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PREPARATION OF CELL-SIZE UNILAMELLAR LIPOSOMES WITH HIGH CAPTURED VOLUME AND DEFINED SIZE DISTRIBUTION

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Cell-size unilamellar vesicles were made by removing solvents from microscopic chloroform spherules containing smaller water droplets within. The average diameter of the vesicles in a typical preparation was 9.2 μm, comparable to that of human erythrocytes (7 μm). The standard deviation of the size distribution was 3.0 μm. The unilamellarity and bilayer unit membrane of vesicles were demonstrated by transmission electron microscopy. Materials so far successfully incorporated into vesicles include glucose, sucrose, Arsenazo III and Ponceau S dyes, thymidine triphosphate, methotrexate, agarose, collagen, ferritin, polyadenylic acid, DNA, and whole bacteria. The captured volume per milligram of lipids (up to 144 μl/mg) was almost an order of magnitude greater than the highest value reported in the literature to date (up to 15.6 μl/mg) (Szoka, F.C. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4194–4198).

Introduction

Liposomes or lipid vesicles have been widely studied as models for biological membranes and as microcapsules for introducing various materials into cells and tissues [1–6]. For microencapsulation of water-soluble drugs and other biologically active materials, high capture efficiency and defined size distribution are desirable characteristics. For use as a model for plasma membranes, unilamellar vesicles that approximate cell size are needed. Existing methods of liposome preparation [7–16] have been only partially successful in making liposomes with the above characteristics. We here report a relatively simple procedure for preparation of cell-size unilamellar vesicles with high capture efficiency and defined size distribution. Such preparations may prove especially useful for chromosome mediated gene transfer experiments [17].

For abbreviations see Table I on p. 3.

Materials and Methods

Lipids and other reagents

Soy phosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, cholesterol, phosphatidylethanolamine from *Escherichia coli*, cardiolipin, sphingomyelin, dioctylsulfosuccinate, triolein, DL-α-tocopherol, cholesterol oleate, poly(adenylic acid), and Arsenazo III dye were purchased from Sigma, St. Louis, MO. Phosphatidylglycerol, dipalmitoylphosphatidylglycerol, and ferritin were from Cal Biochem, La Jolla, CA. Chloroform was from Fisher Scientific, and diethyl ether was from J.T. Baker Co. Ponceau S was from Reeve Angel Ltd. London; methotrexate was from Lederle, Wayne, NJ; [*Me*-^{3}H]-thymidine 5′-triphosphate, [U-^{14}C]glucose, [U-^{14}C]phosphatidylcholine from algae, and Trixton X-100 were from New England Nuclear, Boston, MA; and [3′,5′,7-^{3}H]-methotrexate, poly([8-^{3}H]adenylic acid), and [U-^{14}C]sucrose were from Amersham, Arlington Hts, IL. All phospholipids were kept at −20°C under

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nitrogen in glass vials with aluminum-lined screw caps until use. Glassware were used exclusively for all steps involving lipids and lipid solvents. All the commercially available materials were used without further purification. (^{3}H-Proline)-labelled collagen from cultured diploid human fibroblasts was a gift from Dr. P. Bornstein; it was heat-denatured by incubating at 100°C for 30 min. *Streptococcus salivarius* was a gift of Dr. C.A. Evans. [*Me*-^{3}H] thymidine-labelled DNA and unlabelled DNA were isolated from a HeLa cell line by the method of Pellicer et al. [18]. One-dram glass vials (with screw caps) were from Acme Glass Co., Los Angeles, CA. The mechanical shaker was a vortex mixer ('Vari-whirl') purchased from Van Waters & Rogers, Inc.

