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A novel method for encapsulation of macromolecules in liposomes

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Hemoglobin and alkaline phosphatase were each encapsulated in phosphatidylcholine liposomes using a dehydration-rehydration cycle for liposome formation. In this method, liposomes prepared by sonication are mixed in aqueous solution with the solute desired to be encapsulated and the mixture is dried under nitrogen in a rotating flask. As the sample is dehydrated, the liposomes fuse to form a multilamellar film that effectively sandwiches the solute molecules. Upon rehydration, large liposomes are produced which have encapsulated a significant fraction of the solute. The optimal mass ratio of lipid to solute is approx. 1:2 to 1:3. This method has potential application in large-scale liposome production, since it depends only on a controlled drying and rehydration process, and does not require extensive use of organic solvents, detergents, or dialysis systems.

Introduction

Liposomes are defined as vesicular lipid bilayers that enclose a volume of aqueous solution. The ability of liposomes to trap various solutes and to interact with cells by endocytosis or fusion has led to their application as a vehicle for intracellular delivery. Although several different methods are now available for liposome preparation [1,2] there remains some difficulty in adapting existing methods to efficient encapsulation of macromolecules. For instance, sonication of a lipid suspension [3–5], with proteins or nucleic acids may mechanically denature the dissolved macromolecules. The organic solvents used in reverse phase [6] and solvent vaporization methods [7] can also damage certain proteins, and methods involving detergent dialysis [8] and freeze-thaw steps [9] are not readily adaptable to large-scale preparations. It therefore seemed worthwhile to develop a relatively gentle and simple encapsulation procedure that could potentially be used in production of liter quantities of liposomes. To this end, we tested the possibility of using a dehydration-rehydration cycle for encapsulating macromolecules in which small unilamellar vesicles prepared by sonication or the French press method [10] are mixed with the solute to be encapsulated, followed by drying under nitrogen. These conditions cause the liposomes to fuse and form multilammelar structures that sandwich the solute. Upon rehydration, the multilayered material swells to form large vesicles, and these trap a significant fraction of the original solute. This method was first established for encapsulation of 6-carboxyfluorescein and DNA [11], and in the present study we have extended it to proteins. Hemoglobin was chosen as a model protein because it could be conveniently measured by absorption spectrophotometry, and alkaline phosphatase was chosen to determine whether an enzyme could remain active through the encapsulation process.

Materials and Methods

1-Palmitoyl-2-oleoylphosphatidylcholine was obtained from Avanti Phospholipids, Inc. Bovine

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Abbreviation: POPC, 1-palmitoyl-2-oleoylphosphatidylcholine.

intestinal alkaline phosphatase, fluorescamine, Triton X-100, and trypsin were purchased from Sigma. Human hemoglobin was prepared by hyposmotic hemolysis of washed cells, followed by centrifugation to remove contaminating plasma membranes.

Alkaline phosphatase activity was monitored by the increase in absorption at 410 nm as *p*-nitrophenylphosphate is hydrolysed to *p*-nitrophenol [12]. An aliquot was taken from each liposome sample and added to an assay mixture containing 0.8 mM *p*-nitrophenylphosphate, 2.0 mM $MgCl_2$, 0.01 M Triton X-100, and approx. 25.5 mM Tris adjusted with NaOH to pH 10, the pH optimum of the enzyme.

Fluorescamine was used to assay for protein both external and internal to the liposomes. For assays of protein outside the liposomes, an aliquot of the sample was added to a total assay volume of 3 ml containing 0.16 M sodium borate at pH 9.0 (made by adjustment of boric acid with NaOH) and 0.045 mg fluorescamine. The concentration of derivatized protein was measured as a function of fluorescence at 480 nm, with an excitation wavelength of 390 nm, and compared with standard samples of the same protein. For assays of protein inside the liposomes, the external protein was measured and then subtracted from the total protein concentration present in the aliquot. The total protein assay mixture contained 0.14 M sodium borate, 0.01 M Triton X-100, and 0.045 mg fluorescamine, again in a 3 ml assay volume.

