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# LIPOSOMES OF CONTROLLABLE SIZE IN THE RANGE OF 40 TO 180 nm BY DEFINED DIALYSIS OF LIPID/DETERGENT MIXED MICELLES

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## Summary

Liposomes, in the size range of 40—180 nm, are formed when lipid and additives are solubilized with detergent, yielding defined mixed micelles, and the detergent is subsequently removed by controlled dialysis. Their most important properties are that they are indeed unilamellar with usefully large encapsulated volumes and are homogeneous in size. Liposomes have been formed from both natural and synthetic phospholipids with cholesterol and charged molecules added. This relatively simple technique may be particularly useful for encapsulating drugs, enzymes and other macromolecules and in studies of reconstitution of membrane proteins.

## Introduction

Artificial lipid vesicles, termed liposomes, are widely used as models for biological membranes, as recipients for drugs and other pharmacologically active compounds and for various other biological applications [1]. Different methods of liposome preparation are known. The most important are: formation of large multilamellar vesicles [2], small single-shelled liposomes prepared by sonication [3] or by gel filtration of mixed lecithin/cholate micelles [4], and larger single-shelled liposomes prepared by injection methods [5—7], by removal of detergents from mixed detergent/lipid micelles by dialysis [8—11], by a French pressure cell [12], by reverse-phase evaporation [13] or by a 'fractionated extrusion technique' [14].

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The major aim of this work was to develop a new preparation method which is able to control the size of the formed liposomes, to maintain the single bilayer structure and to attain highly homogeneous preparations.

Size and size homogeneity are two of the most important parameters of liposomes affecting their behaviour in blood circulation and tissue distribution [15]. Unfortunately, most of the investigations reported involving interactions of liposomes with biological systems are performed with liposomal preparations of undefined physicochemical properties. Consequently, much more attention should be paid to the physicochemical properties of prepared liposomes and reasonable standards of size distribution should be declared.

The preparation of liposomes by the detergent dialysis method described earlier [9] yields vesicles with an extremely narrow size distribution. The purpose of this work was to improve upon this detergent dialysis method so that unilamellar liposomes of a range of sizes and lipid compositions could be prepared. It was desirable to have a variety of liposome compositions so as to include a range of substances such as charged lipids, cholesterol, hydrophilic compounds such as inulin and hydrophobic substances with biological activity such as chlorpromazine.

Carey and Small [16] and Mazer et al. [17] observed a dependency of the bile salt/egg phosphatidylcholine mixed micellar size on the lipid : detergent molar ratio. According to their results, Mazer et al. [17] have proposed an improved 'Small-Dervichian' model of mixed micellar structure called the 'mixed disc' model. Until now no investigation has been reported on whether the mixed micellar size determines the size of the resulting liposomes. Therefore, it is necessary to remove the detergent by controlled and standardized dialysis, performed in this work with the Lipoprep dialyzer. Observations reported by Brunner et al. [4] suggest that the kinetics of detergent removal from mixed micelles causally affect liposomal size.

A reliable estimation of size and size distribution of liposomes affords measurements by different independent methods. Therefore, we applied dynamic laser light scattering, sedimentation velocity centrifugation with the analytical ultracentrifuge, electron microscopy and gel chromatography.

In contrast to small unilamellar vesicles and multilamellar vesicles, the internal volume : lipid ratio of large unilamellar vesicles prepared by the reported method is further increased, which affects the amounts of entrapped hydrophilic marker.

## Material and Methods

*Phospholipids and other reagents.* Phosphatidylcholine was isolated from fresh egg yolk according to the method of Singleton et al. [18] and characterized as described before [9]. Phosphatidylethanolamine from egg yolk, phosphatidylinositol from wheat germ, and phosphatidic acid from egg lecithin were obtained from Lipid Products Ltd., Nutfield, U.K. Cerebroside, type II, from bovine brain (Sigma) and the synthetic lipid, dimyristoyl phosphatidylcholine (Fluka), were used without further purification whereas stearylamine (Fluka) was recrystallized before use. [1-$^{14}$C]Dipalmitoyl phosphatidylcholine (100 mCi/mmol), 1-α-dipalmitoyl[2-*palmitoyl*-9,10-$^{3}$H]phosphatidylcholine (13 Ci/

