

Vaccine Entrapment in Liposomes

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The use of liposomes as carriers of peptide, protein, and DNA vaccines requires simple, easy-to-scale-up technology capable of high-yield vaccine entrapment. Work from this laboratory has led to the development of techniques that can generate liposomes of various sizes, containing soluble antigens such as proteins and particulate antigens (e.g., killed or attenuated bacteria or viruses), as well as antigen-encoding DNA vaccines. Entrapment of vaccines is carried out by the dehydration–rehydration procedure which entails freeze-drying of a mixture of “empty” small unilamellar vesicles and free vaccines. On rehydration, the large multilamellar vesicles formed incorporate up to 90% or more of the vaccine used. When such liposomes are microfluidized in the presence of nonentrapped material, their size is reduced to about 100 nm in diameter, with much of the originally entrapped vaccine still associated with the vesicles. A similar technique applied for the entrapment of particulate antigens (e.g., *Bacillus subtilis* spores) consists of freeze-drying giant vesicles (4–5 μm in diameter) in the presence of spores. On rehydration and sucrose gradient fractionation of the suspension, up to 30% or more of the spores used are associated with generated giant liposomes of similar mean size. © 1999 Academic Press

There is an urgent need for effective and safe immunological adjuvants that could promote appropriate immune responses to vaccines in human immunization programs (1, 2). This is especially true for most of the synthetic peptide and subunit vaccine antigens which, in addition to being costly or only available in small quantities (e.g., recombinant DNA products), can be weakly or nonimmunogenic. However, immunological adjuvants presently available, e.g., Freund's complete and incomplete adjuvants, bacterial endotoxins, poly-anions, mineral adsorbents, induce local or systemic

toxicity, form unacceptable granulomas, lack efficiency, or have short-term effects. Another possible hazard with some of these adjuvants is the production of allergic reactions to vaccines in a minority of recipients, for instance, those already sensitized to the antigen (1, 2).

The immunological adjuvant property of liposomes was first established (3) when strong humoral immune responses to diphtheria toxoid (entrapped in liposomes) were obtained after injection into mice. Unlike other adjuvants, there were no granulomas at the site of injection (3, 4) and no hypersensitivity reactions were observed in preimmunized animals when the antigen was given in the entrapped form (5). Furthermore, liposomes composed of the appropriate phospholipid (e.g., egg phosphatidylcholine) do not develop antibodies against their phospholipid component (6) nor have they produced any side effects in repeatedly injected patients (7–9). Extensive work in this and other laboratories in the last 25 years has shown that liposome adjuvanticity applies to a large variety of bacterial, viral, protozoan, tumor, and other antigens (6, 10). In many experiments (10) protection of animal models was achieved by immunization with the relevant liposome-entrapped antigens.

Recently, a possible alternative to conventional vaccines has emerged in the form of genetic immunization (11–13). It is now known that intramuscular injection of naked, antigen-encoding plasmid DNA leads to humoral and cell-mediated immune responses against the antigen. It appears (11–13) that immunity follows uptake of DNA by muscle cells, DNA expression, extracellular release of the generated antigen, and its uptake up by antigen presenting cells (APCs). It is also feasible that some of the injected DNA is taken up directly by APCs. Disadvantages of naked DNA immunization include uptake of DNA by only a minor fraction of muscle cells;

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exposure of DNA to deoxyribonuclease in the interstitial fluid, thus necessitating the use of relatively large quantities of DNA; and, in some cases, the need to inject into regenerating muscle to enhance immunity (11–13). Work from our laboratory (12, 14) suggests that such problems could be circumvented by the use of liposome-entrapped DNA. This approach would eliminate the involvement of muscle cells and facilitate, instead, uptake of DNA by APCs infiltrating the site of injection or in the lymphatics (8, 10), at the same time protecting DNA from nuclease attack (15). In addition, transfection of APCs with liposomal DNA could be promoted by the judicious choice of vesicle surface charge, size, and lipid composition or by the coentrainment, together with DNA, of plasmids expressing appropriate cytokines or immunostimulatory sequences. Indeed, it has already been shown that immunization of mice by a variety of routes with (cationic) liposomal (or niosomal) DNA leads to humoral (splenic interleukin-4 and plasma IgG subclasses) and cell-mediated (splenic interferon γ) immune responses much greater than those obtained with naked DNA or DNA complexed with preformed similar liposomes (12, 14, 16–18).