Preparation of chloroform spherules

Amphipathic lipids totaling 3.33 μmol plus small amounts of neutral lipids (triolein, tocopherol, or cholesterol oleate) were combined in a one-dram vial (1.4 cm × 4.5 cm, screw cap lined with aluminum foil), and chloroform was added to bring the volume to 1 ml. Many lipids can be used to form liposomes as shown in Table I. Material to be trapped, dissolved in 1 ml of 150 mM aqueous sucrose solution, was added to the chloroform-lipid solution. The vial was immediately shaken with a standardized mechanical shaker for 45 s (see legend for Fig. 3) to produce a water-in-chloroform emulsion. It was essential when the aqueous phase was being added to chloroform to avoid producing a chloroform-in-water emulsion. This was achieved by adding the aqueous phase in three divided portions with manual swirling after the addition of each aliquot. The emulsion, after mechanical shaking, was divided into two equal portions and each was transferred into a new vial containing 'mother liquor'.

The mother liquor was prepared by addition of 0.5 ml of lipid solution in diethyl ether (3.33 μmol total of amphipathic lipids plus small amount of neutral lipid per ml of ether) to each of two new vials containing 2.5 ml of 200 mM sucrose solution, and then shaking the vial mechanically for 15 s.

After transfer of the water-in-chloroform emulsion into the mother liquor, the vial was immediately shaken mechanically for 10 s to produce microscopic chloroform spherules, most of which contained smaller water droplets within.

Evaporation of chloroform and ether to form liposomes

The chloroform spherule suspension in mother liquor was layered on the bottom of a 250 ml filtration flask (bottom diameter 8 cm). A stream of nitrogen gas at 1.5 l/min was introduced into the flask through a piece of glass tubing protruding 5 cm into the mouth of the flask. The flask was gently swirled every 15–30 s to keep the chloroform spherules suspended.

Throughout the solvent evaporation, which took about 45 min, the flask was kept in a 37°C water bath. Essentially complete evaporation of chloroform and ether was indicated by a marked decrease in turbidity. Remaining traces of ether and chloroform may be removed by incubating the liposome preparation at 100°C in a loosely capped vial for 10 min.

For separation of liposomes from the untrapped material and lipid debris, an equal volume of 5% glucose solution was added to the liposome preparation and the liposomes were pelleted in a clinical centrifuge at 600 × *g* for 5 min. The supernate was removed and the liposome pellet was resuspended gently into 5% glucose solution or phosphate-buffered saline (0.15 M, pH 7.4).

Results

Only a small proportion of the amount of lipids initially used eventually became a part of the liposomes and the rest was separated from the final liposome preparation as 'lipid debris' in the supernate when liposomes were pelleted in a clinical centrifuge at 600 × *g*. When ^{14}C-labelled phosphatidylcholine was added as tracer to the lipids (PC/C/PG/TO, 4 : 4 : 2 : 1, molar ratio) in chloroform, only 35% of the radioactivity was in the liposome pellet and 65% was in the supernate. Adding ^{14}C-labelled phosphatidylcholine to the lipid solution in ether gave 7.6% radioactivity in liposome pellet and 92.4% in supernate. Therefore, out of 6.67 μmol of total amphipathic lipids initially used (3.33 μmol in chloroform and 3.33 μmol in ether), 1.42 μmol finally became incorporated into the liposomes.

The captured volume was 52 l/mol of lipids used for PC/C/PG/TO system (see Table I). However, if the amount of lipid that was removed as lipid debris was not included and only the amount that actually

TABLE I

LIPOSOMES OF VARIOUS LIPID COMPOSITION

Capture efficiency was measured by trapping poly(adenylic acid) (0.1 mg/ml) and Ponceau S dye (0.1%). Captured volume expressed in μl/mg or l/mol is the amount (volume) of the original aqueous solution trapped in vesicles per unit quantity of lipids used. Percent encapsulated is the percentage of starting material incorporated into vesicles. The standard procedure described in the text was used except for No. 3 in which the mechanical shaking to form chloroform spherules was done at half the usual volume per vial and the duration of shaking increased form 10 s to 30 s because of foaming. Soy phosphatidylcholine (PC), cholesterol (C), phosphatidylglycerol (PG), triolein (TO), cardiolipin (CL), dioctylsulfosuccinate (DOSS), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylcholine (DPPC), sphingomyelin (SM), dioleoylphosphatidylcholine (DOPC), DL-α-tocopherol (VE), phosphatidylethanolamine (PE), and cholesterol oleate (CO) were the lipids. DPPG and DPPC were not completely soluble in the solvents; suspensions of fine lipid particles were therefore used. n.d., not done.