mmol), [4-$^{14}$C]cholesterol (54 mCi/mmol), [2,4-$^{3}$H]cholic acid (14 Ci/mmol), [*glucose*-U-$^{14}$C]-*n*-octyl-β-D-glucopyranoside (300 mCi/mmol), [G-$^{3}$H]inulin (145.4 mCi/g) and [*benzene ring*-$^{3}$H]chlorpromazine hydrochloride (31 Ci/mmol) purchased from New England Nuclear or Applied Science Laboratories and showed a radiochemical purity of greater than 98.5%. Sodium cholate, inulin and all other reagents used (Merck) were of analytical grade. Cholesterol (Fluka) was recrystallized twice from methanol whereas the chemical purity of 1-*O*-(*n*-octyl)-D-glucopyranoside (Sigma) and chlorpromazine hydrochloride were greater than 99.8%.

*Preparation of mixed lipid/detergent micelles.* The lipid film from ethanolic or methanolic solutions was prepared by evaporation of the solvent during 1 h at 30° C. To avoid auto-oxidation and chemical degradation, all solutions were kept under an $N_2$ atmosphere throughout the preparation. The dried lipids were dispersed in 1 mM phosphate buffer, pH 7.3, adjusted to 0.16 ionic strength with NaCl at room temperature. Variable amounts of detergent were added to the lipid dispersion to obtain lipid : detergent molar ratios in the range of 0.2—1.15. Mixed micelle solutions with lipid : detergent ratios higher than 0.6 had to be gently stirred for 12 h at room temperature, and provided they were stored at 7°C and protected from light they could be used over a period of about 6 days after preparation. All other solutions with lipid : detergent ratios lower than 0.6 were stored until clear at room temperature for minutes up to 1 h before detergent removal. These micelles were stable over weeks.

*Preparation of liposomes using the flow-through dialyzer Lipoprep.* The Lipoprep apparatus is essentially based on the flow-through dialysis method described earlier [9] with various technical improvements. Fig. 1 shows the

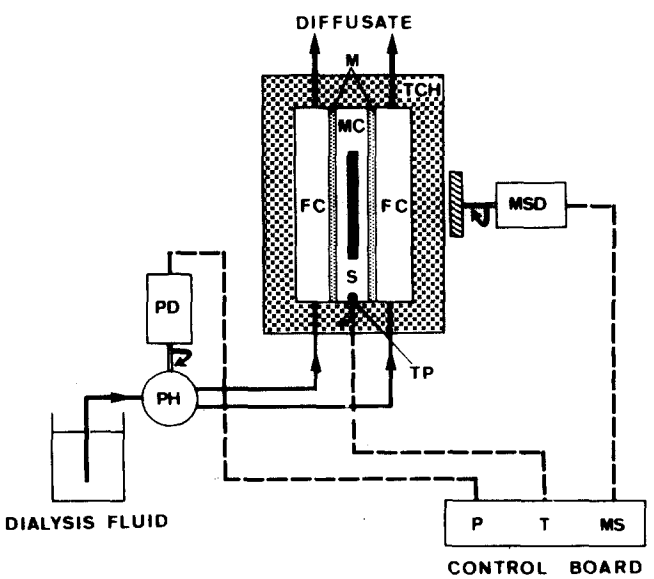


**Fig. 1. Instrumental set-up of the triple-cell dialyzer: Two flow-through cells (FC) and middle cell (MC) containing the mixed micelle solution, two semipermeable membranes (M), stirring bar (S), micro temperature probe (TP), temperature-controlled cell holder (TCH), magnetic stirrer (MSD) and pump drive (PD), two pump heads (PH) and control board (P = flow-rate control, T = temperature control, and MS = stirrer-speed control).**

triple-cell dialyzer with the internal thermostat allowing the preparation of liposomes of various lipids and common mixtures above the transition temperature. Routinely, 6 ml of the mixed micelle solution were injected into the middle cell which is in contact with two cellulose membranes of high permeability (molecular weight cut-off 10 000, Diachema Ltd., Rüschlikon, Zürich). Stirrer speeds in the range 75—100 rev./min and flow rates of 0.3—3.0 ml/min for each flow-through cell turned out to be optimal conditions for detergent removal. After dialysis with phosphate buffer up to 22 h, above the corresponding transition temperature, the formed liposomes can be taken from the middle cell. Dust particles are removed by centrifugation to avoid perturbation of the light-scattering measurements. No loss of lipids could be detected after this procedure. These liposome preparations were stored at 7° C or, when synthetic lipids were used, at a temperature above the transition temperature.

