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A VERY MILD METHOD ALLOWING THE ENCAPSULATION OF VERY HIGH AMOUNTS OF MACROMOLECULES INTO VERY LARGE (1000 nm) UNILAMELLAR LIPOSOMES

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Using detergent removal by dialysis we have succeeded in making liposomes under very mild conditions, compatible with the introduction of macromolecules such as antibodies or RNA. We observed that in the presence of detergents, Bio-Beads SM-2® adsorb both octylglucoside and Triton X-100 as well as phospholipids. We used octylglucoside, which can be removed by dialysis more quickly than any other detergent, and can be completely eliminated, unlike the detergents ordinarily used, such as Triton X-100 and sodium deoxycholate. Bio-Beads allowed the complete removal of detergent when placed outside the dialysis bag. Under these conditions, dialysis resulted in the formation of large unilamellar liposomes. Their size depended mainly on the composition of the lipids. The largest liposomes corresponded to a lipid mixture of phosphatidylcholine, phosphatidylserine and cholesterol in a molar ratio of 1 : 1 : 1. Entrapment measurements and electron microscopic studies showed that these unilamellar liposomes were very large (1000 nm diameter) and that 45–50% of the macromolecular solutions could be entrapped with 13 mM phospholipids (35 l/mol lipid). These liposomes exhibited all the properties required for the delivery of macromolecules into cultured cells.

Introduction

During the past 10 years, many studies have shown that liposomes can be used to transfer material into cells (for reviews see Refs. 1, 2). For this purpose, phospholipid vesicles were prepared mainly by physical techniques (sonication, French press), use of organic solvent (reverse-phase evaporation, ether injection) or detergent removal. Our objective was to find a method for preparing large unilamellar liposomes capable of fusing with membrane in order to inject macromolecules (proteins or nucleic acids) into cells. This method would have to be very mild and, more specifically, would have to avoid contact with organic solvents which denature proteins or high energy, such as ultrasound or pressure, which breaks down nucleic acids. The use of the detergent removal technique seemed the best way of preserving the structure and activity of these macromolecules.

Removal of detergent by dialysis or gel filtration supplies unilamellar liposomes [1]. The internal volume of these liposomes varies with the nature of the detergent [3], the molar ratio of detergent to phospholipids [3], the lipid composition of the vesicles [2] and the rate of dialysis [4]. The size of the vesicles has been reported to be the

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

same both after dialysis and after gel filtration [3]. Nevertheless, the scarcity and/or the cost of the molecule to be encapsulated (antibodies, enzymes, purified genes or messenger RNA) necessitate a very high rate of encapsulation and preclude techniques which require large amounts of encapsulation material such as detergent removal by gel filtration.

The method we developed involves an improvement of the technique of detergent removal by dialysis. We describe below the experimental conditions necessary to obtain very large vesicles in a short time, without detectable amounts of detergent. This technique combines octylglucoside, dialysis, and Bio-Bead SM-2® adsorption.

Materials and Methods

Materials

Triton X-100 was purchased from Merck (Darmstadt, F.R.G.), *n*-octyl-D-glucopyranoside (octylglucoside) from Sigma (St Louis, MO). [*glucose*-^{14}C]Octyl-β-D-glucopyranoside (314 mCi/mmol) and [*phenyl*-^{3}H]Triton X-100 (1.58 mCi/mg) were obtained from New England Nuclear (Boston, MA).

Egg lecithin (phosphatidylcholine) came from Lipid Products (South Nutfield, U.K.), phosphatidylserine and cholesterol from Sigma, and stearylamine from P.L. Biochemicals (Milwaukee, WI). L-α-[*dipalmitoyl*-1-^{14}C]Phosphatidylcholine (60–100 mCi/mmol) was purchased from New England Nuclear.

All radioactive samples were counted in a scintillator 299 from Packard in an Intertechnique SL30 scintillation counter. Phospholipid concentration was determined by the Bartlett method for phosphate determination [5].

Bio-Beads SM-2® were obtained from Bio-Rad (Richmond, CA). The beads were washed extensively before use with different mixtures of solvent: water, water/methanol (1 : 1), methanol/acetone (1 : 1), acetone, methanol/ acetone (1 : 1), methanol, methanol/water (1 : 1), water × 3, 0.05 M acetic acid and finally water × 3, according to the procedure of Holloway [6]. The material to be entrapped was fluorescein-labeled X-immunoglobulin from Lancer (Foster City, CA) or messenger RNA.