No.	Lipids (molar ratio)		Polyadenylic acid			Ponceau S dye		
			Captured vol.		% Encapsulated	Captured vol.		% Encapsulated
			μl/mg	l/mol		μl/mg	l/mol	
1.	PC/C/PG/TO	(4 : 4 : 2 : 1)	104	70	51	77	52	38
2.	PC/C/CL/TO	(4 : 4 : 1 : 1)	105	76	51	109	79	53
3.	PC/C/DOSS/TO	(4 : 4 : 2 : 1)	104	63	46	93	56	41
4.	PC/C/DPPG/TO	(4 : 4 : 2 : 1)	58	38	28	60	40	29
5.	DPPC/C/CL/TO	(4 : 4 : 1 : 1)	n.d.	n.d.	n.d.	32	22	15
6.	SM/C/CL/TO	(4 : 4 : 1 : 1)	n.d.	n.d.	n.d.	73	51	34
7.	DOPC/C/CL/TO	(4 : 4 : 1 : 1)	105	76	51	123	90	60
8.	PC/C/CL/VE	(4 : 4 : 1 : 1)	n.d.	n.d.	n.d.	114	78	52
9.	PE/C/CL/VE	(4 : 4 : 1 : 1)	144	96	64	124	82	55
10.	PC/C/CL/CO	(4 : 4 : 1 : 1)	81	57	38	110	78	52
11.	PC/C/CL/TO	(4 : 4 : 1 : 0.25)	117	84	52	104	75	46
12.	PC/C/CL/TO	(4 : 4 : 1 : 0.10)	88	63	38	97	69	42
13.	PC/C/CL/TO	(4 : 4 : 1 : 0.05)	111	80	48	88	63	38

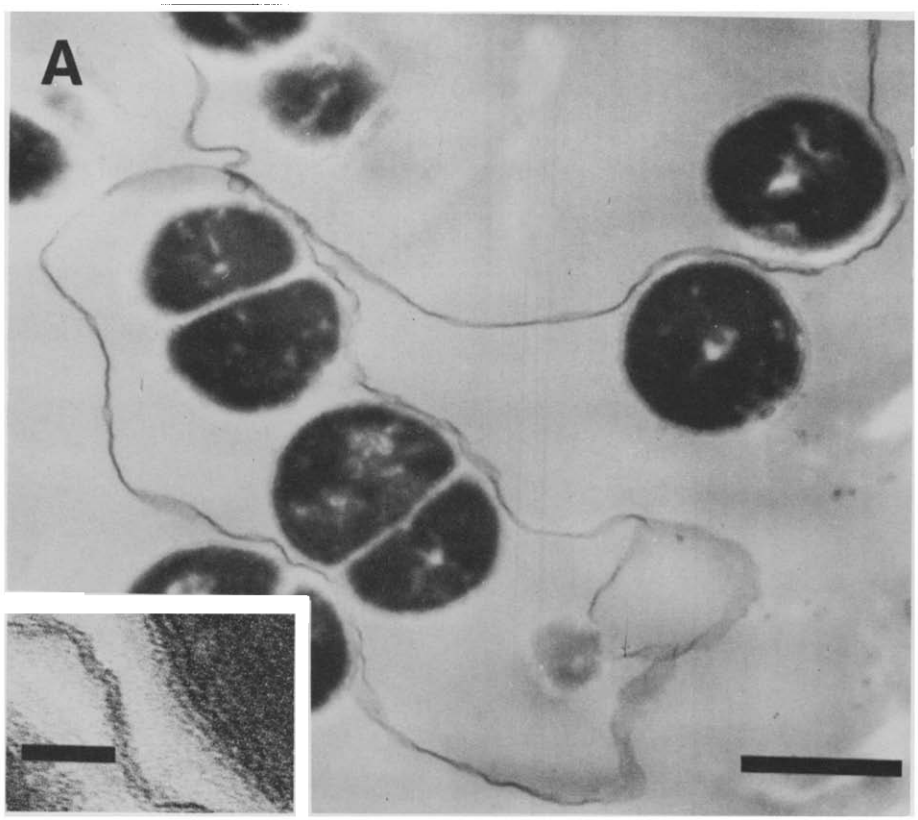
formed liposomes was used for calculation, the captured volume was 243 l/mol. The expected diameter of unilamellar liposomes (PC/C/PG/TO) was calculated as described in Ref. 14 from the captured volume (243 l/mol). The calculated diameter of 10 μm agrees well with the measured diameter of 9.2 ± 3.0 μm.

