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Entrapment of Proteins in Phosphatidylcholine Vesicles†

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ABSTRACT: The trapping efficiency of globular proteins in four different types of phosphatidylcholine vesicles was systematically studied. Vesicles were generated in a mixture of ^{125}I-labeled proteins of various molecular weights. The trapped proteins were separated from untrapped proteins by gel filtration and ultrafiltration and subsequently analyzed by gel electrophoresis and autoradiography. Entrapment of proteins was demonstrated by their resistance to trypsin digestion. The relative amount of each entrapped protein species was then compared to that of the original protein solution. In multilamellar vesicles and large unilamellar vesicles, proteins of molecular weight up to 97 000 had the same trapping efficiency as sucrose. In small unilamellar vesicles generated by either sonication or ethanol injection, however, the relative trapping efficiency of protein decreased progressively as the molecular weight of the protein became greater. For example, the trapping efficiency of α-amylase (M_r 97 000) was only half of that for sucrose. The apparent decrease in trapping efficiency with the protein's molecular weight in small unilamellar vesicles can be accounted for by the combination of the bound water layer at the vesicle's internal surface and the steric hindrance when protein is captured during vesicle formation.

In recent years, phospholipid vesicles (liposomes) of various sizes and composition have been used as carriers, both in vitro and in vivo, to introduce biologically active substances into cells [for a recent review, see Pagano & Weinstein (1978)]. In particular, phospholipid vesicles offer an attractive method for the enzyme replacement therapy (Cohen et al., 1976; Roerdink et al., 1976; Tyrrell et al., 1976; Belchetz et al., 1977). In these experiments, enzymes are trapped within the internal aqueous space of the vesicle which is subsequently exposed to cells. It is therefore of crucial importance that the amount of enzyme trapped in the vesicle be quantitative and well controlled. However, it has been the experience of the investigators that trapping of proteins in lipid vesicles, particularly small unilamellar vesicles (SUV),[1] is difficult and sometimes impossible. It is the purpose of this communication to report a systematic study on the trapping of proteins of various molecular weights in four different types of phospholipid vesicles. The results clearly showed a deviation from the ideal trapping of large proteins in SUV but not in larger vesicles.

Materials and Methods

Materials. Total lipids were extracted from hen yolks with $CHCl_3$–MeOH (2:1 v/v) by the method of Folch et al. (1957). Phosphatidylcholine was purified by silicic acid column chromatography (Litman, 1973). [^{3}H]Dipalmitoyl-PC was synthesized by a catalytic hydrogenation with tritium gas on purified dipalmitoleoyl-PC at the New England Nuclear, Inc., and subsequently purified by silicic acid chromatography. All lipids were stored in sealed ampules under N_2 at −70 °C and periodically examined for purity by TLC. Lysozyme (Worthington), chymotrypsinogen and peroxidase (Miles), conalbumin (Sigma), and α-amylase (Calbiochem) were purchased from commercial sources and used without further purification. Proteins were iodinated with Na^{125}I (New England Nuclear) by using chloramine-T (Hunter & Green-

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[1] Abbreviations used: SUV-son, small unilamellar vesicles prepared by sonication; SUV-EtOH, small unilamellar vesicles prepared by the EtOH injection method; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PC, phosphatidylcholine; $NaDodSO_4$, sodium dodecyl sulfate.

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wood, 1962) and purified by gel filtration on Bio-Gel P-4 (Bio-Rad). The specific activities of proteins ranged from 2 × 10^8 to 4 × 10^8 dpm/mg. [^{14}C]Sucrose (8.7 × 10^{14} dpm/mol) was purchased from New England Nuclear. Radioactivities of ^{3}H, ^{14}C, and ^{125}I were counted in a liquid scintillation counter. ^{125}I was also counted in a Beckman Biogamma II counter.