Preparation of liposomes

A typical preparation of liposomes was performed according to the following general conditions. Lipid products, stored at −30°C in 2 : 1 (v/v) chloroform/methanol, were mixed in different proportions and sometimes radioactive [^{14}C]phosphatidylcholine was added as tracer (about $2 \cdot 10^5$ cpm). The mixture (final concentration 10–30 mM) was dried to a thin film under a stream of nitrogen, then in vacuo for 1 h to remove residual traces of organic solvent. Materials to be encapsulated were introduced with buffer L (10 mM Hepes (pH 7.4)/1 mM EGTA/150 mM NaCl) and after vortex shaking, the lipids were hydrated 30 min at a temperature above the highest transition temperature of the components in the mixture. Detergent was finally added with, when necessary, $2 \cdot 10^5$ cpm/ml of radioactive detergent ([^{14}C]octylglucoside or [^{3}H]Triton X-100). Then the volume of the samples was adjusted to 0.625 ml with buffer L. After vigorous shaking, the detergent was removed as described below and the liposomes were collected.

Three different techniques were used to remove detergent. (i) Sample in a dialysis bag 1 cm wide was dialyzed against 1000 ml of 10 mM Tris-HCl (pH 7.4)/1 mM EDTA/150 mM NaCl with four changes of the medium. The bag was adapted to a Pasteur pipette in order to facilitate sampling for radioactive assays of phospholipid or detergent. (ii) The volume of dialysis medium was reduced to 100 ml and Bio-Beads were added outside the bag in the buffered medium. The medium was not changed. (iii) In some experiments, Bio-Beads were added directly to the liposome preparation in a test-tube, and placed on a rotary mixer running at 10 rpm for at least 3 h.

When necessary, the liposome suspension was passed through a Sepharose 4B column to remove the non-encapsulated material.

Measurement of turbidity in phospholipid/detergent mixtures

The lipid film (4–5 mM) was hydrated with 1 ml buffered medium and vigorously mixed with a Vortex before being placed in a spectrophotometer cell of 1 cm optical length. The absorbance at 380 nm was measured in a Cary 118 spectrophotometer after each addition of 10 μl of a 171 mM

octylglucoside solution. Results were expressed as a function of absorbance and the molar ratio of detergent to phospholipids. There was no correction for sample dilution.

Electron microscopy and determination of size distribution of liposomes

Liposomes were prepared with a standard procedure. Vesicle suspensions were applied to carbon-coated grids (300 mesh) and washed with stain solution (0.1–1% uranyl acetate in water). Excess fluid was removed with filter paper and the grids were immediately examined in a JEOL JEM 200 CR electron microscope at 100 kV. The diameters of individual vesicles were measured and the corresponding spherical volumes were calculated. The size distribution of the liposomes was determined by counting at least 200 vesicles in three separate experiments.

Entrapment efficiency and internal volume

Estimations of encapsulation efficiency (percentage of original solute entrapped in the liposome preparation) were carried out by measuring the concentration of entrapped material (fluorescein-labeled antibody or RNA from HeLa cells) in the samples after gel permeation on Bio-Gel A-15m or Sepharose 4B equilibrated with buffer L. The elution peak of the liposomes was monitored at 400 nm, then the absorbance of each fraction was measured before and after treatment with 1% sodium dodecyl sulfate (495 nm for fluorescent immunoglobulin G or 260 nm for RNA). Entrapment efficiency was evaluated on the basis of the ratio of moles of solute entrapped to moles of total solute and lipids of liposomes.

The internal volume of liposomes was calculated on the basis of a surface area of 75 $Å^2$ per phospholipid molecule [7] with the following equation:

$$\% \text{ encapsulation} = 37 \times M \times d \times 10^2 \quad (1)$$

where M is the concentration of phospholipids (mol/liter) and d the diameter (μm).

Results and Discussion

Solubilization of lipids by octylglucoside

One of the main problems encountered in liposome formation by detergent removal is the residual detergent entrapped, even after extensive dialysis (a few percent for deoxycholate or cholate and around 10% for Triton X-100 [8,9]). Thus we choose to use β-D-octylglycoside which can be removed almost completely (less than 0.05%) [3,10], and quickly because of its very high critical micellar concentration, which facilitates rapid removal from mixed micellar complexes. It is currently accepted that a molar octylglucoside : egg phosphatidylcholine ratio of 4–5 [3,10] is effective for obtaining a clear solution. Our studies have yielded another optimal detergent : lipid ratio for several lipid mixtures. Fig. 1 shows typical sigmoidal curves of turbidity disappearing as a function of detergent addition for neutral, negatively charged and positively charged vesicles, whether or not they contain cholesterol.