*Physicochemical characterization of mixed micelles and liposomes.* Homogeneity and hydrodynamic properties were measured by analytical ultracentrifugation (Beckman L-65 with schlieren accessories) and dynamic laser light scattering [9]. Liposomes were further characterized by freeze-fracturing and freeze-substitution electron microscopy, gel filtration on Sepharose 4B, and the internal volume of the vesicles experimentally determined using the water-soluble marker inulin.

## Results and Discussion

*Formation and properties of lipid/detergent mixed micelles*

As there are few experimental data on the size, shape or homogeneity of lipid/detergent mixed micelles, we have measured the mean hydrodynamic radii of lipid/detergent mixed micelles over a wide range of conditions with the technique of quasielastic light-scattering spectroscopy [9]. For egg phosphatidylcholine/cholate mixed micelles, we find that at low egg phosphatidylcholine : cholate molar ratios (0.2 up to 0.4) the time of micelle formation is very short but the system is heterogeneous, probably reflecting the coexistence of simple and mixed micelles. At high egg phosphatidylcholine : cholate molar ratios (greater than 0.6), the resulting systems are extremely homogeneous, suggesting that only mixed micelles are present. From Fig. 2, it is evident that the values of the mean hydrodynamic radii of the mixed micelles appear to diverge as the egg phosphatidylcholine : cholate molar ratio approaches the micellar phase limit for the system studied which is in contrast to the theoretical predictions of Carey and Small [16]. This divergence is consistent with a new model for the structure of the phosphatidylcholine/bile salt micelle called the mixed disc model postulated by Mazer et al. [17]. This model proposes that the egg phosphatidylcholine/cholate micelle is a disc in which egg phosphatidylcholine and cholate exist within the interior of the disc in the ratio of the phase limit, while cholate constitutes the disc's perimeter. Based on the dimensions of the lamellar liquid crystalline phase, it can be assumed that the thickness of the disc is about 5 nm.

Using the detergent *n*-octyl-β-D-glucopyranoside, the optimal range of egg phosphatidylcholine : detergent molar ratios is between 0.15 and 0.3 and the resulting mixed micelles are also very homogeneous but show large hydrodyna-

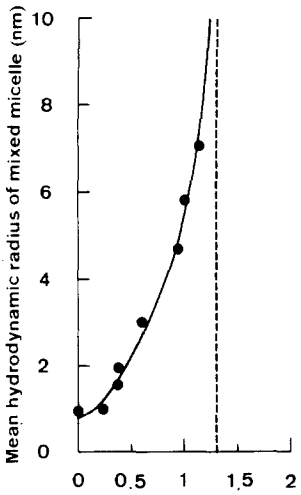


Fig. 2. Mean hydrodynamic radii of egg phosphatidylcholine/cholate mixed micelles with different egg phosphatidylcholine; cholate molar ratios measured by dynamic laser light scattering (argon ion laser, 514.5 nm, Spectra Physics at a scattering angle of 90° and a temperature of 23°C) at constant lipid concentration (16.93 mM).

mic radii of 6.2 ± 0.3 nm. The structure and homogeneity of these mixed micelles seem to be in accordance with those of egg phosphatidylcholine/bile salts.

*Formation and properties of unilamellar liposomes*

Solutions of mixed micelles were dialyzed against phosphate buffer with the Lipoprep instrument as described in Material and Methods. Fig. 3 shows the course of liposome formation starting with homogeneous egg phosphatidylcholine/cholate mixed micelles by controlled removal of the detergent studied by sedimentation-velocity measurements. The transformation of the micelles into homogeneous bilayer liposomes is characterized by an intermediate state in which mixed micelles and liposomes appear to be at equilibrium. Constant kinetics of detergent removal are essential and prevent the formation of heterogeneous liposomal systems. A perturbation of the kinetics of detergent removal at concentrations of cholate residues higher than about 5 mg/ml results in an extremely heterogeneous vesicle size distribution. This could be confirmed by separating the $^{14}C$-labeled species on Sepharose 2B and 4B and by size-distribution studies from electron microscopy. The rate of detergent dialysis influences the vesicle size. A 5-fold decrease of this rate leads to a significant increase of the mean vesicle radius of about 40% which could be shown by using cellulose membranes of very low permeability.

