Biochimica et Biophysica Acta, 943 (1988) 103–107
Elsevier

BBA 70567

BBA Report

The captured volume of multilamellar vesicles

Walter R. Perkins [a], Sharma R. Minchey [a], Marc J. Ostro [a], T.F. Taraschi [b] and Andrew S. Janoff

[a] *The Liposome Company, Inc., Princeton, NJ, and* [b] *Department of Pathology and Cell Biology, Thomas Jefferson University, Philadelphia, PA (U.S.A.)*

(Received 11 April 1988)

Key words: Vesicle; Captured volume; Intravesicular volume; Solute distribution; Multilamellar vesicle; Phospholipid vesicle; Liposome; ESR

Characterization of classical 'hand-shaken' multilamellar lipid vesicles (MLVs) confirmed that these systems exclude solute during formation thus confounding previous captured volume measurements which typically have utilized solute as a marker of the occluded aqueous space. We used solvent rather than solute to determine the captured volume of these systems and obtained values at least twice those previously reported. We present here a captured volume and lamellarity profile of 'hand-shaken' MLVs and suggest that these parameters are dependent on the lipid concentration present during hydration.

Multilamellar lipid vesicles (MLVs) are commonly and easily prepared by hydrating diacyl phospholipids in excess buffer. The importance of such structures as membrane models or in drug delivery has been well documented [1]. These applications rely on the optimization of a number of experimentally relevant parameters including captured volume (usually expressed as the amount of aqueous volume sequestered per μmol of lipid). Normally this quantity is determined by dispersing lipid in an aqueous medium containing a relatively impermeable isotopic solute such as ^{22}Na or $^{3}H/^{14}C$ carbohydrate. Isolation of the vesicles from the external radioactivity by either centrifugation, dialysis or gel filtration is then used to ascertain the proportion of solute trapped which in turn is used to calculate the captured volume. Recently it has been found that MLVs exclude solute during formation [2,3]. As a result of this, solute entrapment techniques to measure captured volumes yield artifactually low values. Here we report that when MLV captured volume is based on the determination of solvent rather than solute distributions, the values that are obtained are at least a factor of two higher than those previously reported.

Our approach to this problem began by expressing the captured or internal volume, V_i, of a liposome suspension in terms of the partial volumes present. Thus

$$V_T = V_o + V_i + V_L \quad (1)$$

Where V_T is the total sample volume, V_o the external aqueous volume and V_L the volume occupied by the lipid. V_L was calculated from the amount of lipid present multiplied by its partial specific volume; for egg PC we used a value of 0.983 ml/g [4].

Fig. 1 compares the captured volumes of egg PC MLVs and freeze-thawed MLVs (FAT MLVs) determined by either solvent or solute distributions. For solvent distribution we adapted a tech-

Correspondence: A.S. Janoff, The Liposome Company, Inc., 1 Research Way, Princeton, NJ 08540, U.S.A.

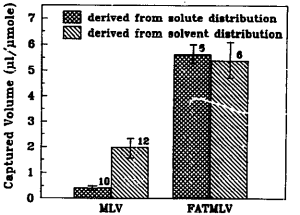

Fig. 1. Captured volumes ±S.E. of egg PC MLVs and freeze-thawed MLVs as determined isotopically by solvent or solute distribution. Numbers indicate replicas. Solvent: Either 50 mg egg PC per 500-ml round bottom flask (thin film) or 300 mg per 100-ml round bottom flask (thick film) were dried overnight under vacuum (100 mTorr). The film was rehydrated with shaking in 5.0 ml Tris buffer (10 mM Tris-HCl, 150 mM NaCl, 13 mM glucose) containing 10^6 dpm of ^3H_2O. The suspension was then transferred to pre-cooled tared 15 ml Corex tubes and kept on ice for at least 10 min. Next 10^6 dpm [^{14}C]glucose was added and aliquots retained so that the specific activity of both isotopes could be determined. The sample was centrifuged at $10000 \times g$ for 15 min and the pellet weighed, vortexed and a sample retained for phosphate analysis. The remainder of each pellet was then dissolved in 5 ml scintillation cocktail (Beckman HP) and transferred to 10 ml poly Q vials (Beckman). The tubes were rinsed with 5 additional ml of cocktail and the rinse added to the vial. V_T and V_o were calculated as the ratio of dpm values arising from ^3H or ^{14}C in the pellet to the specific activity of that isotope in the sample. From these values and the concentration of phosphate [10] V_i could be calculated from Eqn. 1. Solute: Liposomes were prepared as above ($T = 4°C$) except that the rehydration buffer contained 10^6 dpm [^{14}C]glucose and no ^3H_2O. [^{14}C]Glucose was not added after formation. The liposomes were separated from their formation buffer by centrifugation as above and washed twice in non-labelled buffer. Captured volume was calculated as the ratio of dpm values recovered in the pellet to the specific activity of glucose in the initial preparation divided by the phosphate recovered in the pellet. Freeze-thawed MLV (FATMLV): 100 mg lyophilyzed egg PC were dispersed in 1 ml Tris buffer by vortexing until the suspension was homogeneous. The resulting MLV were subjected to five freeze-thaw cycles as previously described [3]. Captured volumes were determined as described above for MLV.