LIPOSOMES AS DRUG CARRIERS

Liposomes are vesicles consisting of one or more concentric bilayers alternating with aqueous compartments (Fig. 1). They are usually made up of

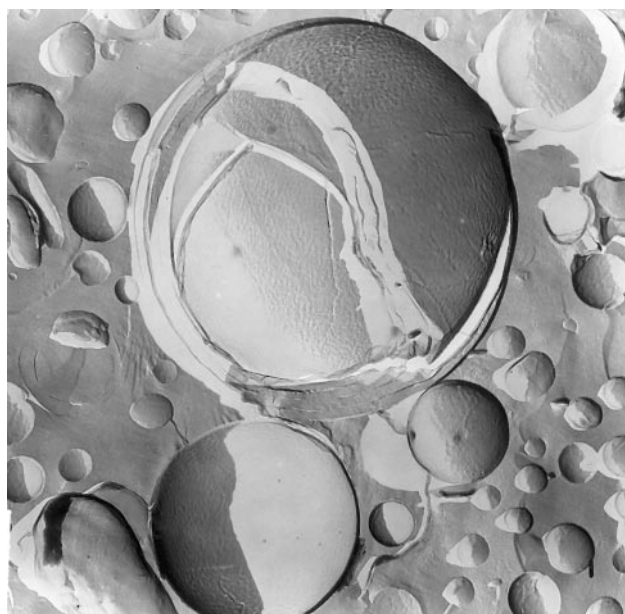


FIG. 1. Scanning electron micrograph of a mixture of small unilamellar and multilamellar liposomes of various sizes.

phospholipids or other amphiphiles such as nonionic surfactants. Depending on the nature of the amphiphile, bilayers can be in a “fluid” or “rigid” state at ambient temperature (T_a). The fluid state is achieved with amphiphiles that have a gel–liquid crystalline transition temperature (T_c , the temperature at which the fatty acid chains melt) below the T_a , whereas the rigid state requires amphiphiles with a T_c above T_a . Because of their ability to incorporate water-soluble and lipid-soluble molecules in their aqueous and lipid phases, respectively, liposomes have been used since 1970 (19) as a means to deliver a great variety of pharmacologically active agents to specific sites in the body in need of pharmacological intervention. In this way, many of the problems associated with direct drug use (e.g., toxicity as a result of indiscriminate drug action, premature drug inactivation or excretion, and inability of drugs to reach the target intracellularly) can be circumvented. Applications in therapeutic and preventive medicine, both in experimental animals and clinically, include antimicrobial and cancer therapy, vaccines, metal detoxification, gene therapy, enzyme or hormone therapy, and as a blood surrogate (by employing hemoglobin-containing liposomes) (8). Several injectable liposome-based products (including a vaccine) have already been licensed in the United States and/or Europe and elsewhere (8). The *in vivo* use of liposomes has been made by every conceivable route, including the intravenous, intramuscular, subcuta-

TABLE 1

Entrapment of Peptides and Proteins in DRV Liposomes

Material to be entrapped ^a	Amount (mg)	Phospholipid (μ mol)	Entrapment (%)
Tetanus toxoid	2.00	16	40–82
Bovine serum albumin	2.00	16	40–45
RIVE	0.05	16	29–31
A/Sichuan	0.05	16	38–45
rHBsAg	0.20	16	31–33
LV39	0.20	16	74–82
Interleukin-2	Up to 10^6 units	16	60–70
Poliovirus 1-VP2 peptide	0.22	16	74–82
Poliovirus VP2 peptide	0.22	16	62–68
HBsAg S peptide	1.00	32	42–45
HBsAg pre-S1 peptide	1.00	32	46–48

^a Materials were entrapped as described in the text. RIVE, Reconstituted influenza virus envelopes; A/Sichuan, A/Sichuan influenza virus hemagglutinin and neuraminidase; rHBsAg, recombinant hepatitis B surface antigen; LV39, *Leishmania major* antigen (mixed isolate); HBsAg, full-length hepatitis B surface antigen. Synthetic S peptide is the 110–137 amino acid sequence; synthetic pre-S1 peptide sequence is 15–48.

neous, intrathecal, intratracheal, oral, intranasal, and topical (skin and a variety of mucosal tissues) routes (8).