Formation of Lipid Vesicles. SUV-son was prepared according to Huang (1969). Ten milligrams of [^{3}H]PC in $CHCl_3$ was evaporated to a thin film and suspended in 5 mL of aqueous buffer containing a marker(s). Sonication was carried out in a Heat Systems sonicator (Model W-375) operating at step 3 for 30 min at 1 °C under argon. A 0.5-in. probe equipped with a sapphire tip was used. SUV-EtOH was prepared from 45 mg of [^{3}H]PC by the procedure of Batzri & Korn (1973) with marker(s) added in the aqueous buffer. MLV was prepared from 10 mg of [^{3}H]PC according to Bangham et al. (1974). LUV was prepared by a modified procedure of Deamer & Bangham (1976). Briefly, 5 mL of [^{3}H]PC (2 mg/mL) in diethyl ether was injected into 10 mL of aqueous buffer which was kept at 48 °C by a circulating water jacket. The injection rate was 1 mL/min. A mild vacuum was applied to the aqueous phase to facilitate the evaporation of diethyl ether. In all four types of vesicle preparation, a trace of [^{3}H]dipalmitoyl-PC was added to PC to give a final specific activity of (0.7–3) × 10^4 dpm/mg. Marker in the aqueous buffer (0.15 M KCl and 10 mM Tris, pH 7.4) was [^{14}C]sucrose (2.1 × 10^6 dpm/mL), [^{125}I]lysozyme (2.3 × 10^6 dpm/mL), or a mixture of ^{125}I-labeled proteins [(6.4–7.4) × 10^6 dpm/mL for conalbumin and α-amylase, 5.2 × 10^6 dpm/mg for all others].

Separation of Trapped Marker from Untrapped Marker. After vesicles were prepared, they were concentrated to 1–1.5 mL by ultrafiltration before application to a Sepharose 4B column (2.6 × 37 cm) equilibrated with 0.15 M KCl and 10 mM Tris, pH 7.4, and eluted with the same buffer. Fractions were counted for both [^{3}H]PC and radioactive marker (^{14}C or ^{125}I). The desired fractions containing vesicles (as judged by ^{3}H counts per minute) were pooled and washed with the same buffer on Pellicon membranes, Type PTHK (Millipore, average molecular weight cutoff 10^5), under a pressure of 10 psi. Markers of molecular weight less than 80 000 passed through the membrane without any retention, while markers of molecular weight greater than 80 000 (α-amylase) showed partial retention on each wash. Washing was repeated until no markers appeared in the filtrate. Usually three to four washes (8 mL of buffer each) were sufficient. The recoveries of the vesicles and the trapped marker were greater than 95%. The vesicle suspension was finally concentrated to 1–1.5 mL. A gel filtration on a Sephadex G-100 column (0.6 × 45 cm) was sometimes done to check if any free, untrapped marker still contaminated the vesicle preparation.

Trypsin Digestion of Marker Proteins. Vesicles with trapped markers were digested with trypsin (1 mg/mL, Worthington) with or without 1% Triton X-100 for 45–60 min at 37 °C. Digestion was terminated by adding soybean trypsin inhibitor (1.5 mg/mL, Sigma).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. The $NaDodSO_4$–Tris gel system of Weber & Osborn (1975) was used with modification. Slab gels were prepared on a Hoefer apparatus (Model 500). Stacking and separating gels were made with 3 and 12% acrylamide, respectively. Although 0.1% $NaDodSO_4$ was included in the electrophoresis buffer, there was no $NaDodSO_4$ present in the gel slab. This modification improved the sharpness of the individual bands. It was essential to incubate the protein markers in a boiling water bath for 5 min in the presence of β-mercaptoethanol to precipitate phospholipids; excess lipids did interfere with the electrophoretic mobility of the proteins. Electrophoresis was run at a constant voltage of 40 V, and the gel temperature was kept at ~15 °C by circulating tap water. After the electrophoresis was done, the gel slab was fixed in 20% trichloracetic acid at 60 °C for 1 h before staining and destaining with Coomassie Brilliant Blue. The destained gel was dried on a piece of filter paper by using a Hoefer gel drying apparatus (Model 540). A sheet of Kodak NS-5T X-ray film was overlayed by the dried gel, and exposure for autoradiography was carried out for 2 to 3 days in the dark. The relative intensity of bands on the autoradiogram was determined by a scanning densitometer equipped with a peak area integrator (ORTEC, Model 4310). For a test of the accuracy and linearity of the method, solutions containing various amounts of ^{125}I (up to 5 × 10^4 dpm) were applied to a filter paper in bands which simulated the protein bands on the gel slab. The filter paper was air-dried and autoradiographed. The relative intensities of bands as determined by the peak areas of the densitometer scan were plotted against the amount of radioactivity applied to the filter paper. Linearity was observed up to 3 × 10^4 dpm, indicating that this method of quantitation was acceptable for ^{125}I-labeled proteins of up to 3 × 10^4 dpm. The amount of ^{125}I-labeled protein per band in our gel slabs never exceeded this limit.