nique used to determine the matrix volume of cellular organelles [5] which takes advantage of the rapid permeability of ^3H_2O across membranes and the limited permeability of ^{14}C-labeled carbohydrates [6]. We hydrated a thin film of egg PC in ^3H_2O and after liposome formation marked the external aqueous volume, V_o, by the addition of [^{14}C]glucose. By determining the specific activities of each isotope in the sample, we were able to determine V_T and V_o in the pellet after removal of most of the buffer phase by centrifugation and calculate V_i from Eqn. 1. This solvent-dependent procedure gave a captured volume about two-fold higher than if the captured volume was determined by hydrating the thin film in the presence of [^{14}C]glucose and relying on solute to mark the internal aqueous phase. That this discrepancy arose due to the nonequilibrium distribution of [^{14}C]glucose could be demonstrated because after freeze-thaw cycling both techniques gave identical values. Freeze-thawing has been shown to result in equilibrium solute distribution across multilayered liposomal systems [3].

Continuing this approach we next developed an electron spin resonance (ESR) method for determining captured volume based upon either solvent or solute distribution. Here we relied on 4-trimethylammonium TEMPO, a label that neither permeates nor binds appreciably to egg PC bilayers. V_o was determined by adding a known amount of label to preformed vesicles and measuring its concentration in the outer aqueous phase by use of a standard curve comparing label concentration to the amplitude of the $m_I = +1$ resonance peak arising from the probe in buffer. The increment in label concentration measured compared to that which would arise in the absence of liposomes represented the extent that the label was excluded from V_i. Thus:

$$V_o = \frac{M}{C} \qquad (2)$$

where M is the number of moles of label added and C its measured concentration. Thus with a knowledge of the amount of phosphate in the preparation, V_i and captured volume could be calculated from Eqn. 1. For comparison we also calculated volume by forming MLVs in the presence of label. For these experiments we made the classical assumption, albeit incorrect, that solute is distributed ideally throughout the aqueous phase and label$_{in}$/label$_{out}$ = volume$_{in}$/volume$_{out}$. Distribution of the label was determined as before from the ESR spectral amplitude (peak to peak) of

the $m_I = +1$ resonance. By taking advantage of the fact that externally added Mn^{2+} does not penetrate liposomal systems but broadens to insignificance this peak height we were able to construct the following relationship:

$$\frac{A - A'}{A'} = \frac{L_o}{L_i} = \frac{V_o}{V_i} \tag{3}$$

where A' and A are the amplitudes of the $m_I = +1$ line with and without Mn^{2+} present, respectively, and L_o/L_i is the ratio of label outside to inside the vesicles. From rearrangement of Eqn. 1 we get

$$V_i = \frac{V_T - V_L}{1 + [(A - A')/A']} \tag{4}$$

allowing calculation of that internal volume marked by the trapped label.

Fig. 2 compares the two techniques. The solute distribution derived values are in agreement with literature values [7] but comparison to the solvent distribution derived values suggested that in fact they underestimated the captured volume at all lipid concentrations by a factor of two. Again multiple freeze-thaw cycling confirmed that these discrepancies arose because of the nonequilibrium distribution of the solute label. Table I shows that after freezing and thawing the MLVs five times both techniques gave identical values, the expected result if an equilibrium solute distribution were reached.

A further consequence of the fact that MLVs are solute depleted is that they exist under osmotic compression. Thus we compared the solvent derived captured volume of these vesicles to solvent derived captured volumes of vesicles resulting from the hydration of a thin film of lipid co-deposited with solutes already present. Such systems called