Much of the progress in biomedical and other uses of liposomes can be largely attributed to advances in related technology (20). This has evolved from the original "classic" method of the 1960s to a variety of sophisticated techniques developed to meet particular needs. Some of these techniques are characterized by a high entrapment yield (i.e., a high drug-to-lipid mass ratio) and are amenable to scaleup. However, only a few techniques are applicable to all water-soluble drugs, regardless of size, charge, solubility, and other physical characteristics. Indeed, the variety and complexity of techniques for liposome production are now so great that no one laboratory has hands-on experience with all of them. In this article, some of the liposome technology, as developed and applied in our laboratory over the last 15 years, is described (15, 21–29). Such technology is characterized by high-yield drug entrapment in vesicles of an average size ranging from about 100 nm to several micrometers under conditions that, generally, preserve the activity of labile drugs (e.g., antigens, plasmid DNA, or attenuated microbes).

DESCRIPTION OF METHOD

Materials

Materials that may be needed for the preparation of vaccine-containing liposomes include the following:

egg phosphatidylcholine (PC)
 phosphatidic acid (PA)
 phosphatidylglycerol (PG)
 phosphatidylserine (PS)
 distearoylphosphatidylcholine (DSPC) (more than 99% pure)
 cholesterol (CHOL)
 triolein (TO)
 stearylamine (SA)
 1,2-bis(hexadecylcycloxy)-3-trimethylaminopropane (BisHOP)
N-[1-(2,3-dioleoyloxy) propyl]-*N,N,N*-triethylammonium (DOTMA)
 1,2-dioleoyloxy-3-(trimethylammonium propane) (DOTAP)
 3β-(*N,N*-dimethylaminoethane)carbonylcholesterol (DC-CHOL)
 Sepharose CL-4B
 polyethylene glycol 6000 (PEG 6000)

TABLE 2

Incorporation of Plasmid DNA into Liposomes by Dehydration–Rehydration Method^a

Liposomes	Incorporated plasmid DNA (%)					
	pGL2	pRc/CMV HBS	pRSVGH	pCMV4.65	pCMV4.EGFP	VR1020
PC, DOPE ^a	44.2	55.4	45.6	28.6		
PC, DOPE ^b	12.1		11.3			
PC, DOPE, PS ^a	57.3					
PC, DOPE, PS ^b	12.6					
PC, DOPE, PG ^a			53.5			
PC, DOPE, PG ^b			10.2			
PC, DOPE, SA ^a	74.8					
PC, DOPE, SA ^b	48.3					
PC, DOPE, BisHOP ^a	69.3					
PC, DOPE, DOTMA ^a	86.8					
PC, DOPE, DC-Chol ^a		87.1	76.9			
PC, DOPE, DC-Chol ^b			77.2			
PC, DOPE, DOTAP ^a		80.1	79.8	52.7	71.9	89.6
PC, DOPE, DOTAP ^b		88.6	80.6	67.7		81.6
PC, DOPE, DODAP ^a			57.4			
PC, DOPE, DODAP ^b			64.8			

Note. ³⁵S-Labeled plasmid DNA (10–500 μg) was ^aincorporated into or ^bmixed with neutral (PC, DOPE), anionic (PC, DOPE, PS, or PG), or cationic (PC, DOPE, SA, BisHOP, DOTMA, DC-Chol, DOTAP, or DODAP) dehydration–rehydration vesicles (DRVs). Incorporation values for the different amounts of DNA used for each of the liposomal formulations did not differ significantly and were therefore pooled (values shown are means of values obtained from three to five experiments). PC (16 μmol) was used in molar ratios of 1:0.5 (neutral) and 1:0.5:0:25 anionic and cationic liposomes). PC, Egg phosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; SA, stearylamine; BisHOP, 1,2-bis(hexadecylcycloxy)-3-trimethylaminopropane; DOTMA, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-triethylammonium; DC-Chol, 3β-(*N,N*-dimethylaminoethane)carbonylcholesterol; DOTAP, 1,2-dioleoyl-3-(trimethylammonium)propane; DODAP, 1,2-dioleoyl-3-dimethylammoniumpropane. Plasmid DNAs used encoded luciferase (pGL2), hepatitis B surface antigen (S region) (pRc/CMV HBS), human growth hormone (pRSVGH), *Mycobacterium leprae* protein (pCMV 4.65), fluorescent green protein (pCMV4.EGFP), and *Schistosoma* protein (VR1020).