Table I lists the physicochemical properties of the unilamellar liposomes prepared by defined kinetics of detergent removal starting from egg phosphatidylcholine/cholate mixed micelles of different size. It is evident that the hydrodynamic radius, the apparent sedimentation coefficient, the particle weight and internal volume of the liposomes increase with increasing egg phosphatidylcholine : cholate molar ratio of the mixed micelles at constant lipid concentra-

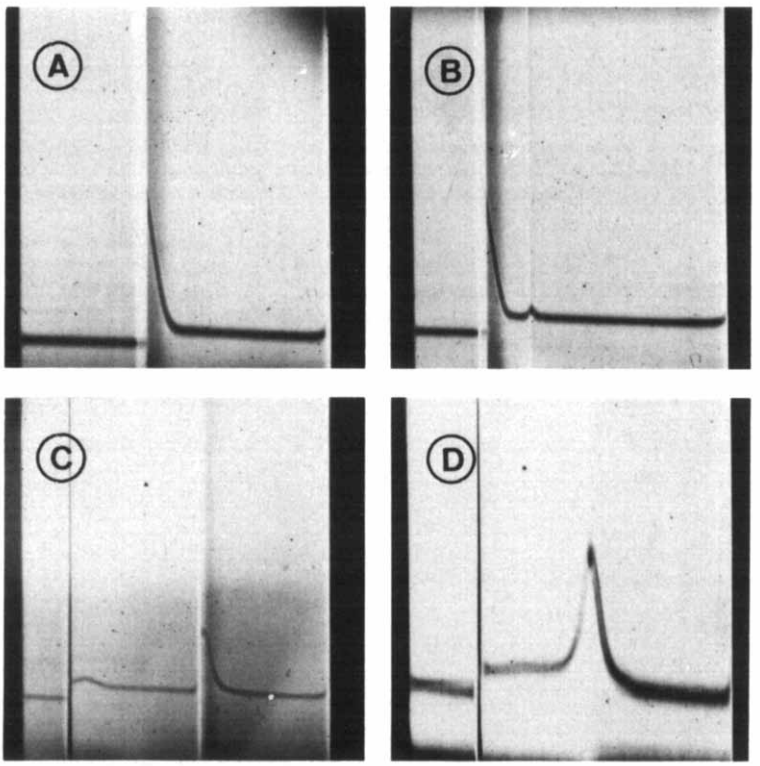


Fig. 3. Sedimentation schlieren patterns of the formation of homogeneous unilamellar liposomes during detergent removal at 20°C and 42 000 rev./min. (A) Mixed micelles after 10% cholate removal. (B) Mixed micelles and formed liposomes after 30% cholate removal. (C) Disappearing mixed micelles and growing liposomal fraction after 60% cholate removal. (D) Liposomes, greater than 99% cholate removed during 22 h.

tion. The preparations show comparable cholate residues and from the Sepharose 4B patterns it can be concluded that the remaining cholate appears completely within the liposomal fraction. It is not clear whether the residual cholate is entrapped in the hydrophilic and/or lipophilic vesicle phase. All the lipid found after Sepharose 4B gel filtration appears in the homogeneous unilamellar liposome fraction which means that these preparations are free of any multilamellar vesicles. The quality parameter of monodispersity from laser light-scattering measurements of the prepared liposomes before gel filtration is clearly better than those of sonicated unilamellar liposomes. The very high homogeneity of our liposome preparations is illustrated in Fig. 4 and the vesicle sizes evaluated from electron microscopy confirm the laser light-scattering data.

The vesicles produced by the method described are indeed unilamellar. This is confirmed by the estimation of the encapsulated volume with the water-soluble marker inulin and the freeze-substitution electron microscopy of egg phosphatidylcholine vesicles containing an iron-dextran complex termed Imferron (see Fig. 5). Actually, the bilayer is visible and multilamellar vesicles are not present. Inulin-encapsulated liposomes and corresponding empty liposomes show the same size in vesicle diameter of 65.8 ± 3.2 nm and the estimated

TABLE I

INFLUENCE OF THE EGG PHOSPHATIDYLCHOLINE : CHOLATE MOLAR RATIO OF MIXED MICELLES ON THE PHYSICOCHEMICAL PROPERTIES OF UNILAMELLAR LIPOSOMES AT A CONSTANT LIPID CONCENTRATION OF 16.93 mM (13 mg/ml)

