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Extemporaneous preparation of large unilamellar liposomes

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Direct contact between lipids solubilized by octyl glucoside and Amberlite XAD-2 beads yielded large liposomes (240 nm diameter) with no residual detergent molecules, in less than 10 min. This extemporaneous preparation of liposomes was prepared with a detergent/bead ratio no higher than 0.12 (μmol/mg) and a phosphatidylcholine/phosphatidylserine/cholesterol molar ratio of 1:1:1. The liposomes were mainly unilamellar, as deduced from thin section and freeze-fracture electron micrographs and from measurement of calcein incorporation into the vesicles. The relatively large internal volume of these vesicles (8.9 l/mol lipid) accounts for the high percentage of entrapped material observed. The percentage increased with lipid concentration, but could not be increased above 20% corresponding to 20 mM total lipids.

Introduction

Liposomes are used to carry drugs and to delay their effects in living organisms, and to deliver macromolecules to cultured cells (reviewed in Refs. 1–3). In both cases, large unilamellar vesicles (LUV) are often needed because they encapsulate large amounts of material and can fuse with cell membrane under favorable conditions [4–5]. But the methods usually employed to prepare these vesicles, such as reverse-phase evaporation [6] or dialysis [7,8], are complex, time-consuming, and sometimes detrimental to the macromolecules being encapsulated (for instance, RNA, protein and DNA) because of the presence of organic solvents, and the length of liposome preparation. Furthermore, the clinical use of liposomes entails difficulties such as preserving for long periods of time.

For these reasons, we here propose a method for preparing LUV in a few minutes, which combines the properties previously described [8], with the rapidity of multilamellar liposome formation. These unilamellar liposomes are homogeneous in size, and have large intravesicular volumes allowing the entrapment of substantial amounts of macromolecules.

Materials and Methods

Materials

n-Octyl D-glucopyranoside (octyl glucoside) was purchased from Boehringer Mannheim (Meylan, France), (glucose-^{14}C) octyl-β-D-glucopyranoside (314 mCi/mmol) was obtained from New England Nuclear (Boston, MA) and (^{3}H)-sucrose (8.9 Ci/mmol) from Amersham, U.K. Ultrogel ACA 202 came from IBF; Sephadex G25, Sepharose 4B and Sephacryl S1000 from Pharmacia. Calcein was purchased from Merck and was used without further purification. Amberlite XAD-2 was obtained from Touzart et Matignon (Vitry-sur-Seine,

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; MLV, multilamellar vesicle; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; ELUV, extemporaneous large unilamellar vesicle.

France). Before use, the beads were extensively washed with different mixtures of solvents as previously described [8] according to the procedure of Holloway [9].

Egg lecithin, phosphatidylserine and cholesterol were obtained from Sigma. L-α-di[1-^{14}C]palmitoylphosphatidylcholine (60–100 mCi/mmol) was purchased from New England Nuclear. All radioactive samples were counted in a Packard 229 scintillator. Phospholipid concentration was determined by Bartlett's method for phosphate determination [10].

Preparations of liposomes

Extemporaneous large unilamellar vesicles were produced as follows. Lipids (10–30 mM) in chloroform/methanol (2 : 1, v/v) were mixed in different proportions, sometimes in the presence of about $2 \cdot 10^5$ cpm of [^{14}C]phosphatidylcholine for tracing. After organic solvent elimination under a nitrogen stream, and then in vacuo, lipids were wet with buffer A (10 mM Hepes (pH 7.4), 1 mM EGTA, and 150 mM NaCl) and left to swell for a half hour with frequent vigorous shaking. In some experiments, materials to be encapsulated were put into solution with buffer A. A 20% octylglucoside solution was finally added in a detergent-lipid molar ratio of 10 [8] with, when necessary, $2 \cdot 10^5$ cpm of radioactive detergent. Unless otherwise stated, the final volume was adjusted to 1 ml with buffer A.

To remove detergent, nonpolar polystyrene-divinylbenzene beads (Amberlite XAD-2) in buffer A were directly added to the liposome preparation after draining excess of buffer by aspiration. The sample was placed on a rotary mixer at 10 rpm for 10 min. The liposomes were collected by gentle centrifugation or directly harvested from the suspension with a syringe. In encapsulation experiments, the liposome preparation was passed through a Sepharose 4B or Sephacryl S1000 column to remove the unencapsulated material. The column was preequilibrated with a dilute solution of phosphatidylcholine in buffer A to saturate the gel, as recommended by Reynolds et al. [11].