Liposomes prepared were studied by transmission electron microscopy and light microscopy (Figs. 1 and 2). The electron micrographs showed that liposomes are unilamellar vesicles, composed of bilayer unit membranes about 75 Å thick, quite similar in appearance and thickness to natural plasma membranes. By light microscopy, the liposomes were spherical vesicles with attached 'buttons', presumably representing neutral lipids; not having water-soluble 'head' groups, these would not be expected to become a part of the thin-bilayer membrane.

By varying the duration of mechanical shaking, liposome size could be adjusted to produce bigger or smaller mean size (Figs. 2 and 3). For the three preparations shown in Figs. 2 and 3, the mean diameters and standard deviations were 9.2 ± 3.0 μm, 5.9 ± 1.8 μm, and 4.3 ± 1.0 μm, respectively.

Table I shows capture efficiencies of liposomes made from various lipid combinations. Four types of lipid were used in each preparation. (1) Amphipathic lipids with net zero charge (PC, SM, PE). (2) Cholesterol. (3) Amphipathic lipids with net negative charge (PG's, CL, DOSS). (4) Neutral lipids without any hydrophillic head group (TO, VE, CO). All four types of lipids are necessary and omitting any one of them usually results in no liposomes or low encapsulation efficiency. The amount of neutral lipids can be reduced to 0.55% (Table I, No. 13) with slight decrease in trapping efficiency but complete omission gives poor results.