Liposomes were made by dispersing phosphatidylcholine in water (1.0 mg/ml) using probe sonication at 40 W for 1 min with a Biosonik III probe sonicator. (Longer sonication times did not improve the final preparations.) The protein solutions, also in water, were added to varying amounts of the lipid dispersion to a protein concentration of 2 mg/ml for both alkaline phosphatase and hemoglobin. The mixture was then dried at 37°C in the rotating flask of a flash evaporator under a stream of dry nitrogen gas. The films of lipid and protein was rehydrated in the original volume of the sample by addition of water, followed by 30-min incubation in the rotating flask. If desired, further uniformity of the preparation could be obtained at this point by centrifugation or filtration through polycarbonate filters. Aliquots of the rehydrated mixture were then assayed for enzyme activity and total protein content, and the remaining volume was centrifuged at $43\,000 \times g$ for 18 h to concentrate the liposomes. After the liposomes were pelleted, they were resuspended in water and assayed for activity and protein content.

Liposome formation under the above conditions was observed by phase microscopy, and the process was compared for pure lipid dispersions and for lipid/protein mixtures. A drop of the suspension was dried on a microscope slide and 10 μl of water or buffer was then placed on the dried film, followed by a cover slip. Swelling of the hydrated film and subsequent liposome formation were photographed over periods up to 2 h, and compared with aliquots taken from the flask during a preparation run.

Results

Phase microscopy

When a dispersion of phosphatidylcholine prepared as sonicated liposomes was dried and rehydrated, myelin figures of lipid were observed to grow from the film during hydration (Fig. 1). This is commonly observed in hydrating phospholipid systems, and clearly demonstrated that the liposomes fused during drying. If the myelin figures were gently agitated, the cylindrical structures,

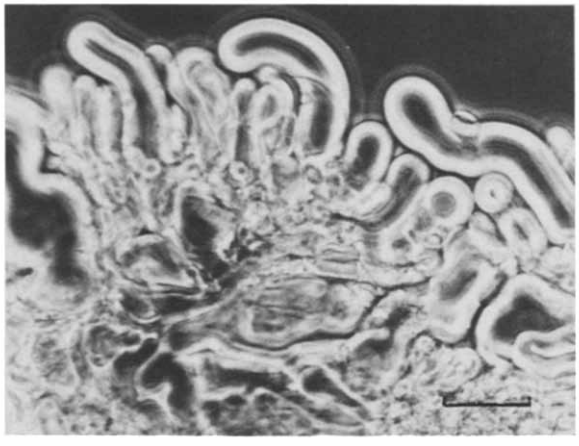

Fig. 1. Myelin figures form during rehydration of pure lipid films. 1.0 mg/ml POPC was sonicated in water to produce liposomes, and a 10-μl aliquot was dried on a microscope slide. During dehydration, vesicles fuse to produce a multilamellar film, and when water or buffer is added the rehydrating lipid produces characteristic myelin figures. Bar shows 20 μm.