Hydrodynamic radius was measured by dynamic laser light scattering using an argon ion laser (514.5 nm) and a scattering angle of 90° at 23°C. Homogeneity: quality parameter of monodispersity from laser light scattering, ideal spheres $Q = 0.05$. Apparent sedimentation coefficient was obtained from sedimentation-velocity experiments using the analytical ultracentrifuge with schlieren optics, 42 000 rev./min at 20°C. Particle weight was calculated by incorporation of experimentally estimated $s$ and $D$ values into the Svedberg equation using a partial specific volume of 0.9848 ml/g. The internal volumes of liposomes were determined from the experimentally estimated particle weight assuming an outer hydration layer of 0.7 nm and a bilayer thickness of 3.7 nm according to Huang and Mason [19].

| Egg phosphatidylcholine : cholate molar ratio | Cholate residue (mg/ml) | Hydrodynamic radius (nm) | Homogeneity ($Q$) | Apparent sedimentation coefficient (S) | Particle weight ($\times 10^{-7}$ dalton) | Internal volume (l/mol lipid) |
|---|---|---|---|---|---|---|
| 0.60 | 0.12 | 27.4 ± 1.4 | 0.06 ± 0.02 | 6.9 ± 0.4 | 1.297 | 1.75 |
| 0.76 | 0.15 | 33.7 ± 1.7 | 0.05 ± 0.01 | 12.8 ± 0.7 | 3.392 | 2.19 |
| 0.95 | 0.12 | 34.3 ± 2.4 | 0.09 ± 0.04 | 14.4 ± 0.8 | 4.019 | 2.21 |
| 1.15 | 0.10 | 40.3 ± 2.0 | 0.08 ± 0.03 | 16.5 ± 0.9 | 5.070 | 2.39 |
| Sonicated liposomes prepared according to the method of Huang and Mason [19] | | 10.5 | 0.15—0.25 | — | 0.188 | 0.25 |

encapsulated volume of 1.904 l/mol lipid agrees very well with the calculated value (1.978 l/mol lipid) according to Huang and Mason [19]. Therefore, it can be assumed that the whole vesicle population is unilamellar.

In addition to egg phosphatidylcholine, the proposed preparation procedure also works with other natural or synthetic phospholipids including additives such as cholesterol as well as stearylamine and sodium dicetyl phosphate to confer positive or negative charge. The total lipid recovery is always higher than 90%. Table II lists the estimated hydrodynamic radii of liposomes of common lipid mixtures and the degree of vesicle homogeneity. The radioactivity ratio of egg phosphatidylcholine and cholesterol in the mixed micelles as well as in the final liposomes is equal. This ratio and the absolute amount of lipid remain nearly unchanged over a period of 20 days. From Fig. 6 it is evident that multilamellar structures are absent and that the cholesterol is completely incorporated into the vesicle bilayer. Homogeneity and vesicle size estimated from electron microscopy confirm the data of the different liposomes listed in Table II. As the detergent *n*-octyl-β-D-glucopyranoside shows a very high rate of dialysis, the residual detergent concentration of the final liposome preparation is less than 0.1% compared with preparations using sodium cholate (0.5—1.5%).

As the volume of the aqueous space within the liposomes is a very important factor influencing drug encapsulation, the usefulness of the method described could be confirmed using glucose [22] and inulin as highly polar solutes. On increasing the encapsulated vesicle volume from about 0.25 l/mol lipid (sonicated liposomes) up to 1.9 l/mol lipid (vesicle diameter 66 nm) and 6.8 l/mol lipid (vesicle diameter 150 nm), the extent of entrapped glucose and inulin expressed in mg solute/mg lipid increases nearly linearly with increasing encap-