A wide variety of materials were trapped (Table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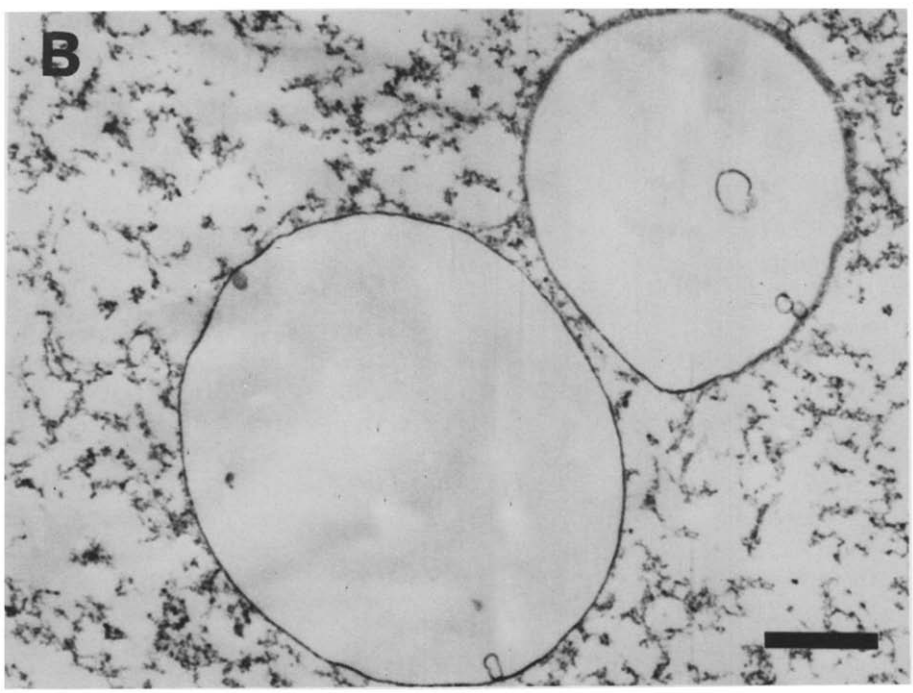
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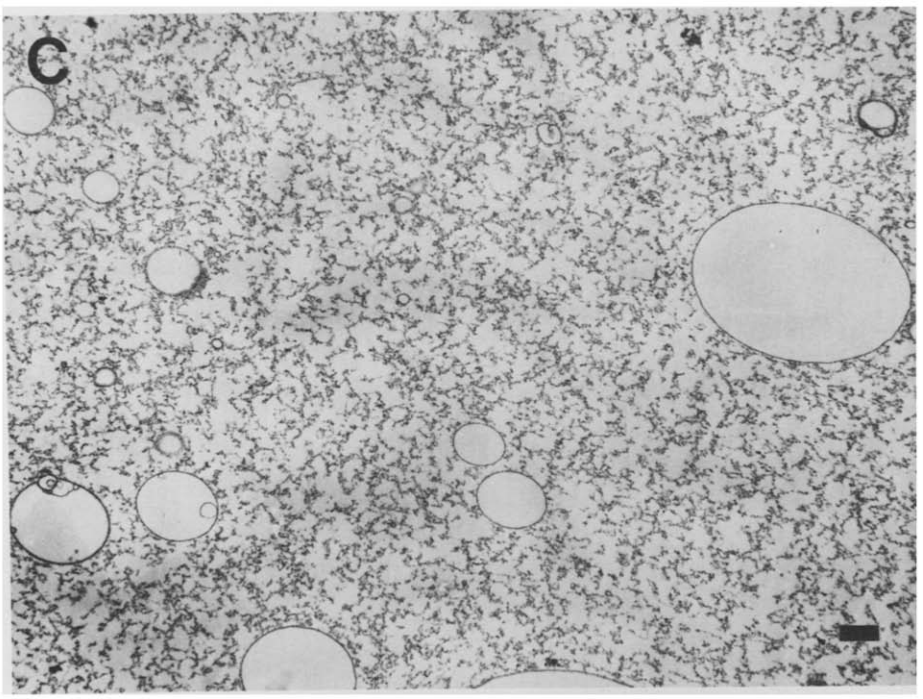