Large unilamellar vesicles (LUV), obtained by dialytic removal of octylglucoside, were prepared as described previously [8]. Small unilamellar vesicles (SUV) were made according to Ref. 12 and multilamellar vesicles (MLV) were made by wetting dried lipids with buffer A, followed by vigorous vortexing for 10 min. All these liposome preparations contained the same lipids (phosphatidylcholine/phosphatidylserine/cholesterol) in the same ratio (1 : 1 : 1), except for liposomes of pure lecithin.

Determination of the physicochemical properties of the liposomes

In order to visualize the liposomes formed during this procedure and to evaluate their size-distribution we applied different electron microscopy techniques. For negative staining, a dilute suspension of the liposomes was sprayed onto carbon-coated grids. Samples were stained with 0.1–0.5% uranyl acetate [8]. For thin sectioning, concentrated aliquots of liposomes were fixed in 3% osmium tetroxide in veronal buffer (pH 7.4) for 12 h at 4°C. Prior to dehydration in cold-graded acetone solutions, they were stained in 2.5% aqueous uranyl acetate for 5 h, infiltrated into mixtures of propylene oxyde and Epon and embedded in Epon. Thin sections were stained with alkaline lead citrate.

For freeze-fracturing, single small droplets of concentrated liposome suspension were placed onto gold discs and frozen in overcooled liquid nitrogen (−206°C). Freeze-fracturing was carried out at −110°c, with no etching, in a Balzers' instrument. Surface replicas wee made by carbon-platinum shadowing followed by carbon reinforcement. The replicas were cleaned in bleach, washed in double distilled water, monted on 400 mesh grids and examined in a Hitachi 600 electron microscope. The size-distribution of the liposomes was determined by evaluating the diameter of at least 500 vesicles, in three separate experiments.

The entrapment efficiency of extemporaneous liposomes was determined by measuring the concentration of entrapped material (calcein, or [^{3}H]sucrose) in the samples after gel filtration on Sepharose 4B or Sephacryl S1000, equilibrated with buffer A. Fractions of the elution peaks were pooled, and ^{3}H radioactivity was counted, or the calcein concentration was measured (excitation, 490 nm; emission, 520 nm) in a Perkin Elmer model MPF 3L spectrofluorimeter, after treating the liposomes with 1% sodium dodecyl sulfate. The

entrapment efficiency was evaluated on the basis of the ratio of moles of trapped solute to moles of total solute and to moles of liposome lipids. The concentration of encapsulated material was calculated from the total lipid content of the liposomes and the mean vesicle diameter measured by electron microscopy. Liposome diameters were also calculated as described earlier [8].

Results and Discussion

Kinetics of detergent removal from the lipid-octyl glucoside mixture by direct contact with Amberlite XAD-2

Amberlite XAD-2 beads are a neutral macroporous polymeric adsorbent with large surface areas, recommended for the removal of detergent from mixtures of many organic and inorganic compounds in solution, as described by the manufacturers. Although they have previously been used in the presence of lipids [13–15], the study of their effects on liposome preparations does not appear to have been systematically carried out. Fig. 1 shows the results of a typical experiment of detergent elimination from a mixture containing lipids (PC/PS/Chol molar ratio, 1 : 1 : 1, 20 mM), octylglucoside (lipid/detergent molar ratio, 1 : 10) and various amounts of Amberlite XAD-2 beads in detergent/bead ratios (μmol/mg) equal to 0.12, 0.086 and 0.053.

Detergent was completely removed in 10 min, irrespective of the amount of beads, as long as the detergent/bead ratio was less than 0.12. In these conditions the remaining detergent, expressed as detergent/lipid molar ratio was always less than 1/100, often included in the range 1/200–1/500. At higher ratios, detergent removal could not be improved [8] and the liposomes always contained non-negligible amounts of octylglucoside. It is interesting to note that the rate of detergent elimination increased with the quantity of beads.