Entrapment of Peptide, Protein, and DNA Vaccines

Solutions

1. Thirty-two micromoles of phospholipid and 32 μmol of CHOL are dissolved in chloroform (2–5 ml). For negatively charged liposomes 3.2 μmol of PA, PS, or PG, and for positively charged liposomes 3.2–8 μmol of SA, BisHOP, DOTMA, DOTAP, or DC-CHOL, is added. Greater amounts of charged lipids can be used depending on the amount of vesicle surface charge required.

2. Up to 10 mg of the water-soluble vaccine is dissolved in 2 ml distilled water (H_2O) or 10 mM sodium phosphate buffer, pH 7.2 [phosphate buffer (PB)], if needed. The nature of buffers with respect to composition, pH, and molarity can be varied as long as this does not interfere with liposome formation or the yield of entrapment. The amount of added vaccine can be increased proportionally to the total amount of lipid used.

Procedure (15, 21, 24)

A. The solution of lipids is placed into a 50-ml round-bottomed spherical Quick-fit flask and the solvent is removed using a rotary evaporator at 37°C. The thin lipid film formed on the walls of the flask is then flushed for about 60 s with oxygen-free nitrogen (N_2) to ensure complete solvent removal and to replace air.

B. Distilled H_2O (2 ml) (solution 2 instead if step C below is not detrimental to the vaccine) is added to the flask, if needed together with a few glass beads, and the mixture is shaken vigorously by hand or mechanically until the lipid film has been transformed into a milky suspension. This process is carried out above the T_c of the phospholipid ($>T_c$) preferably by prewarming H_2O or solution 2 before placement into a prewarmed

flask within a water bath endowed with a shaking facility. The emulsion is left to stand at $>T_c$ for about 1–2 h, whereupon multilamellar liposomes of diverse sizes are formed.

C. After the removal of glass beads, the milky suspension is sonicated at $>T_c$ (with frequent intervals of rest) using a titanium probe slightly immersed in the emulsion. Sonication is carried out under N_2 , achieved by the continuous delivery of a gentle stream of the gas through thin plastic tubing. A slightly opaque to clear suspension of small unilamellar vesicles (SUVs) about 30–80 nm in diameter is produced. The time required to produce SUVs varies and depends on the amount of lipid used and the diameter of the probe. For the amounts of lipid in solution 1, a clear or slightly opaque suspension is usually obtained within up to four sonication cycles, each lasting 30 s with 30-s rest intervals inbetween using a probe 0.75 in. in diameter.

D. The sonicated suspension of SUVs is allowed to rest at $>T_c$ for about 1–2 h, mixed with solution 2 (when water is used in step B), rapidly frozen in liquid nitrogen, and freeze-dried overnight under vacuum (<0.1 Torr) in a Hetosicc freeze-dryer.

E. H_2O (0.1 ml per 32 μmol of phospholipid) prewarmed at $>T_c$ is added to the freeze-dried material which is swirled vigorously at $>T_c$. The volume of H_2O added must be kept at a minimum, i.e., enough H_2O to ensure complete dissolution of the powder. The sample is kept at $>T_c$ for about 30 min. The process is repeated with 0.1 ml H_2O and 30 min later at $>T_c$, with 0.8 ml PB (prewarmed at $>T_c$) and the sample is allowed to stand for 30 min at $>T_c$.

F. The suspension containing multilamellar liposomes [dehydration–rehydration vesicles (DRVs)] with entrapped and nonentrapped vaccine is centrifuged at 40,000g for 60 min (4°C). The pellet obtained (vaccine-containing DRVs) is suspended in H_2O or PB and centrifuged again under the same conditions. The process is repeated once again to remove the remaining non-entrapped material. The final pellet is suspended in 2 ml H_2O or PB. When the liposomal suspension is destined for *in vivo* use (e.g., intravenous injection), NaCl is added to a final concentration of 0.9%.