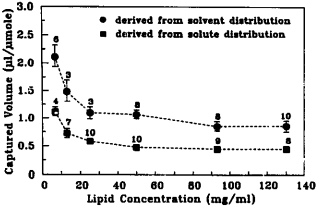

Fig. 2. Captured volumes ±S.E. of egg PC MLVs at various initial lipid concentrations determined by solvent or solute distributions. Numbers indicate replicas. MLVs were formed by dispersion of an egg PC/$CHCl_3$ mixture (10–20 mg/ml) into 500-ml roundbottom flasks followed by rotoevaporation to a film (1 torr). For the preparations containing 300 mg or more of egg PC, the films were dried further at approx. 200 mTorr for 4–10 hours depending upon the sample. Where small quantities of lipid were used the amount of $CHCl_3$ that the lipid was dried from was adjusted to a minimum volume of 5–6 ml (this was to ensure that the lipid film was dispersed evenly over the walls of the flask). Generally a buffer solution containing 10 mM Tris-HCl, 150 mM NaCl, with or without 4-trimethylammonium TEMPO was then added to the lipid and the mixture swirled until all of the lipid was removed from the walls of the flask. Frozen and thawed MLVs (FATMLVs) were made from aliquots of the MLV preparations as previously described [3]. Solvent distribution: 20 μl of a 10 mM solution of 4-trimethylammonium TEMPO was added to 980 μl of a liposome solution (adjusted to approx. 50 mg/ml lipid) for a final sample volume of 1.0 ml. The sample was vortexed, the lipid pelleted by centrifugation, the aqueous phase removed and the concentration of label determined by comparing the peak height of the $m_I = +1$ resonance line recorded on a Bruker ER 100D spectrometer to a standard curve. V_o was then determined by Eqn. 2 and V_i determined by Eqn. 1, taking V_T as the total sample volume before centrifugation. The phosphate content of the sample was then determined [10] allowing calculation of the captured volume. This technique was found to be superior to other ESR techniques for determining captured volume relying on the spin label TEMPONE [11,12] because the amount of probe added was minimal and osmotic balancing of the internal and external aqueous spaces was not necessary. Solute distribution: MLVs were prepared with 200 μM 4-trimethylammonium TEMPO present in the buffer solution. Aliquots of the sample were diluted 1:1 into the same buffer solution void of label or into a solution containing 200 mM $MnSO_4$, 250 mM EDTA, and 50 mM Mops at pH 7.0. The ESR spectral amplitude (peak to peak) of the $m_I = +1$ resonance was then used to quantitate the distribution of label by Eqn. 3 and V_i determined by Eqn. 4 allowing the calculation of captured volume. Both solute and solvent distribution determination required that the spin label did not bind or permeate the bilayer. This was found to be the case. The ESR line shape arising from the probe was found to be unaffected by the presence of lipid. In addition, when MLVs containing label were washed to remove external label and combined with sufficient Mn^{2+} to broaden the signal of any probe leaking out, no decrement in peak height could be determined over the experimental interval.

monophasic vesicles have been shown to exist in a noncompressed fashion [2]. Thus at 40 mg/ml, MLVs gave a captured volume of 1.1 $\mu l/\mu mol \pm 0.03$ ($n = 8$) whereas MPVs gave a captured volume of 2.6 ± 0.04 $\mu l/\mu mol$ ($n = 5$).

A last point concerns the fact that in our hands the captured volume of MLV systems fell as a function of the initial lipid concentration. Although it has been suggested that such profiles could result from nonspecific absorption of marker material to exposed sites on the bilayer [8] our results suggest that this is not the case. In our experiments once vesicles were formed they were all adjusted to approximately the same lipid concentration before solvent distribution determinations were made. Thus we suspected that the concentration-dependent changes in captured volume arose from changes in MLV morphology. To investigate this phenomena we employed ^{31}P-NMR. Mn^{2+} was added to vesicle dispersions at concentrations (5 mM) sufficient to broaden beyond detection the signal arising from those phospholipids facing the external medium. The extent to which the ^{31}P-NMR signal was reduced by this procedure has been correlated with the fraction of lipid exposed to the external aqueous phase and thus the lamellarity of the vesicles [9]. In our hands as the initial lipid concentration rose, lamellarity increased. This was entirely consistent with the fall in captured volume observed (Table II) and indicated that both the lamellarity and captured volume of MLVs are dependent on formation protocols.

In summary we have found that the captured volume of 'hand shaken' multilamellar systems is greater by a factor of two than has been previously assumed. We arrived at this conclusion principally by marking the external aqueous space of various vesicle preparations with a spin label that neither permeated nor bound to the lipid. Similar determinations should be possible using other markers meeting these criteria. The application of such techniques will allow accurate captured volume measurements of multilamellar vesicles to be made regardless of solute distribution anomalies.

TABLE I

CAPTURED VOLUMES OF FROZEN AND THAWED MLVs (FATMLVs) DETERMINED VIA ELECTRON SPIN RESONANCE SPECTROSCOPY UTILIZING EITHER THE SOLUTE DISTRIBUTION OR SOLVENT DISTRIBUTION APPROACH

Lipid (mg/m^3)	Captured volume ($\mu l/\mu mol$)		Ratio
	from solute distribution	from solvent distribution	
6.2	7.08 ± 0.51 *	7.05 ± 0.64	1.00
12.4	7.90 ± 0.22	6.70 ± 0.39	1.05
24.4	7.10 ± 0.53	7.03 ± 0.21	1.01
47.6	7.18 ± 0.13	7.16 ± 0.11	1.00
90.9	5.69 ± 0.18	5.71 ± 0.18	1.00
130.0	4.53 ± 0.24	4.52 ± 0.16	1.00

* S.E.

TABLE II

^{31}P-NMR SIGNAL INTENSITY DECREMENTS ARISING FROM MLVs FORMED AT VARIOUS LIPID CONCENTRATIONS AND SUBSEQUENTLY EXPOSED TO 5 mM $MnSO_4$

Values are from a typical experiment.

Lipid (mg/ml)	Captured volume ($\mu l/\mu mol$)	% decrease in ^{31}P-NMR Signal
6.2	2.26	12.3
48	1.41	8.8
131	0.88	4.0

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