Fig. 1 shows moreover that the total amount of phospholipids in solution decreased in proportion to the quantity of beads. These decreases can be attributed either to a binding of lipids to beads as in the case of detergent, or to a dilution of the sample by buffer contained in the internal volume of the beads. We evaluated this internal volume at 0.4–0.45 ml/g dry beads, according to experiments performed by double weighing of drained beads before and after methanol treatment, followed by solvent elimination under a nitrogen stream and 2 h in vacuo. Using this new parameter and assuming the latter hypothesis, we calculated the decrease in phospholipid concentration (due to dilution in the internal bead volume) to be equal to 39%, 47% or 60%, respectively, when 1.65, 2.3 or 3.75 g of beads were used. These values are in close agreement with the data in Fig. 1. Additional experiments confirmed these results. The same samples as in Fig. 1, but treated with 2.2 g beads, showed decreases in lipid content of 50%, 34%, 25% and 20% when the final volumes were 1, 2, 3 and 4 ml, respectively. We further checked that cholesterol behaves like the phospholipids (curve C). These results preclude any selective binding of

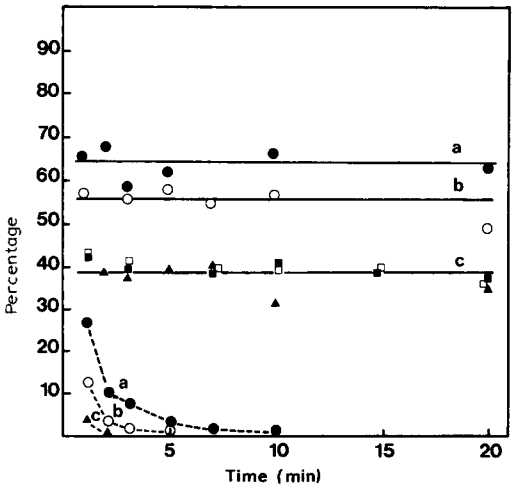

Fig. 1. Detergent removal with Amberlite XAD-2. 20 μmol of PC/PS/Chol (1 : 1 : 1, M/M) were dried, hydrated by buffer A, then mixed with octylglucoside in a detergent/lipid ratio equal to 10 : 1 in a final volume of 1 ml, as indicated in Materials and Methods. Samples contained about $2 \cdot 10^5$ cpm of either [^{14}C]PC (solide lines) or [^{14}C]octylglucoside (dotted lines). The clear solutions were mixed with various amounts of drained beads: 1.65 g (●), 2.3 g (○), and 3.75 g (▲), respectively, in experiments a, b and c. The test tubes were then placed on a rotary mixer at 10 rpm for 10 min. Aliquots of 10 μl were collected at various time intervals and analyzed for radioactivity. Percentages of lipids (solid lines) and detergent (dotted lines) remaining in solution are expressed without correcting for the dilution provoked by bead additions. In one experiment (c), phospholipids (□) and cholesterol (■) were simultaneously labeled by $2 \cdot 10^5$ cpm of [^{14}C]PC and $1.75 \cdot 10^5$ cpm of [^{3}H]cholesterol.

lipids by the beads. They clearly suggest that the decrease in lipid content in the presence of beads is due to the occupancy of all the internal volume of the beads. Two kinds of experiments support this hypothesis. The absorption of lipids into the beads was independent of the lipid/detergent ratio, implying that no competition occurred. On the other hand, liposomes that are larger than the bead pores cannot penetrate, and consequently are not damaged by the presence of beads. This was demonstrated in the following experiment. A liposome sample, prepared as in Ref. 8, with unencapsulated material removed (see Methods), was kept in contact with the beads for 10 min (detergent/bead ratio of 0.54 μmol/mg), and then chromatographed again. The leakage of fluorescent material was evaluated at 0.01% by this technique, which proves that the liposomes were in no way modified by the beads. Thus, these two experiments corroborated the hypothesis of lipid dilution in the internal volume of the beads, inducing the decrease in lipid concentration available in the sample.

Hence the lipid concentration in the presence of wet beads can be expressed by the following equation:

$$C = C_0 \frac{V}{V + 0.4P} \quad (1)$$

where C_0 and C are lipid concentrations (mM) before and after bead addition, V is the initial volume of the sample (ml), and P the amount of beads (g).

Since we know that the percentage of encapsulated material depends on the size of the vesicles, which itself depends on the lipid concentration [16], the above results are useful in fixing the best experimental conditions for producing liposomes with preselected characteristics.

Characteristics of extemporaneous liposomes

In order for the technique of extemporaneous liposome preparation to be more useful than multilamellar vesicle (MLV) preparation the internal volume of the LUV and consequently their entrapment capacity must be larger than that of the MLV. Several experiments were performed in order to evaluate this capacity, while defining the main characteristics of these liposomes.