Calculation of Trapping Efficiencies of Protein Markers from $NaDodSO_4$–Polyacrylamide Gel Electrophoresis Autoradiography. The percent of trapping efficiency of a given protein marker was compared to that of lysozyme and was calculated according to the equation

$$\% \text{ trapping efficiency} = \frac{A \times b}{B \times a} \times 100\% \qquad (1)$$

where A and a are the relative amounts of lysozyme in the original solution and in the vesicle-entrapped form, respectively, and B and b are the relative amounts of a given protein marker in the original solution and in the vesicle-entrapped form, respectively. The relative amount of a protein is proportional to the band intensity of the protein in the autoradiogram.

Results

Vesicle Preparation and Separation of Trapped Marker from Untrapped Marker. We have examined the entrapment of proteins in four different types of phospholipid vesicles. Two of them were small and unilamellar ones (SUV-son and SUV-EtOH). The other two types of vesicles were larger and more heterogeneous (MLV and LUV). When a single, small molecular weight marker was used, the separation of the trapped marker from the untrapped marker by Sepharose 4B column chromatography was quite sufficient. When large molecular weight proteins were to be entrapped, there was substantial overlap in the elution of trapped proteins and untrapped ones. Figure 1 shows a typical elution profile of SUV-EtOH with a collection of protein markers in the original buffer solution. The apparent shift of the elution position of the entrapped ^{125}I-labeled proteins from that of the [^{3}H]PC was due to a substantial overlap from the untrapped proteins. The fractions (descending region of the major ^{3}H peak) enriched with homogeneous SUV were pooled and extensively washed by ultrafiltration until all the contaminating untrapped proteins were removed. All other vesicle preparations were processed similarly to ensure a complete removal of any untrapped proteins.

Entrapment of Protein Markers in PV Vesicles. For the purpose of this study, it is very important to make sure that

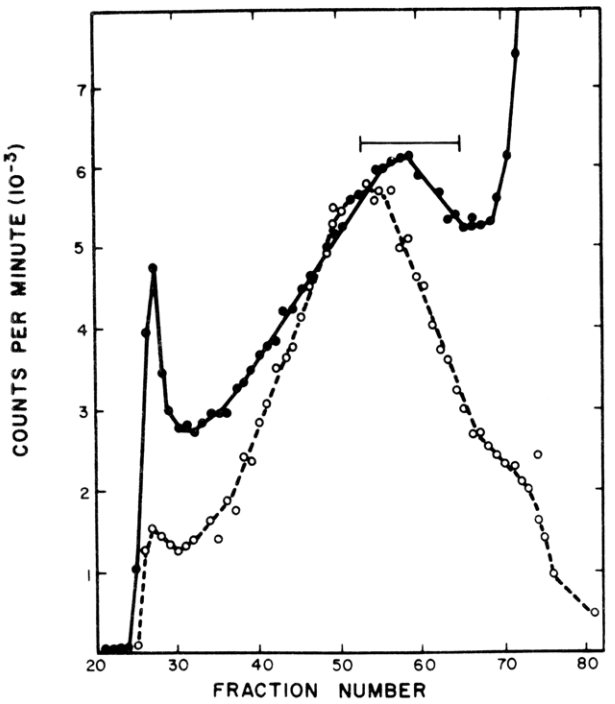

FIGURE 1: Elution profile on a Sepharose 4B column of SUV-EtOH prepared in the presence of a ^{125}I-labeled protein mixture. [^{3}H]PC (dashed line) and ^{125}I-labeled protein (solid line) are shown. Fractions indicated by the bar were pooled for further analysis.