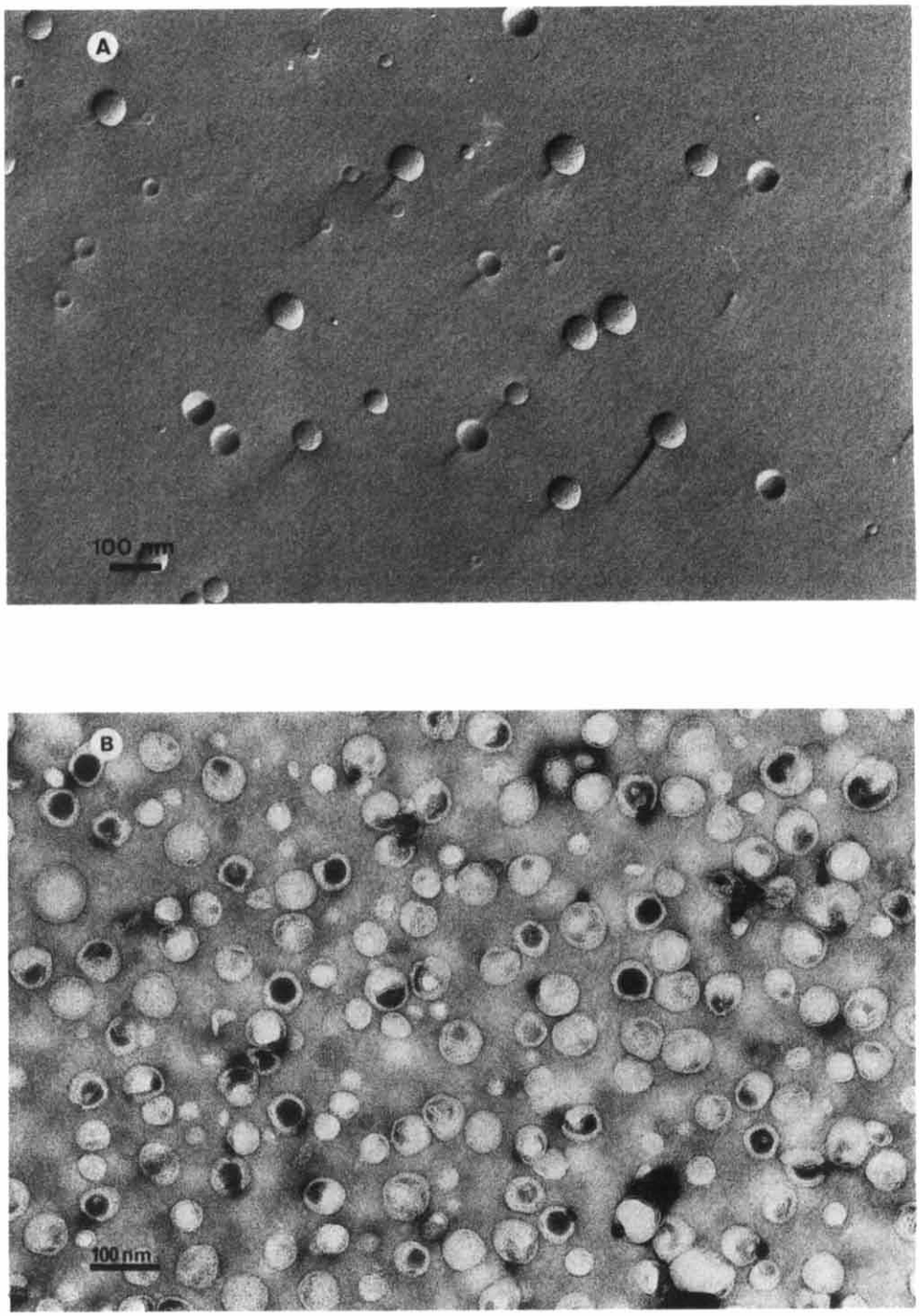


Fig. 4. (A) Freeze-fracture electron microscopy of egg phosphatidylcholine liposomes (13 mg/ml). The detergent removal from the mixed micelle solution was performed in the presence of ferritin (0.5%). (B) Electron microscopy of egg phosphatidylcholine liposomes with a mean diameter of 55 nm applying a combined freeze-substitution-negative staining technique.