The liposomes exhibited a very homogeneous size distribution, as shown in Fig. 2. Their mean diameter, measured on micrographs from negative staining (Fig. 2a) and thin sectioning (Fig. 2b) was smaller than that of liposomes prepared with the same lipids and detergent in the same proportions by the dialysis method [8]. We computed a mean diameter of 240 nm for extemporaneous vesicles (Table I), compared to 800 nm for LUV prepared by dialysis [8]. Under the same conditions, extemporaneous PC liposomes had a mean diameter of 170 nm, instead of 400 nm when prepared by dialysis [8]. Thus, the rate of detergent removal also seems to play a major role in the liposome size distribution, along with the lipid concentration [16]. These results are comparable to the upper values found by Schendener et al. [18] who used the dialysis method and a Lipoprep device.

Fig. 2b and c show that the vesicles are unilamellar. This was observed with both microscopic techniques. This liposome property was confirmed in different ways. The encapsulation assays showed that the percentages of entrapped material were very close to those predicted by the relation giving the percentage of encapsulation by unilamellar liposomes as a function of lipid concentration and vesicle diameter [8]. We computed percentages of 8.9% and 17.6% for lipid concentrations of 9 and 20 mM, respectively, and a mean diameter of 240 nm. These values are very close to 8% and 19% in Table I. Conversely, using the same relation, we can calculate vesicle diameters from the encapsulation assay data (calculated diameters in Table I). Table I establishes a clear correspondence between the diameters (or volumes) measured by electron microscopy and those calculated on the basis of the unilamellar liposomes form. Experiments with calcein similarly demonstrated that the intraliposomal concentration of this fluorescent negatively charged compound agrees with a unilamellar vesicle form. Liposomes (SUV, MLV and extemporaneous-vesicles) were prepared in the presence of 10 mM calcein, and the phospholipids and calcein were then assayed. After gel filtration, the vesicle diameters were measured by electron microscopy. Assuming that the lipids were distributed as in unilamellar vesicles, we found a calcein concentration of 8 mM in extemporaneous liposomes (Table I), 8.9 mM in SUV (50 nm in diameter), and 2.2