These curves show that the optimal molar detergent : lipid ratio ranges between 5–8 (M/M). The

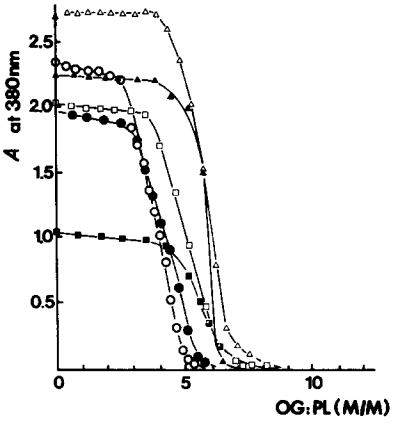

Fig. 1. Turbidity of liposome preparation as a function of the molar ratio of detergent to lipids. Multilamellar liposomes prepared in the absence of encapsulated material from different mixtures of lipids, as described in Materials and Methods, were placed in a spectrophotometer cell of 1 cm pathlength, then a 171 mM octylglucoside solution (O.G.) was added directly to the cell liposome preparation. The composition (M/M) and whole concentration of lipids were: ▲, PC 5.3 mM; △, PC/cholesterol (2 : 1) 8 mM; ●, PC/PS (1 : 1) 5.3 mM; ○, PC/PS/cholesterol (1 : 1 : 1) 8 mM; ■, PC/stearylamine (2 : 1) 4 mM; and □, PC/stearylamine/Chol (2 : 1 : 1.5) 6 mM.

solubility was at its highest with negatively charged vesicles and decreased with positive and neutral vesicles. For egg phosphatidylcholine, we found a molar detergent : lipid ratio higher than previously reported [3,10,11], which would explain why Mimms et al. [3] recommended the use of a greater molar ratio, about 10, to obtain a better vesicle-preparation, with more than 90% of the lipids in vesicular form. It is interesting to note that cholesterol did not modify the solubilization of lipid mixtures.

For the following experiments, a detergent : lipid ratio of 10 : 1 to 12 : 1 was used.

Removal of the detergent

Bio-Beads SM-2® adsorb Triton X-100, a neutral detergent [6], as well as deoxycholate, a cationic one [12]. We tested the capacity of Bio-Beads to bind octylglucoside, another non-ionic detergent.

Fig. 2 shows the adsorption of octylglucoside as a function of different detergent-to-bead ratios. By comparison with the corresponding adsorption of Triton X-100, it can be noted that octylglucoside is bound more rapidly than Triton X-100. Moreover, there is an optimal detergent-to-bead ratio beyond which detergent removal cannot be improved. This optimal ratio is equal to 0.11 for octylglucoside (1 μmol detergent/9 mg Bio-Beads) and corresponds to a complete exclusion of detergent from medium, whereas this same optimal ratio is 0.05 for Triton X-100 (1 μmol detergent/20 mg beads) but with 0.25% residual detergent which cannot be reduced by additional beads (29 mg beads/μmol detergent). These results confirm previous data [8,9] showing the difficulty of eliminating Triton X-100. Nevertheless, they constitute a marked improvement of this technique.

In another series of experiments, we also analyzed the binding capacity of Bio-Beads with phospholipids during a typical preparation of liposomes using direct contact between mixed micelles of detergent-phospholipid and Bio-Beads. We also compared the effects of Triton X-100 and octylglucoside on this phenomenon. For this purpose, an identical amount of lipids (PC/PS, molar ratio 1 : 1) was solubilized by each of these detergents, in a detergent : phospholipid molar ratio of 8 (Fig. 1, Ref. 3), and then the samples were mixed with