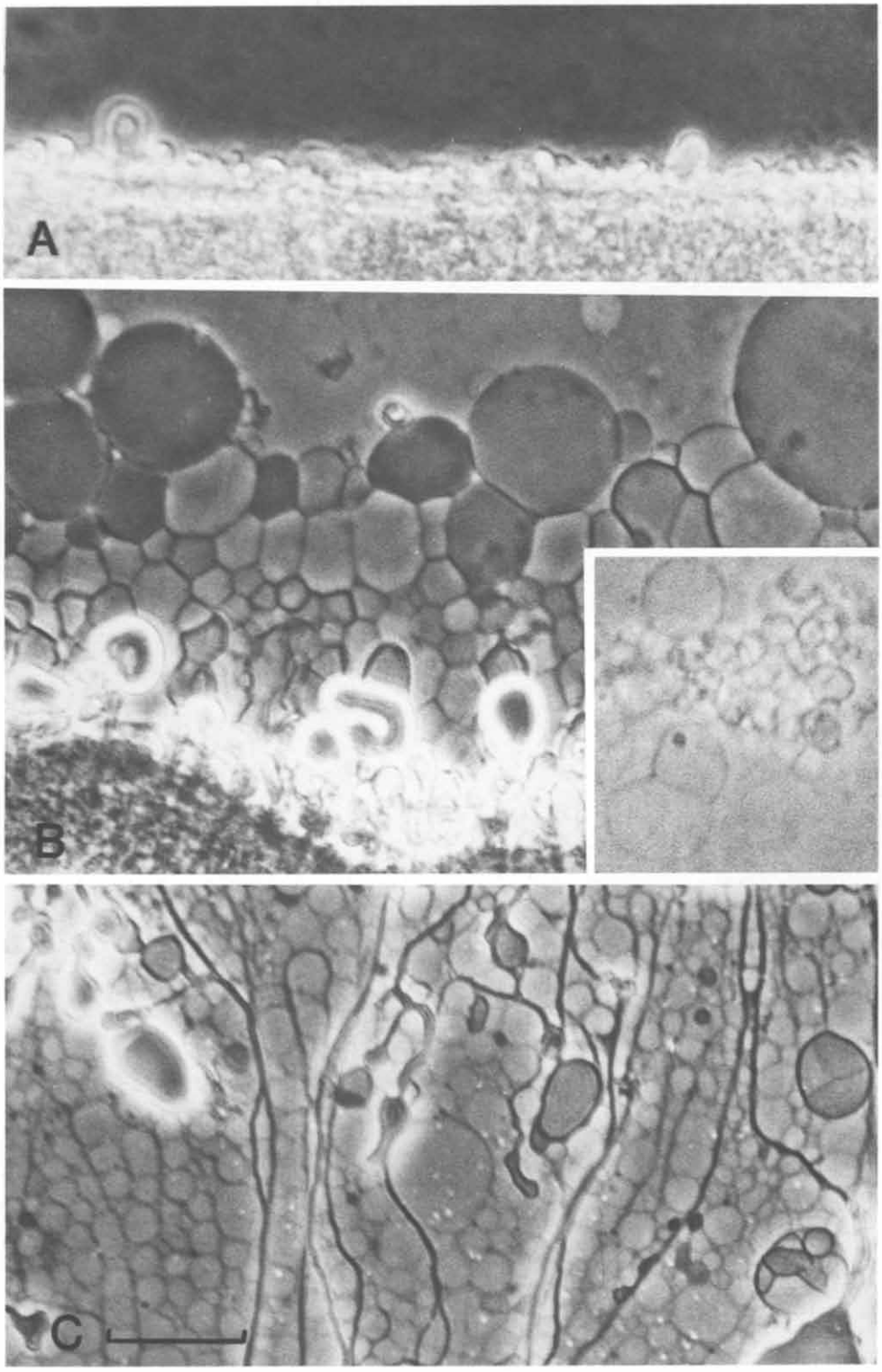

Fig. 2. Large vesicles are produced when phospholipid/protein mixtures are rehydrated. POPC liposomes were prepared as in Fig. 1, but were mixed with hemoglobin in mass ratios of 1 : 2 (POPC : hemoglobin) before drying. (A) The edge of the dried film 1 min after addition of water; (B and C) the same region 30 and 60 min later. The inset of (B) is a sample taken during an actual preparation run, 3 min after rehydration is initiated. Bar shows 20 μm.