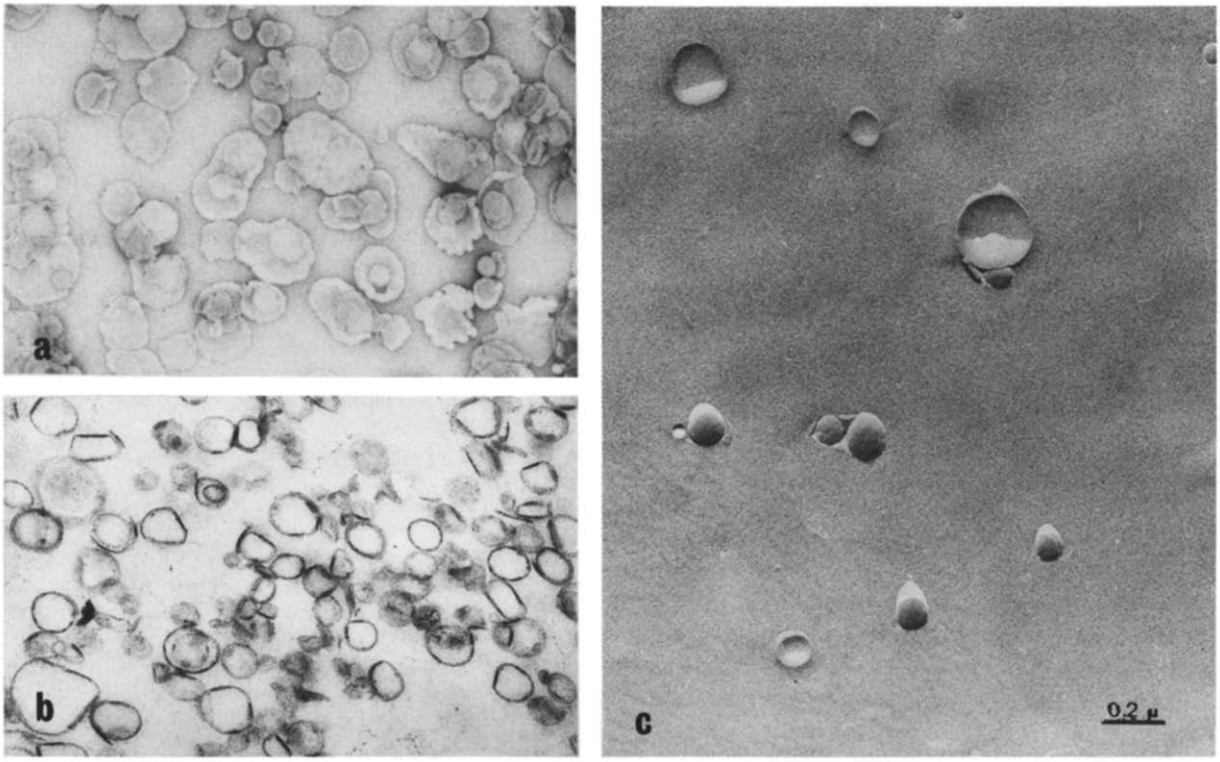

Fig. 2. Electron micrographs of negatively stained (a), thin-sectioned (b) or freeze-fractured liposomes (c). Liposomes were prepared as in Fig. 1 (0.12 μmol detergent per mg beads). The final lipid concentration after liposome formation was 10 mM.

TABLE I

CHARACTERISTICS OF EXTEMPORANEOUS LIPOSOMES

Unless otherwise stated, the final lipid concentration was 10 mM. The preparation of liposomes (PC/PS/Chol, 1:1:1, M/M), the entrapment assays, and the electron microscopic studies were carried out as described in Materials and Methods. The figures in brackets are the mean percentages of liposome diameters included in the range. Internal volume was calculated on the basis of the following assumptions: the vesicles are spherical and the area per phospholipid molecule is 0.7 nm^2 [16].

Parameters	Values
Diameter measured by electron microscopy (nm)	170–280 (81)
Calculated diameter (nm)	256
Percentage of material entrapped	
in 9 mM lipids	8
in 20 mM lipids	20
Internal volume (l/mol PL)	8.9
Intraliposomal calcein concn. (mM)	8 ± 0.4

mM in MLV (mean diameter, 1 μm). It is known that a negative charge on liposomes and calcein can reduce the entrapment capacity of different liposomes [19]. Nevertheless, it can be concluded that extemporaneous liposomes are mainly unilamellar (extemporaneous large unilamellar vesicles or ELUV).

Fig. 3 completes the data in Table I dealing with the encapsulation capacity of ELUV. As expected, it increased in proportion to the lipid concentration up to values as high as 20% of the encapsulable material, corresponding to 20 mM lipids. Beyond this limiting concentration, we obtained unreliable results (17% encapsulation, corresponding to 40 mM lipids) because of the experimentals conditions: the lipid-detergent volume was too small to completely wet the amount of beads needed to remove the detergent. Furthermore the ELUV contained relatively large amounts of entrapped material, 8.9 l/mol of lipid, though their

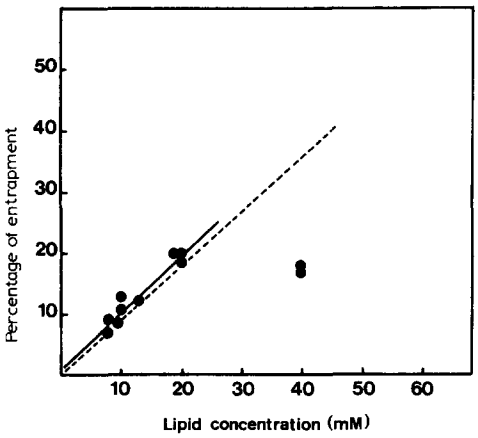

Fig. 3. Percentage of encapsulated material as a function of lipid concentration. Liposomes of PC/PS/Chol (1:1:1, M/M) were prepared as in Materials and Methods with increasing concentrations of lipids in the presence of $2 \cdot 10^5$ cpm [^{3}H]-sucrose. Unencapsulated material was eliminated by gel filtration on Sepharose 4B or Sephacryl S1000. In some experiments this elimination was performed by centrifugation for 30 min, under a pressure of 69 kPa in a Beckman Airfuge. The dotted line correspond to the entrapment calculated from Eqn. 1 in Ref. 8.

capacity was not as great as that of the very large unilamellar liposomes obtained by the dialysis method (35 l/mol · lipid [3]). Nevertheless, ELUV rank among the so-called LUV [20,21].

The method described above combines a very easy technique for liposomal encapsulation with the manufacture of large unilamellar vesicles. The liposome characteristics (ELUV) are suitable for most purposes involving LUV. This extemporaneous method seems particularly advantageous for clinical investigations, since liposomes encapsulating drugs can be quickly prepared just before use.

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