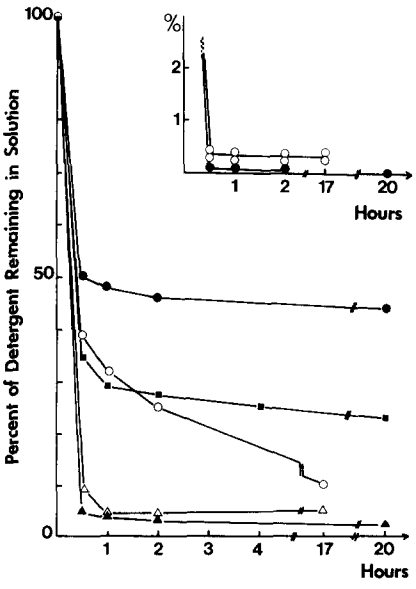

Fig. 2. Detergent removal with Bio-Beads SM-2. Solutions of octylglucoside (filled symbols) or Triton X-100 (empty symbols) in a buffer 10 mM Hepes (pH 7.4)/1 mM EGTA/150 mM NaCl, containing about $2 \cdot 10^5$ cpm of [^{14}C]octylglucoside or [^{3}H]Triton X-100, respectively, were mixed with Bio-Beads in different proportions. Final volume, 1 ml. The beads were continuously maintained in suspension by gentle agitation with a magnetic stirrer. Aliquots of 10 μl were collected as a function of time and analyzed for their radioactivity. Each curve corresponds to one specific detergent : bead ratio expressed in μmol/mg. For octylglucoside, ● corresponds to a ratio 0.65; ■, 0.41; ▲, 0.22 and inset, ● 0.11 – for Triton X-100 0.3 (○); 0.11 (△); 'inset' 0.05 and 0.035 (○).

Bio-Beads and the phospholipid concentrations of the solutions were kinetically analyzed. Under these conditions, liposomes can be made in a very short time but, as illustrated in Fig. 3, a great amount of lipids bind to the beads in both cases. For Triton X-100, a detergent : Bio-Beads ratio of 0.3, which excludes 90% of detergent from medium (data of Fig. 2), also excludes 40% of the phospholipids. Similarly an octylglucoside : Bio-Beads ratio of 0.1 removes 100% of the detergent (Fig. 2) and 55% of the lipids.

These results emphasize the ability of Bio-Beads to adsorb many different compounds with neutral as well as a charged molecules. This property of polystyrene beads has never been previously pointed out, which explains why many authors use Bio-Beads during liposome formation [8] or membrane reconstitution [13,14]. The technique does

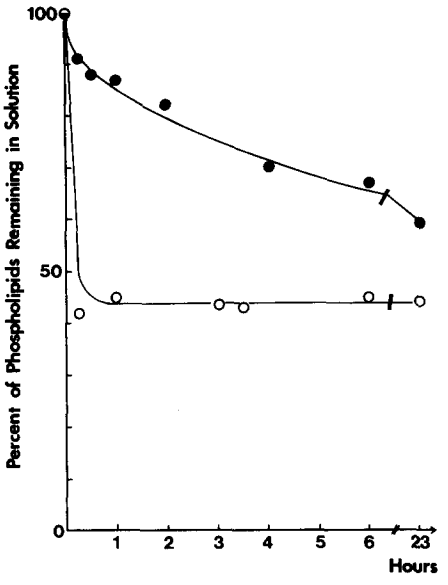

Fig. 3. Phospholipid binding to Bio-Beads SM-2. 19 μmol of PC/PS (molar ratio, 1), containing about $2 \cdot 10^5$ cpm [^{14}C]PC were dried, hydrated by a buffer, then mixed with detergent as indicated in Materials and Methods. The molar ratio of detergent to phospholipid was 0.8. Final volume, 0.625 ml. The clear solutions were mixed with Bio-Beads in the ratio 0.1 for octylglucoside (●) and 0.3 for Triton X-100 (○) (μmol/mg) and the suspensions were continuously stirred. Aliquots of 10 μl were collected as a function of time and analyzed for their radioactivity.