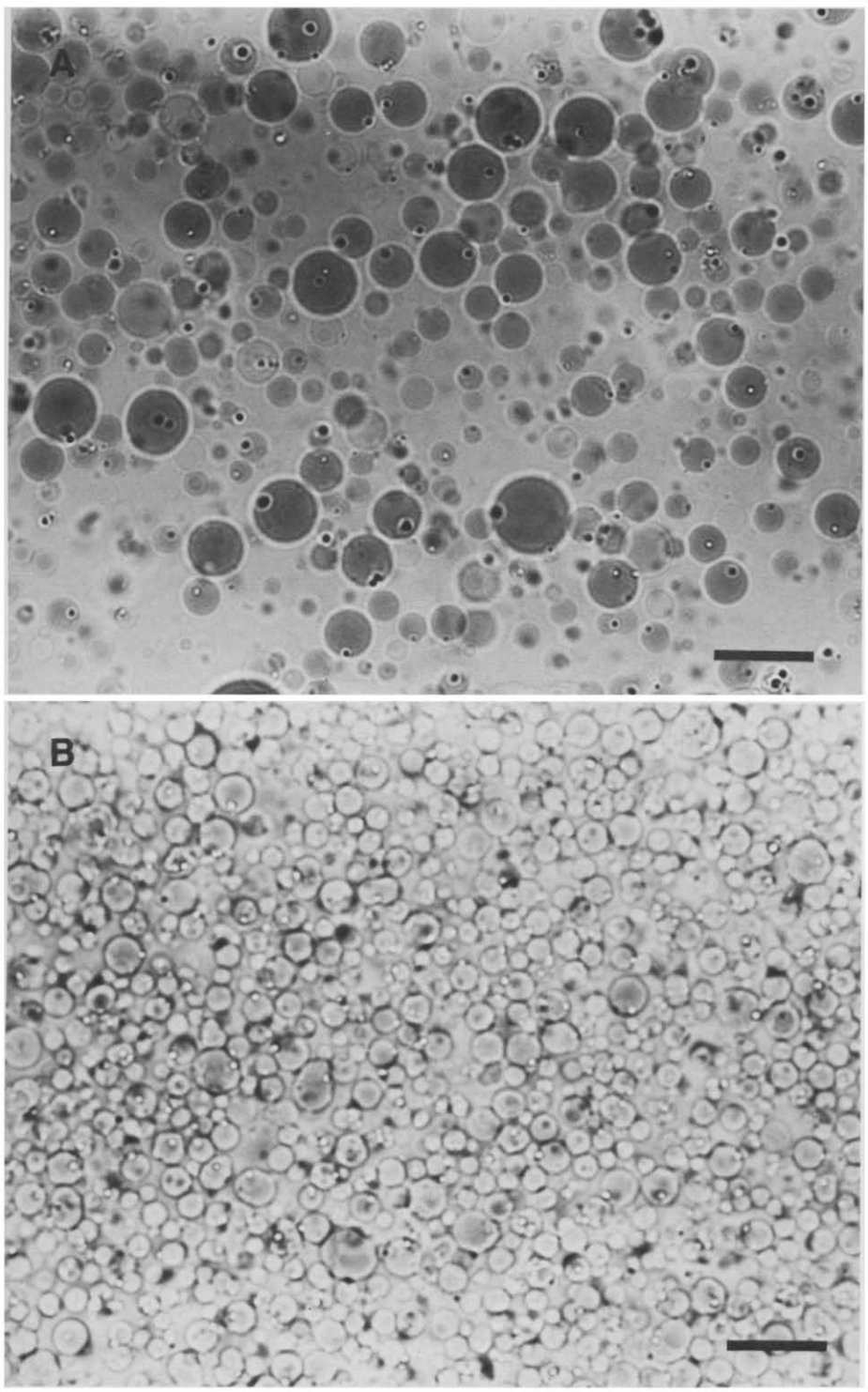
Fig. 1. (A) Transmission electron micrograph of liposomes with *Streptococcus salivarius* trapped within. (B and C) Liposomes containing Ponceau S dye. To the liposome pellet after centrifugation, equal volume of 5% gelatin was added to form the matrix outside vesicles. The liposomes were fixed, stained, and embedded as previously described for mast cells [21]. Scale bar, 0.5 μm; inset scale bar, 0.05 μm.

TABLE II

VARIOUS MATERIALS INCORPORATED INTO LIPOSOMES

A total of 6.67 μmol of lipids PC/C/CL/TO (4 : 4 : 1 : 1) were used in each preparation. The standard method described in the text was used except for No. 6 where 75 mM phosphate buffered saline (PBS) was used in place of 150 mM sucrose, and liposomes were centrifuged up to the top of centrifuge tube through a 0.5 cm thick layer of solution containing 100 mM sucrose and 140 mM glucose. Ponceau S dye, arsenazo dye, and ferritin were quantitated by measuring light absorption at 510 nm, 560 nm, and 425 nm, respectively, after clarification with 0.1% or 0.5% Triton X-100. Trapping of agarose (no. 8) was quantitated indirectly by measuring co-trapped Ponceau S dye. Radioactive tracers were used for quantitating other materials.