G. The extent of drug entrapment in DRV liposomes is monitored by measuring the vaccine in the suspended pellet and combined supernatants. The easiest way to monitor entrapment is by using a radiolabeled vaccine. If a radiolabel is not available or cannot be used, appropriate quantitative techniques should be employed. To determine the vaccine by such techniques a sample of the liposome suspension is mixed with Triton X-100 (up to 5% final concentration) or 2-propanol (1:1 volume ratio) so as to liberate the entrapped material. However, if Triton X-100 or the solubilized liposomal lipids interfere with the assay of the material, lipids must be extracted using appropriate

TABLE 3

z-Average Mean Size of Microfluidized DRVs^a

Medium	Number of cycles				
	1.8	3.5	5.2	7.1	10.6
Washed DRVs					
Water	463.5	149.9	115.0	121.9	114.7
PBS	447.4	198.6	168.1	159.5	155.7
Unwashed DRVs					
Water	473.9	132.9	116.9	116.6	101.9
PBS	456.3	186.2	186.7	169.8	159.9

^a Maltose-containing washed or unwashed DRVs (32 μmol PC) were microfluidized in the presence of water or PBS for up to 10.6 cycles, and samples were measured for vesicle size (diameter in nanometers) by dynamic light scattering (photon correlation spectroscopy). Polydispersity indexes ranged from 0.503 to 0.653 (water) and from 0.517 to 0.653 (PBS).

techniques. Entrapment values range between about 20 and 100%, depending on the amounts of lipid and vaccine used (Table 1). Highest values are achieved when solutes for entrapment bear a net charge that is opposite that of the charged lipid component of liposomes (Table 2). However, as part of the liposome-associated solute may have interacted with the liposomal surface during the entrapment procedure, it is essential that actual entrapment of the solute (as opposed to surface-bound solute) is determined. In the case of DNA or proteins, this can be achieved by the use of deoxyribonuclease (15) and a proteinase (27, 28), respectively, which will degrade most of the external material.

H. This step and the one following are required when vaccine-containing DRV liposomes must be converted to smaller vesicles (down to about 100-nm z-average diameter). To that end, the liposomal suspension obtained in step 5 (prior to separation of the entrapped from the nonentrapped vaccine in step F; unwashed liposomes) is diluted to 10 ml with H₂O and then passed for a number of full cycles through a Microfluidizer 110S (Microfluidics), with the pressure gauge set at 60 psi throughout the procedure to give a flow rate of 35 ml/min. The number of cycles used depends on the vesicle size required (Table 3) or the sensitivity of the entrapped vaccine (e.g., plasmid DNA) (15). However, the greater the number of cycles, the smaller the amount of drug retained by the vesicles (22). Microfluidization of the sample can also be carried out after removal of nonentrapped vaccine as in step F (washed liposomes). However, drug retention in this case is reduced. It appears that the presence of untrapped drug during microfluidization diminishes solute leakage, probably by reducing the osmotic rupture of vesicles and/or the initial concentration gradient across the bilayer membranes (22).

I. The volume of the microfluidized sample (about 10 ml) can, if needed, be reduced to about 1–2 ml by placing the sample (in dialysis tubing) in PEG 6000 within a flat container. Removal of excess H₂O from the

tubing is relatively rapid (within 30–60 min) and it is therefore essential that the sample be inspected regularly. When the required volume has been reached, the sample is treated for the separation of entrapped from nonentrapped vaccine. This is carried out by molecular sieve chromatography using a Sepharose CL-4B column, in which case vaccine-containing liposomes are eluted at the end of the void volume. The content of vaccine within liposomes is estimated as in step G and is expressed as percentage of vaccine in the original preparation obtained in step F. Because the sample is microfluidized following step E, i.e., before the estimation of entrapment, it is necessary that a small portion of the sample to be microfluidized is kept aside for estimation of entrapment according to step F. Vesicle size measurements are carried out by photon correlation spectroscopy as described elsewhere (15, 28). Minimum vesicle diameters obtained after 10 cycles of microfluidization are about 100–160 nm, depending on whether microfluidization was carried out in H₂O or PB, using unwashed or washed liposomes (Table 3).

Entrapment of Large Particles, Viruses, or Bacteria into Giant Liposomes

Solutions Required for Entrapment

1. PC or DSPC, CHOL, PG, and TO (4:4:2:1 molar ratio, 9 μ mol total lipid) in 1.0 ml CHCl₃
2. Lipids as in solution 1 dissolved in 0.5 ml diethyl ether
3. 0.15 M sucrose in H₂O
4. 0.2 M sucrose in H₂O
5. 5% glucose in H₂O
6. 0.1 M sodium phosphate buffer supplemented with 0.9% NaCl, pH 7 (PBS)
7. Discontinuous sucrose gradient prepared by the use of two solutions containing 59.7 and 117.0 g of sucrose, respectively, per 100 ml H₂O (e.g., for *Bacillus subtilis*) in swing-out bucket centrifuge tubes.