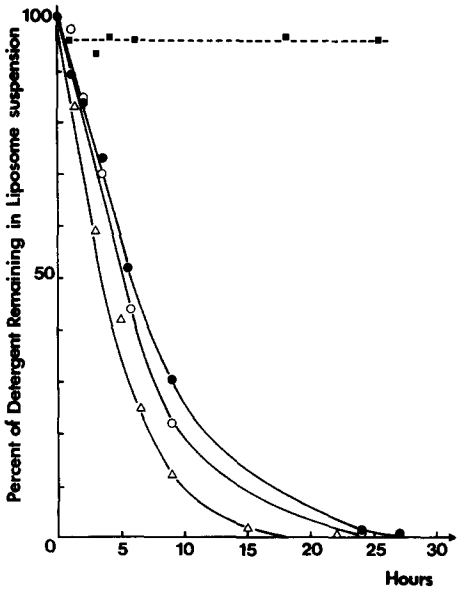

Fig. 4. Percentage of residual detergent in liposome preparation dialyzed against buffer containing Bio-Beads. The same liposome preparations as in Fig. 3 containing either $2 \cdot 10^5$ cpm of [^{14}C]PC or [^{14}C]octylglucoside in a final volume of 0.625 ml were placed in a dialysis bag. ●, Control was dialyzed against four changes of 100 ml of buffer; ○, sample labeled with [^{14}C]octylglucoside dialyzed against 100 ml of buffer containing Bio-Beads (0.1 μmol detergent per mg beads); △, ratio adjusted to 0.04; ■, sample labeled with [^{14}C]PC dialyzed against 100 ml of buffer plus Bio-Beads (ratio 0.04). Aliquots of 10 μl were collected in the bags as a function of time and analyzed for their radioactivity.

not appear to be appropriate to a correct and well-defined entrapment of macromolecules, and direct contact with Bio-Beads should be avoided.

In the following experiments we used octylglucoside and detergent removal with Bio-Beads outside the dialysis bag.

Formation of liposomes by dialysis against buffer containing Bio-Beads

In routine experiments, 20–30 μmol of dried lipids were hydrated either by 0.125 ml of buffer, fluorescent antibody solution (1.25 mg protein), or tRNA solution (400 μg/ml), then were solubilized with octylglucoside containing $2 \cdot 10^5$ cpm of [^{14}C]octylglucoside, using a molar detergent : lipid ratio of 10 : 1, in a final volume of 0.675 ml. The clear solutions were dialyzed against 100 ml of buffer containing different amounts of Bio-Beads (0.04–0.1 μmol detergent/mg bead). Fig. 4 compares results obtained with this technique and with conventional dialysis against four changes of 1000 ml buffer.

For a ratio of detergent to beads lower than 0.1, all detergent molecules were adsorbed in less than 1 h by direct contact between detergent and beads (see Fig. 2). The same results were obtained 24 h later when beads were added to the dialysis medium. As shown in Fig. 4, a ratio lower than 0.1 accelerates detergent removal. The control experiment shows that our technique gives faster removal of detergent with only 100 ml of buffer.

We verified that dialysis using Bio-Beads did not modify the lipid concentration (Fig. 4).

Characteristics of liposomes

Diameters of individual vesicles were measured by electron microscopy. The liposomes exhibit a very homogeneous size-distribution, as generally

TABLE I

CHARACTERISTICS OF LIPOSOMES

Preparation of liposomes, entrapment determinations and electron microscopic studies were carried out as described in Material and Methods. Percentages of entrapped material correspond to 13 mM phospholipids. Figures in brackets are mean percentages of liposome diameters included in the range. n.d., not determined.

Properties	Lipid composition		
	PC	PC/PS (1 : 1)	PC/PS/cholesterol (1 : 1 : 1)
Protein entrapped (%)	26	11 ± 2	47 ± 4
RNA entrapped (%)	n.d.	n.d.	45 ± 4
Calculated diameter (nm)	540	237 ± 50	920 ± 100
Diameter measured by electron microscopy (nm)	300–500(75)	100–240(90)	600–1 100(75)
Internal volume (l/mol PL)	20	8	35

observed with dialysis of detergent [11]. Table I summarizes the results obtained.

The diameter of liposome varies considerably with lipid composition. PC-PS liposomes have a diameter of 200 nm, whereas the addition of cholesterol yields vesicles with about 1000 nm diameter. This effect of cholesterol on vesicle size has been also observed by Rhoden and Goldin [15].