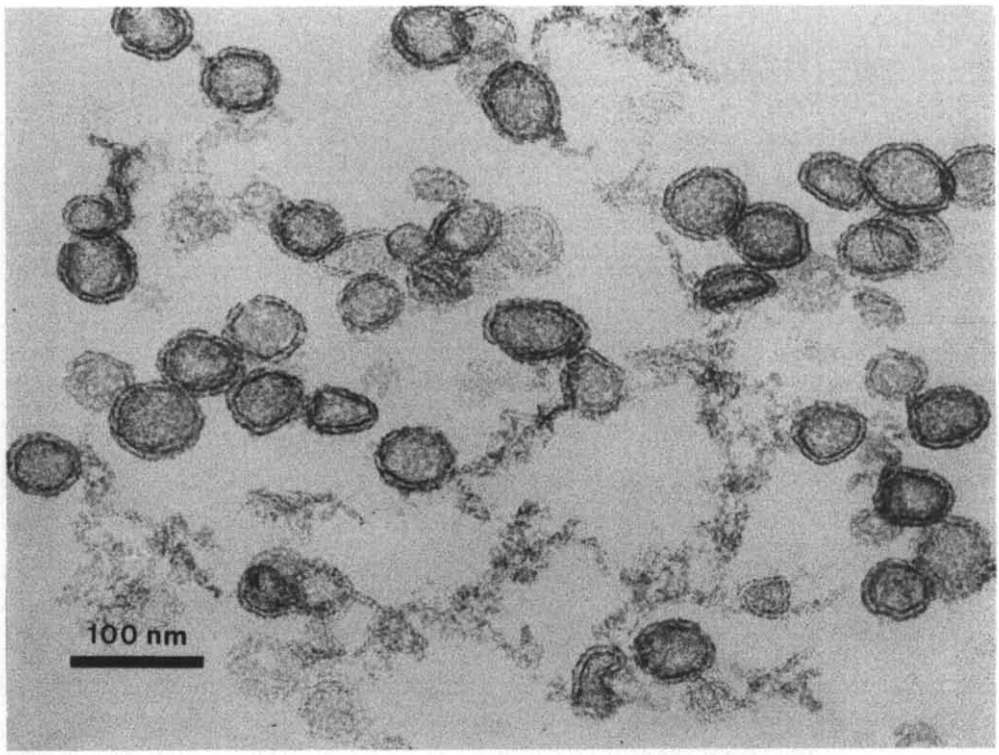


Fig. 5. Freeze-substitution electron microscopy of unilamellar liposomes prepared by defined detergent removal from a mixed micelle solution of egg phosphatidylcholine/cholate (molar ratio 0.60) in the presence of an iron-dextran complex (Imferron, 2.5 mg iron/ml). The liposomes (13 mg lipid/ml) were cryofixed in a propane-jet freezer [20]. Frozen samples were subjected to freeze-substitution at −90°C. The substituent consisted of methanol with 0.5% uranyl acetate, 1% osmium tetroxide and 3% glutaraldehyde. Dehydrated samples were embedded in araldite epon. These sections were stained with uranyl acetate and lead citrate [21].

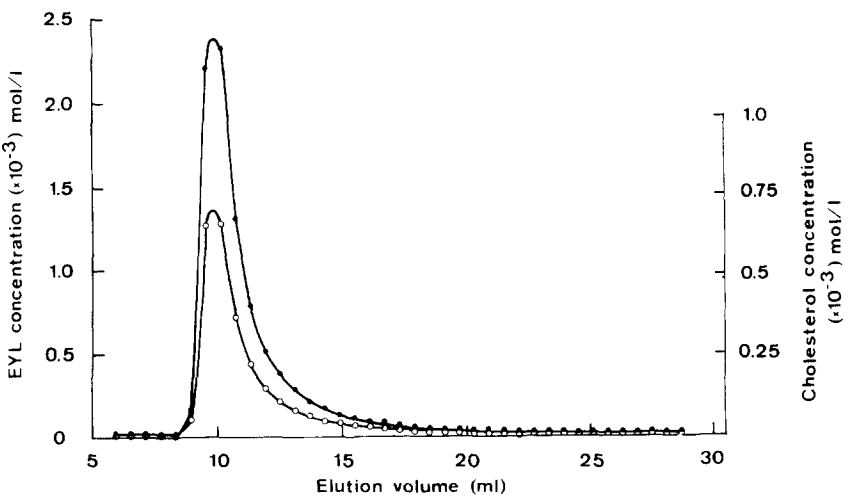


Fig. 6. Elution profile of egg phosphatidylcholine/cholesterol liposomes (molar ratio 8 : 2) on Sepharose 4B. (●) Egg phosphatidylcholine (EYL), (○) cholesterol.

TABLE II

PROPERTIES OF UNILAMELLAR LIPOSOMES OF VARIOUS LIPID MIXTURES PREPARED BY CONTROLLED DETERGENT REMOVAL

Total lipid concentration was varied between 10 and 13 mg/ml; preparations were performed at room temperature. $Q$ and hydrodynamic radius: see Table I.

