville, VA 22901, U.S.A.
** Present address: Department of Biological Sciences, Wayne State University, Detroit, MI 48202, U.S.A.
*** To whom reprint requests should be addressed.

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; NBD-PE, *N*-(4-dinitrobenzo-2-oxa-1,3-diazolyl)-L-α-dimyristoylphosphatidylethanolamine; DiI, 1,1'-dioctadecylindocarbocyanine.

except as noted. Dipalmitoyl-[1-^{14}C]phosphatidylethanolamine was purchased from New England Nuclear (Boston, MA) (sp. act. 95.0 mCi/mmol). Egg PC was purified from fresh hens' eggs according to the procedure of Singleton et al. [4] and stored at −20°C in $CHCl_3$ under argon. Lipids were solubilized in either solvent at 2 mg/ml on ice after complete drying. For the injection process we employed a Sage syringe pump (Orion Research, Cambridge, MA) coupled to a crimped 19-gauge needle with teflon tubing. In addition, the needle could be replaced by teflon tubing which had been crimped at the injection end with a hot hemostat. The condenser was kept at 37°C by a circulating water bath while the entire apparatus was kept in a cold room. An ice bag placed on top of the syringe ensured that the solution did not boil until it reached the condenser. The lipid solvent was injected into an equal volume of aqueous buffer at a rate of 0.2 ml/min.

Lipid vesicles containing unsaturated fatty acids were prepared for negative staining in the following manner. A small volume of a stock solution of vesicles (approx. 5 μmol/ml) in Tris-HCl buffer was fixed with OsO_4 at a final concentration of 0.5% for 30 min on ice. This fixation step was omitted when the vesicle lipids were saturated. The samples were then negative-stained on formvar-coated copper grids with 0.5% uranyl acetate and examined in a Hitachi 11E electron microscope.

For freeze-fracture, single small droplets of concentrated membrane suspension were placed onto gold discs and frozen in Freon-22 cooled by a liquid nitrogen bath. Freeze-fracture was carried out at −110°C with no etching in a Balzers instrument. Surface replicas were made by carbon-platinum shadowing followed by carbon reinforcement. The replicas were then cleaned in bleach, washed in distilled water and mounted onto cleaned, bare 400 mesh grids.

Horseradish peroxidase, type II, was obtained from Sigma Chemical Co. (St Louis, MO). This enzyme at 0.2–3 mg/ml in phosphate-buffered saline, pH 7.4 was entrapped within the vesicles as described above using dichlorofluoromethane as the solvent. The vesicles were washed five times by centrifugation at 12000 × g for 30 min at 4°C and the enzyme was assayed by previously established methods (Steinman et al. [5]). Briefly, in the presence of H_2O_2, horseradish peroxidase catalyzes the oxidation of o-dianisidine whose product has an absorption maximum at 460 nm. The assay conditions were as follows: 0.01% o-dianisidine (w/v in methanol) (Sigma), $6 \cdot 10^{-3}$% H_2O_2 (Baker Chemicals, Phillipsburg, NJ), in 0.1 M phosphate buffer, pH 5.4. Octylglucoside at 25 mM was employed to disrupt the lipid vesicles. The increase in absorbance at 460 nm, which was linear for roughly 1–3 min, was followed with a Cary spectrophotometer. A calibration plot of ΔA/time vs. ng horseradish peroxidase per ml was obtained from a series of standard enzyme concentrations. This assay can accurately measure approx. 2 ng horseradish peroxidase per ml. Following centrifugation, the horseradish peroxidase activity in the supernatant was <2% that in the vesicle fraction and the reagents in the absence of horseradish peroxidase had no measurable activity.

Dichlorofluoromethane (Freon-21) at 0°C is an excellent solvent for lipids; as a result, injection vesicles may be easily made from a wide variety of lipids.

Fig. 1. An electron micrograph of a negative stain preparation of lipid vesicles (bar = 0.5 μm).