No.	Materials trapped	Concentration	Captured vol.		% Encapsulated
			μl/mg	1/mol	
1.	Glucose	30 mM	35	25	17
2.	Sucrose	150 mM	111	81	54
3.	Methotrexate	1.6 mg/ml	78	57	38
4.	Thymidine triphosphate	tracer	103	75	50
5.	Ponceau S dye	0.1%	109	79	53
6.	Ponceau S dye (in 75 mM PBS)	0.1%	68	49	33
7.	Arsenazo III dye	0.02%	97	70	47
8.	Agarose	0.1%	82	60	40
9.	Ferritin	10 mg/ml	25	18	12
10.	Collagen	tracer	64	46	31
11.	Poly(adenylic acid)	0.1 mg/ml	105	76	51
12.	DNA, human	0.01 mg/dl	119	87	58


A
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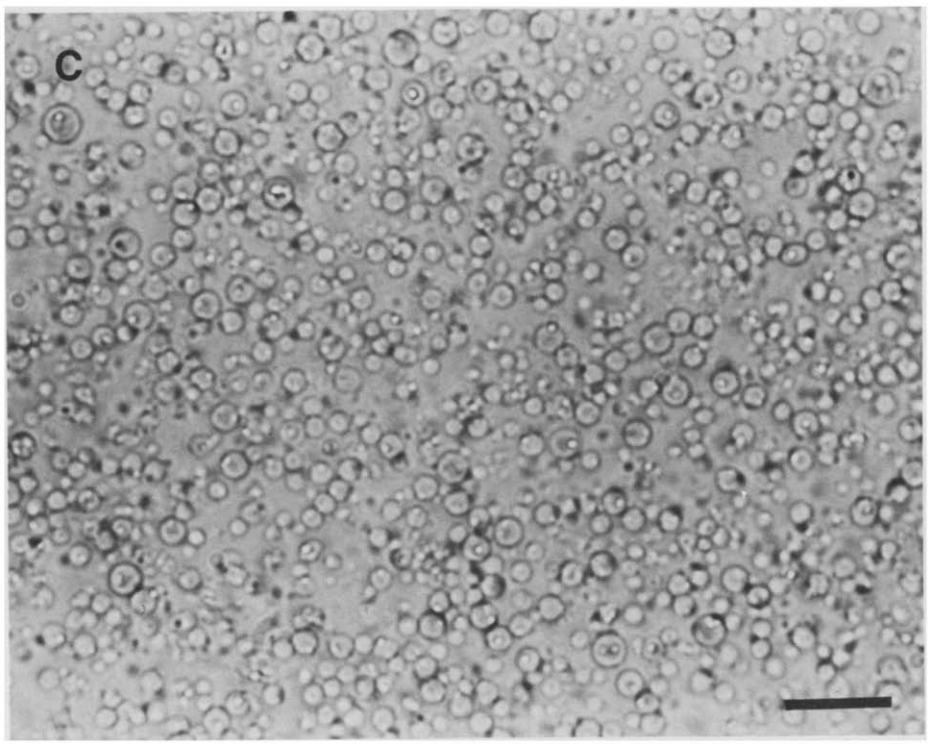

Fig. 2. (A) Light micrograph of a wet mount. The vesicles were prepared by the standard method. The lipid composition was PC/C/PG/TO (4 : 4 : 2 : 1, molar ratio). Vesicles contain 0.3% Ponceau S dye in 0.15 M sucrose. The medium is 5% glucose solution. (B and C) Smaller liposomes prepared by extending the duration of mechanical shaking (Figs. 3B and 3C). Size distributions of Fig. 3 were obtained from such light micrographs. Scale bar, 20 μm.

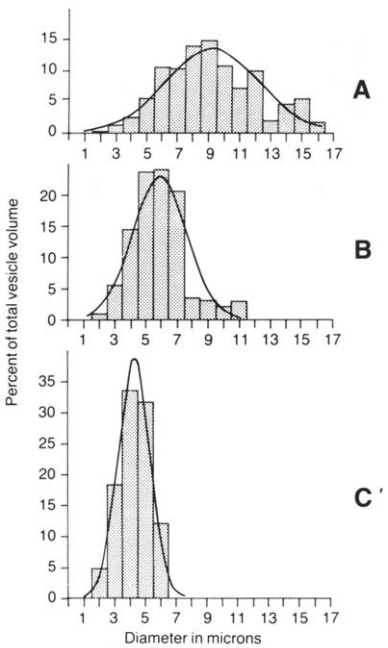

Fig. 3. Distributions of size in three separate preparations made by mechanically shaking for different lengths of time.