TABLE 4

Entrapment of *Bacillus subtilis* and Tetanus Toxoid in Giant Liposomes^a

Liposomes	Entrapped material (% of that used)			
	<i>B. subtilis</i>		Tetanus toxoid	
	A	B	A	B
PC, cholesterol, PG, TO	31.6 \pm 24.2 (12)	26.7 \pm 12.1 (7)	0.0 (4)	8.4 \pm 2.6 (4)
DSPC, cholesterol, PG, TO		21.3 \pm 8.9 (6)	0.0 (4)	11.1 \pm 1.9 (4)

^a ¹²⁵I-Labeled *B. subtilis* and tetanus toxoid were entrapped in giant liposomes as described. Results, based on radioactivity measurements, are expressed as percentages (\pm SD) of material used for entrapment. In one experiment, entrapment of ¹²⁵I-labeled BCG in PC giant liposomes was 27.8%. Numbers in parentheses denote numbers of preparations. PC, phosphatidylcholine; PG, phosphatidyl glycerol; DSPC, distearoylphosphatidylcholine; TO, triolein.

Procedure (3, 29)

A. One milliliter of solution 3 is mixed by vortexing for 45 s with solution 1.

B. The resulting water-in-chloroform emulsion is mixed by vortexing for 15 s with solution 2 and 2.5 ml solution 4.

C. The water-in-oil emulsion formed is placed in a 250-ml conical flask and the organic solvents are evaporated by flushing N_2 at 37°C while the sample is gently agitated in a shaking incubator. This leads to the generation of (sucrose-containing) giant liposomes.

D. The giant liposomes are washed by centrifugation over solution 5 in a bench centrifuge at 600g for 5 min. The liposomal pellet is then resuspended in 1 ml PBS.

E. The suspended pellet of giant liposomes is mixed with 1 ml of a suspension of particulate matter [e.g., killed or live *B.subtilis* spores or killed *Bacille Calmette-Guérin* (BCG) bacteria] and freeze-dried overnight under vacuum (<0.1 Torr) in a Hetosicc freeze-dryer.

F. The freeze-dried material is rehydrated, initially by the addition of 0.1 ml H_2O at 20°C [rehydration of liposomes containing the "high-melting" DSPC at $>T_c$ does not have a significant effect on the percentage entrapment of materials (29)]. The suspension is then swirled vigorously and allowed to stand at $>T_c$ for 30 min. The process is repeated after the successive addition of 0.1 ml PBS and 0.8 ml PBS 30 min later (1 ml total suspension volume).

G. The entrapped particulate material is separated from nonentrapped material (e.g., *B.subtilis*) by sucrose gradient centrifugation as in the step below.

H. The suspension (1 ml) containing entrapped and nonentrapped *B. subtilis* spores or BCG bacteria is placed on top of the sucrose gradient (solution 7) and centrifuged for 1.5 h at 90,000g in a Dupont Combi Plus ultracentrifuge using a swing-out bucket. Following centrifugation, 1-ml fractions are pipetted out from the top of the gradient and assayed for spore or bacteria content. It is convenient to use radiolabeled (e.g., ^{125}I -labeled) spores or bacteria to monitor content. In the case of *B. subtilis* spores or BCG bacteria, these are recovered at the bottom fraction of the gradient when nonentrapped, whereas entrapped material is recovered mostly in the top seven fractions of the gradient in association with liposomes (23).

I. Fractions containing the entrapped spores or bacteria are pooled and dialyzed exhaustively against PBS until all sucrose has been eliminated. The dialyzed material is centrifuged as in step D and the liposomal pellet resuspended in 1 ml PBS for further use. Typical values of *B. subtilis* or BCG entrapment are 21–27% of the material used (Table 4) (23, 29).

CONCLUDING REMARKS

The dehydration–rehydration procedures for the entrapment of vaccines (e.g., peptides, proteins, plasmid DNA, and other macromolecules) or particulates such as spores, bacteria, and viruses as outlined here are characterized by their mildness and are thus compatible with labile materials. Moreover, it has been shown (29) that material-containing liposomal suspensions as prepared here can be freeze-dried in the presence of a cryoprotectant (for storage) without significant loss of material from within the vesicles on reconstitution with 0.9% NaCl. Both procedures are simple to use; however, special care should be taken with the rehydration of the freeze-dried material. It is important that water added during the initial rehydration is kept to a minimum.

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