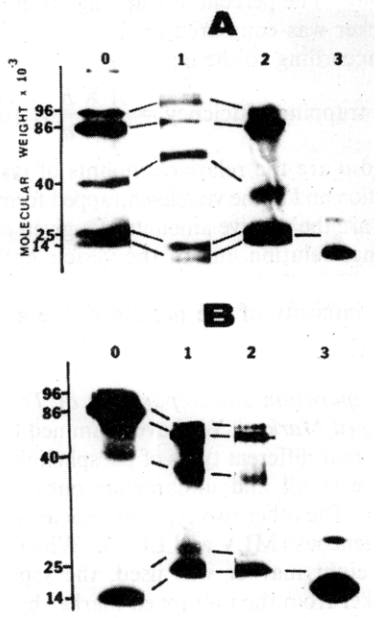

FIGURE 2: Autoradiogram of NaDodSO$_4$ slab gel pattern of ^{125}I-labeled protein markers. Markers in original solution (lane 0); markers entrapped in vesicles (lane 1); entrapped markers after tryptic digestion (lane 2); entrapped markers after tryptic digestion in the presence of Triton X-100 (lane 3). (A) is for SUV-EtOH. (B) is for SUV-son.

all ^{125}I-labeled protein markers associated with vesicles were actually inside the vesicles. It was demonstrated in two ways. First, an aliquot of the ultrafiltered vesicle preparation was chromatographed on Sephadex G-100. All ^{125}I radioactivity coeluted with the ^{3}H radioactivity in the void volume (data not shown). Secondly, all vesicle-associated proteins were resistant to tryptic digestion, as examined by NaDodSO$_4$–polyacrylamide gel electrophoresis and autoradiography (lane 2 of Figures 2 and 3). In controls, tryptic digestion was carried out in the presence of Triton X-100. All protein

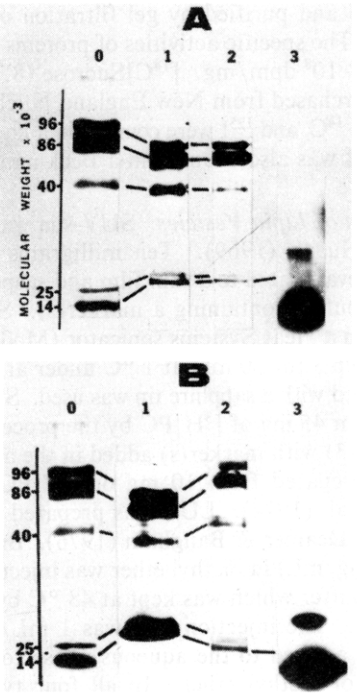

FIGURE 3: Autoradiogram of NaDodSO$_4$ slab gel pattern of ^{125}I-labeled protein markers. The sample applied to each lane is the same as that in Figure 2, except (A) is for LUV and (B) is for MLV.

markers released from vesicles by the action of detergent were digested by trypsin (lane 3 of Figures 2 and 3). Thus, all vesicle-associated protein markers truly resided inside the vesicles.

Entrapments of Sucrose and Lysozyme in SUV-EtOH. Since the trapping efficiencies of various protein markers measured by NaDodSO$_4$–polyacrylamide gel electrophoresis and autoradiography were compared to that of lysozyme (see below), it is important to demonstrate that the trapping efficiency of lysozyme, a relatively small macromolecule, is the same as that of a low molecular weight, water-soluble molecule such as sucrose. We measured it in SUV-EtOH in which [^{14}C]sucrose or [^{125}I]lysozyme was used as a marker. The amount of entrapment was identical for both markers. The trap volume of SUV-EtOH calculated from the data was 1.47 ± 0.14 μL/mg of lipid for sucrose and 1.40 ± 0.15 μL/mg of lipid for lysozyme. These values are also in good agreement with the value of 1.66 μL/mg of lipid obtained by Batzri & Korn (1973), using glucose as a marker. Therefore, the entrapment of lysozyme in small vesicles was not different from that of sucrose.

Trapping Efficiencies of Proteins of Various Molecular Weight. The relative trapping efficiencies of a group of proteins with molecular weight ranging from 14 500 to 97 000 were examined in all four types of vesicles by NaDodSO$_4$–polyacrylamide gel electrophoresis and autoradiography. The autoradiograms of the ^{125}I-labeled proteins after electrophoretic separation are shown in Figures 2 and 3. The relative intensity in the autoradiogram of each protein entrapped in vesicles (lane 2) was compared with that in the corresponding buffer solutions in which vesicles were prepared (lane 0). The percent of trapping efficiency of each protein was calculated according to eq 1. The data for all four types of vesicles are summarized in Figure 4. For the two types of large vesicles (LUV and MLV), all proteins tested could be trapped with an efficiency identical with that of lysozyme. For the two types of SUV, proteins of molecular weight up to 25 000 could be trapped