The strength of mechanical shaking on a vortex was standardized with one-dram vial containing 0.1 ml chloroform with 0.33 μmol of lipids (PC/C/CL/TO in 4 : 4 : 1 : 1 molar ratio) and 4 ml of 200 mM sucrose in water. The machine was set such that after 60-s shaking of the standardization vial, affixed horizontally to the machine head with a piece of adhesive tape, chloroform particles of 7.8 ± 2.9 μm in diameter were produced. All the mechanical shaking was done at this setting and configuration. The duration of shaking was used to control the extent of shaking. Each histogram in this figure represents a single preparation of liposomes. The number of vesicles counted were 711 for (A), 415 for (B), and 234 for (C). The duration of mechanical shaking for making water-in-chloroform emulsion, for making chloroform spherules, and the amount of amphipathic lipids used in each of the three preparations were, respectively, 45 s, 10 s, and 6.67 μmol for (A); 180 s, 40 s, and 6.67 μmol for (B); and 720 s, 160 s, and 20.0 μmol for (C). The ordinates of the histograms were obtained by multiplying the number of vesicles in each size class by the cube of radius and then calculating the percentage of the total volume of vesicles contributed by the various size classes. The best fitting normal distribution curve was superimposed on each histogram.

II), ranging in size from small molecules through proteins and nucleic acids, to particles as large as bacteria. Labile globular proteins (e.g. bovine serum albumin and hemoglobin) did not allow the water-in-chloroform emulsion to form, presumably because of surface denaturation at the water-chloroform interface. Further work is needed to find ways of preventing protein denaturation. One promising method under investigation in this laboratory is the use of labile proteins in microcrystalline form.

The liposomes were found to be sensitive to osmotic pressure, responding by shrinking when the osmolarity of medium was increased. The average diameter of liposomes changed from 9.2 ± 3.0 μm to 7.2 ± 2.3 μm and even further to 5.7 ± 1.8 μm as the osmolarity of medium was increased from 278 mM to 556 mM and then to 834 mM glucose.

Discussion

Our interest in liposomes began when we were searching for an efficient method of introducing genetic materials into cultured mammalian somatic cells. The liposomes prepared by the above method have unique characteristics that would make them suitable for the introduction of DNA or fractionated chromosome into cells via fusogens [19]. The liposomes have unilamellar membranes, so that the contents of vesicles get directly into cytoplasms when liposome membranes fuse with cell membranes. Unlike some of the other methods [14,15], sonication is not used during preparation so that breakage of long DNA molecules is less likely. The comparatively large size of the liposomes should make it possibel for mammalian metaphase chromosomes to be trapped into the liposomes. A simplified method for the transfer of chromosomes into cultured somatic cells should be of special interest to somatic cell geneticists concerned with linkage analysis and the regulation of gene expression [17,20].

Investigators in other fields, however, might also wish to consider using this method. The close similarity of the liposomes to natural plasma membrane in terms of unilamellarity, overall dimensions and osmosensitivity makes these liposomes a very good model for plasma membrane. The defined, yet adjustable size distribution would certainly simplify pharmacological and physico-chemical studies. For encapsulation of biological active materials, high capture efficiency is especially attractive. A 9-fold increase in the captured aqueous volume per milligram of lipid means 1/9 as much lipid is needed for trapping an equivalent volume of dissolved material. Furthermore, the pelletability of liposomes in a clinical centrifuge makes the removal of untrapped material technically easy. The concentration of the liposomes can be easily adjusted and the suspending medium is readily changeable by centrifugation.

Therefore, we believe that the cell-size unilamellar liposomes prepared by the method described here have potentially important applications in many areas of investigation.

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