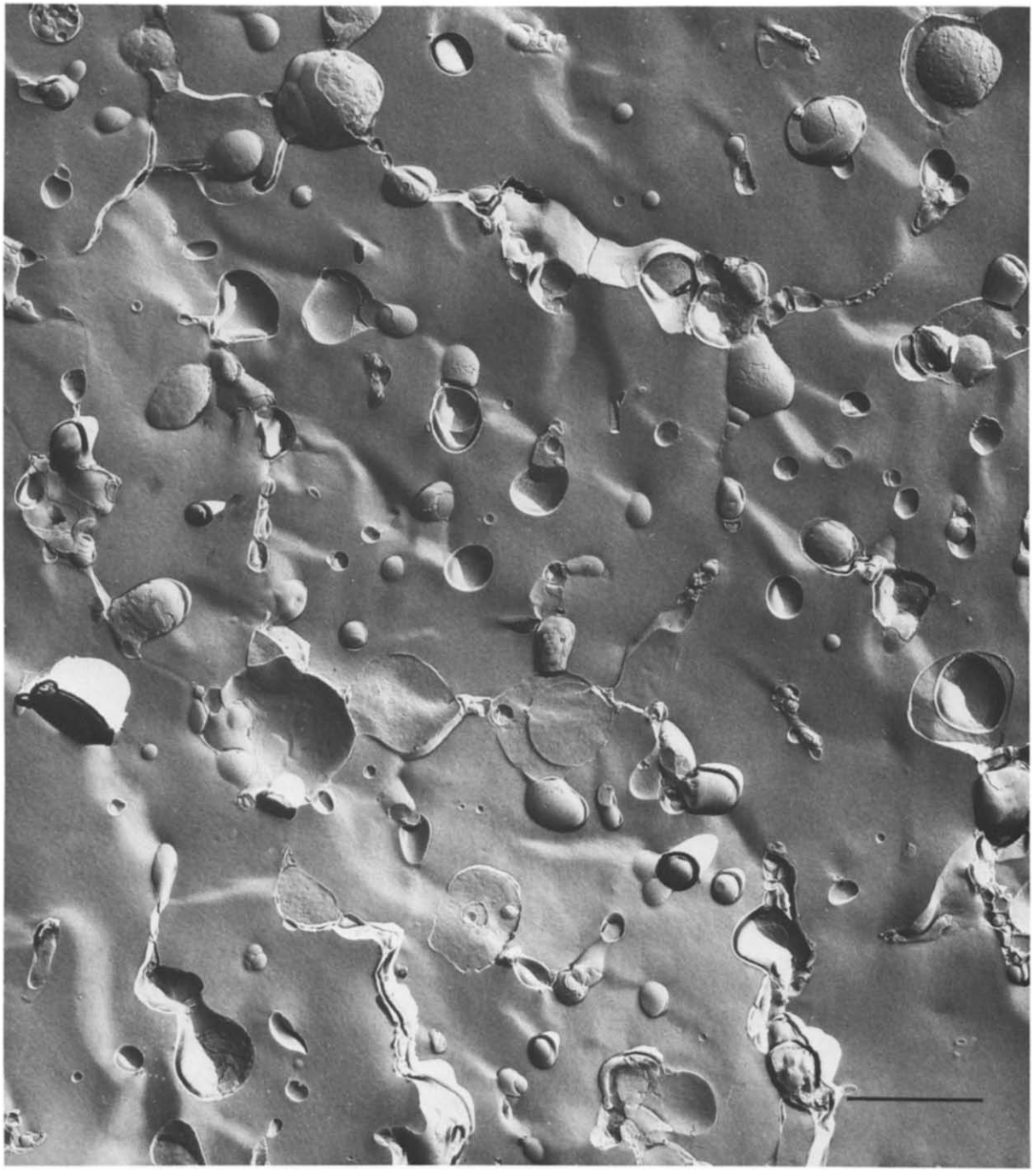

Fig. 2. A representative electron micrograph of a freeze-fracture sample of lipid vesicles. These vesicles are primarily unilamellar, although multilamellar structures can be found (bar = 1.0 μm).

The following lipids were found to be readily soluble in dichlorofluoromethane and have been incorporated into injection vesciles: egg PC, DMPC, DPPC, dicetyl phosphate, dioctadecylamine, cholesterol, phosphatidylserine (PS), cardiolipin, phosphatidylethanolamine (PE), dinitrophenylcaproyl-PE, *N*-(4-dinitrobenzo-2-oxa-1,3-diazolyl)-L-α-dimyristoyl-PE (NBD-PE), and 1,1′-dioctadecylindocarbocyanine (DiI). This list is by no means comprehensive and many other lipids are likely to be soluble in dichlorofluoromethane.

The lipid vesicles formed from dichlorofluoromethane or ethyl methyl ether injection are somewhat heterogeneous in size with an average diameter of approx. 0.5 μm. In Figs. 1 and 2 electron micrographs of typical negative stain and freeze-fracture preparations are shown; these vesicles were composed of egg PC and PS. The majority of vesicles formed by the ethyl methyl ether and dichlorofluoromethane procedures appear to be unilamellar, as shown in the micrographs. Under fluorescence microscopy, larger vesicles (approx. 5 μM) containing 0.1 mol% DiI are also visualized. Among this population of larger vesicles, many appear to be unilamellar.

The enzyme horseradish peroxidase has been entrapped within lipid vesicles. This experiment has two purposes: (1) it demonstrates that enzymatic activity can be maintained by our technique, and (2) it serves as a useful marker for trapping efficiency studies. The trapping efficiency is calculated from the horseradish peroxidase activity per μmol lipid. The total amount of lipid in the centrifuged preparation is obtained from the ^{14}C-tracer experiment. An aliquot of vesicles is assayed for horseradish peroxidase activity. This information, in addition to the original horseradish peroxidase concentration in the injection buffer, may then be employed to determine trapping efficiency. We have found that these vesicles encapsulate roughly 9 μl/μmol lipid in most experiments. This is in good agreement with results obtained by the 65°C method of Deamer and Bangham [1], and somewhat less than some of the largest trapping efficiences reported by Schieren et al. [2] for certain low molecular weight solutes. In addition, we have shown that the horseradish peroxidase activity of the vesicles is linearly dependent upon the concentration of horseradish peroxidase in the injection buffer (data not shown).

We have succeeded in forming unilamellar lipid vesicles at reduced temperatures, 37°C, as opposed to the usual 60–65°C by the use of the low boiling solvents ethyl methyl ether and dichlorofluoromethane. This procedure is clearly a desirable alternative when heat-sensitive materials are to be trapped within lipid vesicles. In addition, dichlorofluoromethane allows the solubilization of a greater range of lipids when compared to diethyl ether. Other workers have suggested to prepare lipid vesicles at lower temperatures (Szoka and Papahadjopoulos [6]); however, we have not found these procedures convenient in our own work. The techniques we have presented are simple and reproducible and may be applicable in the delivery of labile biological materials both in vivo and in vitro. For example, lymphokine delivery to macrophages is a possible application (Sone et al. [7]). However, our main motivation for the preparation of these vesicles has been the study of specific antibody-dependent phagocytosis of vesicles containing trapped ferritin as well as other markers (Petty et al. [8]).

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