Table I: Trapping of Proteins in SUV

protein	M_r	$s_{20,w}$[a]	$\bar{v}$[a]	av radius (Å) calcd[b]	probability of trapping, calcd[c]	rel trapping efficiency (%) calcd	rel trapping efficiency (%) exptl
lysozyme	14 100	1.91	0.703	17.5	0.466	100	100
α-chymotrypsinogen	23 650	2.58	0.721	21.0	0.435	93.5	97.5
peroxidase	39 780	3.48	0.699	26.2	0.390	83.7	77.5
conalbumin	86 180	5.05	0.732	39.1	0.278	59.6	59.5
α-amylase	96 920	6.47	0.717	44.3	0.233	49.9	49.5

[a] Unit for $s_{20,w}$ is 10^{-13} s; values of $s_{20,w}$ and $\bar{v}$ were taken from *CRC Handbook of Biochemistry and Molecular Biology* (1970). [b] Calculated by eq 4. [c] Calculated by eq 3. Vesicle internal radius is taken as 84 Å, and the bound water layer is 13.1 Å thick. Radius of water is 1.65 Å.

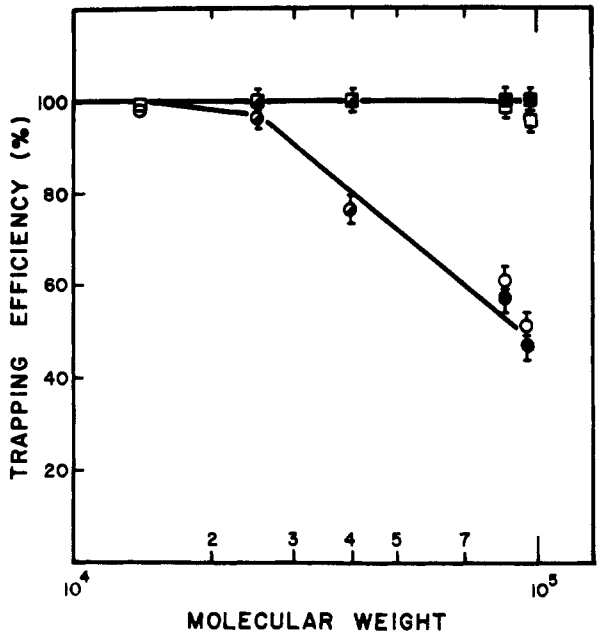

FIGURE 4: Relative trapping efficiency of ^{125}I-labeled protein in SUV-EtOH (○), SUV-son (●), LUV (□), and MLV (■). The bars are standard deviation.

similar to lysozyme. However, proteins of molecular weight greater than 25 000 showed a progressive decrease in trapping efficiency. For example, α-amylase, the largest protein tested, showed an efficiency only ~50% of that of lysozyme. Identical results were obtained for both types of SUV.

Discussion

It is essential to demonstrate that the various markers used in the present study are indeed trapped inside the vesicles and not free in the solution outside the vesicle or adsorbed on the outer surfaces of the vesicles. It is particularly important for SUV-son and LUV preparations, since relatively harsh conditions (i.e., sonication or use of organic solvent) were used which might cause the denaturation or even aggregation of proteins. This possibility is, however, ruled out for all four types of vesicles by the resistance of the vesicle-associated proteins to the tryptic digestion (Figures 2 and 3). We therefore conclude that all protein markers used in this study were indeed trapped in the interior of the vesicles.

The trapping efficiencies of various proteins, as measured by $NaDodSO_4$–polyacrylamide gel electrophoresis and autoradiography, are compared to that of lysozyme, the smallest protein marker used in this study. In the two types of large vesicles, i.e., LUV and MLV, all proteins tested could be trapped with the same efficiency. In the two types of SUV, however, only chymotrypsinogen could be trapped with approximately the same efficiency as lysozyme; proteins of molecular weight greater than 25 000 showed a progressive decrease in trapping efficiency. In other words, the concentrations of these large proteins inside the vesicles are smaller than those outside the vesicles and the difference in concentration becomes greater when the size of the protein becomes larger.