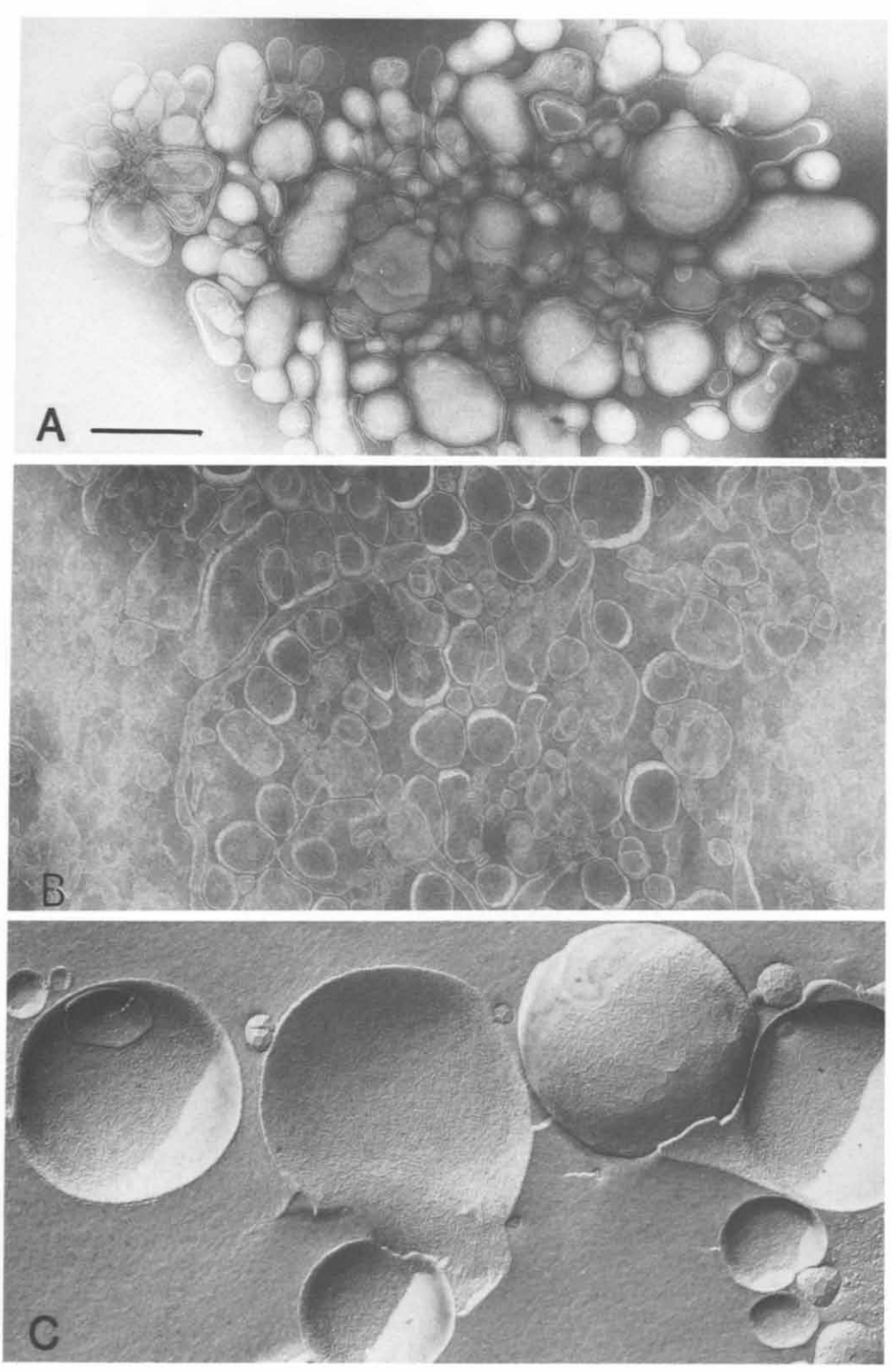

Fig. 3. Electron microscopic appearance of liposomes. Liposomes were prepared by rehydration of protein-lipid films in a rotating flask. (A) POPC : hemoglobin = 1 : 1; (B) POPC : hemoglobin = 1 : 2; (C) POPC : alkaline phosphatase = 1 : 2. Bar shows 0.2 μm.

composed of concentric multilamellar phospholipid, are dispersed in the form of multilamellar liposomes first reported by Bangham et al. [13]. Surprisingly, when a solute like hemoglobin is dried with the lipid in certain mass ratios, a markedly different result is obtained (Fig. 2). Some myelin figures appear but numerous large vesicles, up to 50 μm in diameter, are also observed, reminiscent of those reported by Oku and MacDonald [14]. Over a period of 1 h, the entire dried film dispersed as relatively large vesicles. Similar large vesicles were observed during the first 1–5 min of hydration during a preparation run (inset, Fig. 2B), suggesting that such vesicles are not an artifact of the conditions in which a lipid-protein film is hydrated under a coverslip. This result was highly reproducible, and typical observations are illustrated. After 30-min rehydration in the rotating flask, the larger vesicles were absent, and the resulting small liposomes could not be resolved for photographic purposes by phase microscopy.

If the hydrating lipid-protein film was gently agitated during the swelling process, as occurs during the actual preparation procedure, vesicles in size ranges more typical of large unilamellar vesicles were produced. Fig. 3 shows electron micrographs of such preparations taken as aliquots from a typical run. At suboptimal mass ratios, both unilamellar and multilamellar vesicles were observed (Fig. 3A), while at optimal mass ratios unilamellar vesicles were most commonly observed (Fig. 3B). The optimal mass ratios, defined as those ratios producing the fewest multilamellar vesicles, varied somewhat for different solutes, and for the two proteins tested here it was in the range of 1 : 2 to 1 : 3 (lipid : protein).