Fig. 5 shows that the vesicles are unilamellar. This is confirmed by comparing the volume of the intravesicular compartment from Eqn. 1, with that expected of unilamellar vesicles measured in the same preparation of liposomes by electron microscopy. The intravesicular compartment was measured by entrapping either antibodies coupled to fluorescein, or RNA, and the diameter was deduced according to the vesicle size-dependency of the percentage of volume encapsulated in unilamellar vesicles [1]. Table I establishes a clear

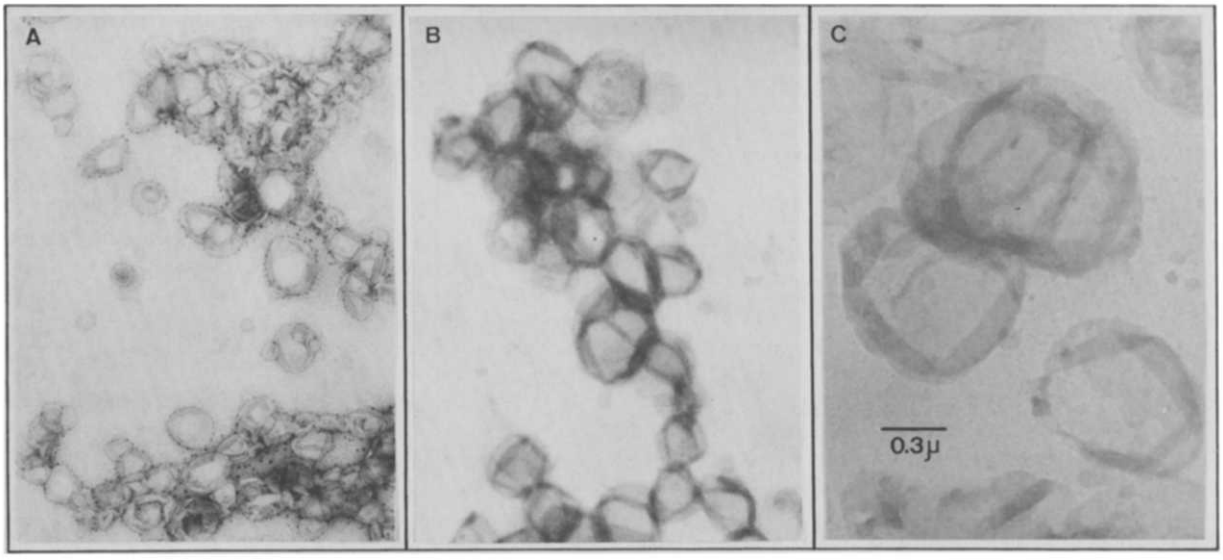

Fig. 5. Electron micrographs of negatively stained liposomes. Liposomes were prepared as in Fig. 4 (0.1 μmol detergent per mg beads) with the beads outside the dialysis bag. (A) PC/PS liposomes (1 : 1; M/M); (B) pure PC liposomes; (C) PC/PS/cholesterol (1 : 1 : 1; M/M).

correspondence between the diameters obtained by these two techniques. Thus the liposomes are mainly unilamellar.

Furthermore, these liposomes contain a very high amount of entrapped material, 35 l/mol of lipid for PC-PS-cholesterol liposomes. This is far greater than that which is obtained by reverse-phase evaporation, 10 l/mol lipid [16]. Furthermore, a reasonable amount of lipid (13 mM) allows the incorporation of up to 50% of macromolecules, which is comparable to the reverse-phase data. We observed that a treatment of liposomes with RNAase after gel permeation did not change the entrapment efficiency (not shown).

Due to the high potential generated in small charged liposomes, entrapment of molecules bearing the same charge is reduced [17]. This eliminates the possibility of using small liposomes containing PS to entrap nucleic acids. Our liposomes avoid this constraint and allow the same entrapment-efficiency with RNA as well as IgG, a slightly positive protein (Table I).

Conclusion

The liposomes we have prepared exhibit all the properties required for the delivery of macromolecules to cultured cells. Large vesicles are more efficient for experiments of macromolecule delivery to cells because they can entrap more material per mass of lipids. For example, one liposome of 1000 nm carries $3 \cdot 10^7$ molecules when loaded with 1 M solution. Thus, the uptake of one liposome per cell seems convenient for most purposes. Furthermore the lipid composition (PC-PS-cholesterol) is not toxic to many cells [18] and even would allow fusion with cellular membrane under conditions known to promote this event, such as the use of poly(ethylene glycol) [19]. In this case, unilamellar liposomes such as these make the process easier [20].

IgG and RNA were not denatured and retained all their activity after entrapment. The very high level of incorporation (up to 50% for 13 mM phospholipid) makes this method ideal for the entrapment of macromolecules which are difficult to obtain in great quantity, such as RNA, monoclonal antibodies or cloned DNA.

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