The above results can be stated in another way. When vesicles are formed in the marker protein solution, the internal volume of the vesicle represents a sampling of the solution. If the vesicles are large, as are LUV and MLV, the sampling volume is significantly larger than the size of the solute (protein) and a faithful sampling of the solution is obtained. If the internal solvent volume of the vesicle approaches that of the solute, as in the case of SUV, deviation from a faithful sampling occurs. The extent of deviation should depend on the average diameter of the vesicle and the size of the solute.

Two factors must be important for the trapping of proteins in SUV: (a) the bound (structure) water layer on the inner surface of vesicles and (b) steric hindrance of trapping large molecules such as proteins. The space occupied by the bound water, resulting from interactions with the ionic groups of phospholipids, is totally nonavailable for solvation. The latter factor, however, gives rise to a reduced possibility of trapping solutes in the remaining space of the vesicle internal volume. The probability of solute exclusion by steric hindrance has been considered by Katz & Diamond (1974) and is given as $(R_p - R_w)/(D - A - R_w)$, where R_p and R_w are the radii of a hydrated protein and water, respectively, D is the radius of the total internal volume of a spherical SUV, and A is the thickness of the bound water layer. Therefore, the probability, P, of a given protein of radius R_p being trapped inside the vesicle is given by

$$P = 1 - \frac{D^3 - (D - A)^3 + [(R_p - R_w)/(D - A - R_w)](D - A)^3}{D^3} \quad (2)$$

or

$$P = \left(\frac{D - A}{D}\right)^3 \left(\frac{D - A - R_p}{D - A - R_w}\right) \quad (3)$$

The relative trapping efficiency of protein is proportional to P.

As an approximation, we have taken the Stokes' radius of a protein for R_p, which is calculated according to

$$R_p = \frac{M_r(1 - \bar{v}\rho)}{N6\pi\eta s_{20,w}} \quad (4)$$

where M_r is the molecular weight, N is Avogadro's number, $s_{20,w}$ is the sedimentation coefficient of the protein, $\bar{v}$ is the partial specific volume of the protein, ρ is the density of water, and η is the viscosity of water. The values of ρ and η were

taken as unity as an approximation.

Table I shows the results of our calculation for the trapping in SUV of the five globular proteins used in this study. The radius of vesicle internal volume was taken as 84 Å (Huang & Charlton, 1971). The radius of water was taken as 1.65 Å. We have varied the thickness of the bound water layer in our calculation and found that the best fit between the calculated and experimental values for the relative trapping efficiency was by assuming the thickness to be 13.1 Å. As evident in Table I, the calculated values are in reasonable agreement with the experimental data. The assumption of a ~13-Å layer of bound water at the internal surface of SUV is not unreasonable. From the data of Bangham et al. (1967), Johnson & Buttress (1973) calculated the thickness of the osmotically inactive water layer to be 11 Å in MLV. The thickness of the layer should be somewhat greater for the internal surface of SUV, due to the high degree of curvature of the phospholipid bilayer.

Although the model presented above is probably an oversimplified one, it does show the strong influences of the bound water lysed and the steric hindrance as the protein is captured during the formation of SUV. For the MLV and LUV, the contributions of these two factors are not as important since the vesicle internal volumes are much larger. For small molecular weight solutes, such as sucrose, eq 3 is also not valid even in the case of SUV. The steric hindrance of trapping in this case must be of negligible importance.

Although the largest protein marker used in the present study is only ~97 000 (α-amylase), preliminary experiments using larger proteins showed even lower trapping efficiency than α-amylase. The relative trapping efficiency in SUV-EtOH for β-galactosidase (M_r 130 000) is only 20–30% of that of sucrose (unpublished experiments). Practically no trapping of goat IgG (M_r 160 000) can be observed in SUV-son; only binding of IgG at the vesicle surface occurs (Huang & Kennel, 1979). These observations further substantiate our conclusion that large proteins cannot be faithfully trapped in SUV.

In conclusion, we have shown that the efficiency of protein entrapment in lipid vesicle depends on the size of the vesicle as well as the size of the protein. It would be naive to assume that the concentration of the protein inside the vesicle is equal to that outside the vesicle. Our study represents a quantitative approach to the problem, which should be of useful information for those studies in which protein entrapment is a central part of the experiment.

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