Captured volume

We went on to determine the effect of varying mass ratios on captured volume, defined as liters encapsulated per mole lipid. The apparent captured volume was measured fluorometrically for both proteins with the fluorescamine assay described in Materials and Methods, by specific activity for the alkaline phosphatase, and by spectrophotometric measurement of hemoglobin. Fig. 4 shows that captured volume decreased markedly with increasing concentrations of phosphatidylcholine for a system containing 2 mg/ml al-

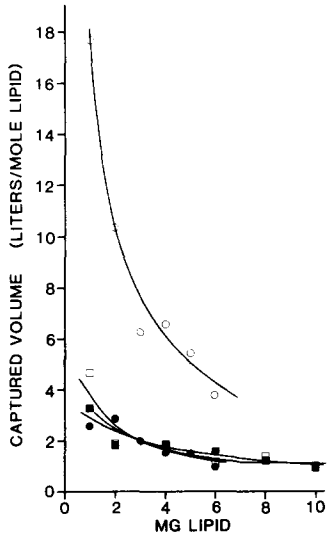

Fig. 4. Relationship between lipid concentration and captured volume. Small unilammelar vesicles/protein mixtures (1 ml) were subjected to one dehydration cycle in the presence of 1 mg/ml hemoglobin or alkaline phosphatase. The captured volume was measured by fluorescamine protein assay. ●, Alkaline phosphatase; ■, hemoglobin; phosphatase activity, ○, or absorbance at 412 nm, □.

kaline phosphatase or hemoglobin. Apparent captured volume measured by the specific activity of alkaline phosphatase was at variance with results obtained by other methods, presumably because substantial amounts of the enzyme adhere to the surface of the vesicles. The activity of the external enzyme cannot be distinguished from the total activity of the pelleted liposomes, since the substrate, *p*-nitrophenylphosphate, is sufficiently permeable so that both internal and external enzyme interact with it. Furthermore, we were unable to find conditions which selectively inactivated the external enzyme. Therefore, the fluorescamine method was used to distinguish between encapsulated and externally bound enzyme, and when this was done, the values for captured volume fell into the same range of those of hemoglobin (Fig. 4).

The encapsulation efficiency, a measure of the fraction of total volume encapsulated, increased gradually with increasing lipid content until the system reached a certain mass ratio of protein to lipid. This ratio was 1 : 3 for hemoglobin and 1 : 2.5 for alkaline phosphatase, suggesting that the

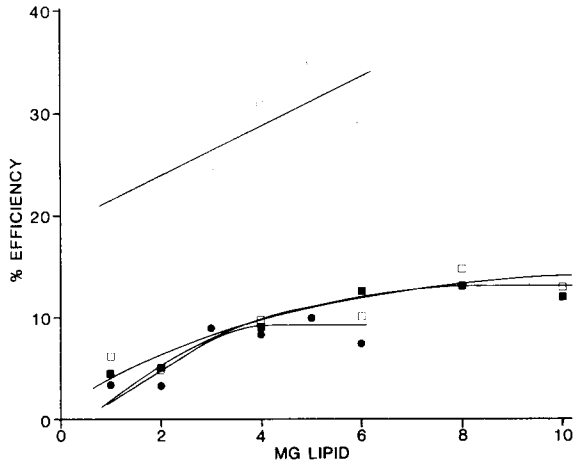

Fig. 5. Encapsulation efficiency as a function of lipid concentration. Small unilamellar vesicle/protein mixtures (1 ml) were subjected to one dehydration-rehydration cycle in the presence of 2 mg/ml hemoglobin or alkaline phosphatase. The percent of total volume encapsulated was measured by fluorescamine protein assay, ●, alkaline phosphatase; ■, hemoglobin; phosphatase activity, ○, or absorbance at 412 nm, □.

total volume encapsulated depended on the ability of the lipid to form unilamellar vesicles. If large amounts of lipid were added, little further increment in encapsulation occurred, probably because the excess lipid simply formed multilamellar structures that did not significantly increase the total encapsulated volume.

Discussion

In an early investigation, Reeves and Dowben [15] observed that slow rehydration of dried lipid films resulted in the formation of relatively large vesicles resembling those described here. However, these workers noted that the presence of ionic solutes or proteins inhibited the success of the method. In contrast, the present study has shown that dehydration-rehydration cycles can be used to prepare liposomes and encapsulate macromolecules such as proteins, but only if the mass ratios of lipid to protein are adjusted to an optimum range. In agreement with Reeves and Dowben, we found that the presence of ionic solutes, for instance, 0.1 M sodium chloride or potassium phosphate, did cause severe aggregation in the present method as well.