| Lipid mixture molar ratio | Detergent | Lipid : detergent molar ratio | Hydrodynamic radius (nm) | Homogeneity ($Q$) |
|---|---|---|---|---|
| Egg phosphatidylcholine/ cholesterol (8 : 2) | cholate | 0.60 | 40.2 ± 2.0 | 0.08 ± 0.04 |
| Egg phosphatidylcholine/ cholesterol (7 : 3) | cholate | 0.52 | 30.6 ± 2.1 | 0.09 ± 0.05 |
| Egg phosphatidylcholine/ phosphatidylethanolamine (3 : 7) | cholate | 0.22 | 18.1 ± 0.9 | 0.03 ± 0.02 |
| Egg phosphatidylcholine/ phosphatidylinositol (8 : 2) | cholate | 0.60 | 29.6 ± 1.5 | 0.09 ± 0.04 |
| Egg phosphatidylcholine/ phosphatidic acid (10 : 2) | cholate | 0.62 | 20.9 ± 1.0 | 0.07 ± 0.02 |
| Egg phosphatidylcholine/ stearylamine (10 : 2) | cholate | 0.62 | 24.7 ± 1.2 | 0.09 ± 0.02 |
| Bovine brain cerebroside/ egg phosphatidylcholine (100 μg/μmol) | cholate | 0.60 | 40.5 ± 2.0 | 0.09 ± 0.03 |
| Dimyristoylphosphatidyl-choline/phosphatidyl-inositol (10 : 2) * | cholate | 1.25 | 71.5 ± 3.5 | 0.08 ± 0.03 |
| Egg phosphatidylcholine | *n*-octyl-β-D-gluco-pyranoside | 0.20 | 88.7 ± 3.6 | 0.07 ± 0.03 |

* Temperature of preparation 30°C.

sulated volume. Similarly, the number of lipophilic chlorpromazine molecules sequestered in each negatively charged liposome increases with increasing bilayer volume [23]. Using the liposome preparation method described, an indispensable prerequisite for any solute to be entrapped when added to the lipid/detergent mixture is the fact that the solute added may not at all perturb the micelle formation.

The usefulness of the proposed standardized and versatile liposome formation technique by detergent removal for drug encapsulation includes the efficiency of drug capture as well as the very accurate drug dosage due to the extremely high vesicle homogeneity compared with multilamellar liposomal drug preparations.

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## References

1 Kimelberg, H.K. and Mayhew, E.G. (1978) CRC Crit. Rev. Toxicol. 6, 25—79
2 Bangham, A.D., Standish, M.M. and Watkins, J.C. (1965) J. Mol. Biol. 13, 238—252
3 Huang, C. (1969) Biochemistry 8, 344—352
4 Brunner, J., Skrabal, P. and Hauser, H. (1976) Biochim. Biophys. Acta 455, 322—331
5 Deamer, D. and Bangham, A.D. (1976) Biochim. Biophys. Acta 443, 629—634
6 Kremer, J.M.H., van den Esker, M.W.J., Pathmamanoharan, C. and Wiersema, P.H. (1977) Biochemistry 16, 3932—3935
7 Batzri, S. and Korn, E.D. (1973) Biochim. Biophys. Acta 289, 1015—1019
8 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477—5487
9 Milsmann, M.H.W., Schwendener, R.A. and Weder, H.G. (1978) Biochim. Biophys. Acta 512, 147—155
10 Enoch, H.G. and Strittmatter, P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 145—149
11 Rhoden, V. and Goldin, S.M. (1979) Biochemistry 18, 4173—4176
12 Barenholz, Y., Amselem, S. and Lichtenberg, D. (1979) FEBS Lett. 99, 210—214
13 Szoka, F., Jr. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4194—4198
14 Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D. (1978) Biochim. Biophys. Acta 557, 9—23
15 Juliano, R.L. and Stamp, D. (1975) Biochem. Biophys. Res. Commun. 63, 651—658
16 Carey, M.C. and Small, D.M. (1970) Am. J. Med. 49, 590—608
17 Mazer, N.A., Kwasnick, R.F., Carey, M.C. and Benedek, G.B. (1979) in Micellisation, Solubilisation and Microemulsions (Mittal, K.L., ed.), Vol. 1, pp. 383—402, Plenum Press, New York
18 Singleton, W.S., Gray, M.S., Brown, M.L. and Withe, J.L. (1965) J. Am. Oil. Chem. Soc. 42, 53—56
19 Huang, C. and Mason, J.T. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 308—310
20 Müller, M., Meister, N. and Moor, H. (1980) Balzers Report BB 800011 DE
21 Reynolds, E.S. (1963) J. Cell Biol. 17, 208—212
22 Milsmann, M. (1980) Ph.D. Thesis, ETH No. 6563
23 Schwendener, R.A. (1979) Ph.D. Thesis, ETH No. 6486