Earlier work showed that DNA and 6-carboxyfluorescein are also encapsulated by dehydration-rehydration cycles [11], and Table I summarizes encapsulated efficiencies and captured volume parameters for all of the solutes that have been tested. The values generally fall short of the theoretical limit of 74% encapsulation that is the limiting value for volume encapsulated by close-packed spheres, and which can be approached by the REV (reverse phase evaporation) method in the presence of 66 mM phospholipid [16]. Instead, encapsulation efficiencies range around 10–15% for most of the solutes. It is not clear why higher efficiencies are not achieved, but it may reflect the physical configuration of the lipid and solute at the point when fusion of vesicles begins to occur. We have not attempted to improve the encapsulation efficiency by varying the conditions of drying, and it is possible that greater efficiencies can be achieved. A related method was recently published

TABLE I

ENCAPSULATION EFFICIENCIES AND CAPTURED VOLUME RANGES FOR VARIOUS SOLUTES

Solute	Solute concn. (mg/ml)	Solute/lipid (mass ratio)	Maximal captured volume (l/mol lipid)	Efficiency range (% volume captured)	Ref.
6-Carboxyfluorescein [a]	0.04	1:250	–	6–16	11
DNA [a]	0.1	1:50	–	9.8–45	11
Hemoglobin [b]	2	1:3	4	4.5–13	–
Alkaline phosphatase [c]	2	1:2.5	3	3–10	–

[a] Egg phosphatidylcholine used. Dried at 60°C.
[b] Average values from protein assay and A_{412} data (eight determinations). Dried at 37°C.
[c] Average values from protein assay (five determinations). Dried at 37°C.

by Kirby and Gregoriadis [17] which involves lyophilization (freeze-drying) to induce liposome fusion and solute encapulation. These investigators obtained encapsulation efficiencies of 14% (carboxyfluorescein) and 39–54% (albumin).

The captured volume of 3 l/mol represents an average vesicle diameter of about 0.1 μm, assuming a homogeneous unilamellar preparation. Fig. 3 shows that the actual vesicle size range is 0.15 ± 0.024 μm for hemoglobin-containing vesicles (negative staining) and 0.23 ± 0.19 μm for alkaline phosphatase-containing vesicles visualized by the freeze-fracture method. Minor fractions of oligolamellar vesicles were observed with both methods, and the captured volume is reasonably consistent with the above dimensions.

Under some conditions, we found that significant amounts of protein may bind to the exterior surface of the liposome, as with alkaline phosphatase. This may represent a limitation to the method if it is necessary that the macromolecules be entirely within the vesicle.

The main advantage of the method is that it does not require organic solvents, detergent dialysis or freezing, which are central features of other liposome preparation methods, and therefore has the potential for being scaled up to preparations of liter volumes of liposomes. To test this, in one run 1.0 g egg phospholipid was dispersed by sonication in 100 ml water and mixed with 100 ml water containing 2.5 g hemoglobin adjusted to pH 7.5. The mixture was dried on the inner surface of a rotating 20-l glass carboy (6 rpm), heated to 40°C while passing nitrogen gas into the flask. Drying required approx. 2 h. The film was then rehydrated by addition of 1 l water, followed by further rotation (30 min) and passage through a 1.2 μm 5 cm Millipore filter. The resulting liposomes were comparable to those described earlier, having a captured volume of 3.9 l/mol and an encapsulation efficiency of 7%. An aliquot of the liposomes was separated from external hemoglobin by gel filtration (Bio-Gel 1.5 m) and the hemoglobin spectral characteristics were determined in the presence and absence of oxygen. The spectra of the encapsulated hemoglobulin underwent the characteristic shift expected for oxygen binding.

A second advantage follows as well, in that the absence of detergents or organic solvents may make it the method of choice for encapsulation of certain macromolecules that are sensitive to these compounds. The requirement for specific mass ratios also could be an important consideration in certain applications. For instance, it may be desired to encapsulate an amount of substance, for instance, an enzyme or drug, that does not permit the most efficient mass ratio to be used. Under these conditions, it is possible to use an inactive carrier compound and Crowe and Crowe (personal communication, see also Ref. 18) have determined that trehalose, a nonreducing disaccaharide that is produced by anhydrobiotic organisms to prevent damage during drying, can be used as a 'filler' to provide the optimal mass ratio.

In summary, the results reported here suggest that dehydration-rehydration cycles can be a useful alternative preparation method for liposomes, particularly if large quantities are desired or if sensitive molecules are to be encapsulated. It is sufficiently gentle so that nearly 90% of the specific activity of alkaline phosphatase can be preserved during preparation. The method requires specific mass ratios of phospholipid to solute, and is limited by the presence of inorganic ions.

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