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Conductivity measurement as a convenient technique for determination of liposome capture volume

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Summary

The reduction in conductivity seen between a buffer solution and a liposome preparation in that buffer was evaluated as a means of measuring liposome capture volume. Using DOPC and DOPG lipid to form negatively charged liposomes, conductivity measurements showed that conductivity of the liposome dispersion decreased as lipid concentrations of liposome preparations increased. Independent measurement of capture volumes by gel filtration chromatography showed that conductivity changes correlated with a liposome concentration dependent increase in capture volume. It is proposed that ions from the hydrating/suspending buffer normally contributing to conductivity were trapped within liposomes upon vesicle formation. These internalized and therefore shielded ions were not able to effectively contribute to conductivity of the liposome dispersion. For multilamellar vesicles (MLVs), capture volume was determined by reduction in conductivity over a large lipid concentration range and a broad buffer ionic strength range. Capture volume could also be determined for small unilamellar vesicles (SUVs). However, the greater number of exposed phospholipid head groups in high surface area SUVs contributed to conductivity of the liposome dispersion thereby limiting range of utility. A much higher ionic strength buffer (relative to MLVs) was required before conductivity of phospholipid no longer influenced conductivity of the dispersion. To expand this study, multilamellar vesicles having either neutral (DOPC) or positive (DOPC/stearylamine) charge were evaluated. Similar correlations were found between reduction in conductivity and mannitol entrapment (capture volume). These studies have confirmed that measurement of reduction in conductivity provides an easy and convenient method for determining liposome capture volume.

Introduction

Several relatively laborious methods for determining liposome capture volume have been developed and are extensively utilized (Weiner et al., 1989; Anzai et al., 1990; Rogers et al., 1990). This paper describes a method whereby liposome capture volume can be rapidly and conveniently measured.

During the course of pharmaceutical evaluation of various liposome preparations, several colligative properties were measured. Conductivity, the measure of electrical current through a solution, was one of those parameters examined. Conductivity measurements consistently showed

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Abbreviations: DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; SA, stearylamine; MLV, multilamellar vesicle; SUV, small unilamellar vesicle.

that liposome dispersions in a given vehicle had a reduced conductivity value when compared to that of the corresponding hydrating buffer alone. Multilamellar vesicles exhibited larger reductions in conductivity than did small unilamellar vesicles as did preparations containing higher liposome concentrations. These observations suggested a possible association between reduction in conductivity and liposome capture volume.

To explore a relationship between reduction in conductivity and liposome capture volume, various liposome compositions were prepared with buffer containing [^{14}C]mannitol; the liposomes consisted of: (1) dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG); (2) DOPC and stearylamine (SA); and (3) DOPC, alone. Mannitol was chosen as the marker for this experiment because this neutral hydrophilic compound will not interact with phospholipid; entrapment of mannitol into the lipid vesicles occurs as a passive process without influence of possible intermolecular attraction. Mannitol entrapment, therefore, is used as a measure of liposome capture volume. Mannitol entrapment and conductivity measurements were performed for liposome preparations which varied as a function of lipid concentration, buffer ionic strength and liposome size.

This paper shows that a definite relationship exists between the reduction in buffer conductivity upon formation of liposomes, and vesicle capture volume. This relationship should prove quite useful for liposome characterization due to the rapid and straight forward means of measurement.

Materials and Methods

Reagents

All chemicals were used without further processing or purification. Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG) were obtained from Avanti Polar Lipid as 20 mg/ml solutions in chloroform. Stearylamine was obtained from Sigma. [^{14}C]Mannitol was supplied by Amersham with a specific activity of 60 mCi/mmol (2.2 GBq/mmol) and a radiochemical purity of 97.3%. Saline, mannitol, and phosphate buffer salts were compendial grade. Sepharose CL-6B gel filtration medium was received from Pharmacia. Liquid scintillation cocktail was obtained from Beckman Instruments. A 10000 μSi/cm (μmho/cm) calibration standard (for the conductivity meter) was obtained from YSI (Yellow Springs Instruments Co., Inc.).

Gel filtration

Sepharose CL-6B was packed into a Pharmacia column (1.6 × 40 cm) as recommended by the manufacturer. After column packing, the gel was presaturated (Nozaki et al., 1982; Reynolds et al., 1983) with an aliquot of empty lipid vesicles (composed of DOPC and DOPG). The buffer system (mobile phase) used to elute liposomes and un-entrapped mannitol from this column was composed of 0.14% sodium phosphate (dibasic), 0.89% sodium chloride, 0.02% potassium chloride, and 0.05% sodium azide, adjusted to pH 7.4.

Liposome preparation

Lipid dissolved in chloroform was placed in a round-bottom flask. Organic solvent was removed by rotary evaporation with a water bath at 60°C. Once bulk solvent was removed, the remaining lipid film continued drying for approx. 1 h to remove trace residual solvent. The dried lipid layer was subsequently hydrated, to form multilamellar vesicles (MLVs), with a phosphate buffer (pH 7.4) as described below. Small unilamellar vesicles (SUVs) were subsequently prepared via sonication for 1 h with a Branson sonifier utilizing a water jacketed cup horn (allowing sonication in a sealed vial). Total lipid concentration in the final preparations varied from 10 to 120 mg/ml with a constant DOPC:DOPG or DOPC:SA molar ratio of 7:3.

Lipid hydrating buffers

Three primary buffers of different ionic strengths were prepared to compare the effect of ionic strength on conductivity of liposome dispersions. The three buffers had ionic strengths (μ) of 0.273, 0.155, and 0.067. The first buffer (μ = 0.273) contained 67 mM (9.5 mg/ml) sodium phosphate (dibasic), 13 mM (1.8 mg/ml) potas-

sium phosphate (monobasic) and 59 mM (3.4 mg/ml) sodium chloride; this concentration of sodium chloride made the preparation isotonic. The second buffer ($\mu = 0.155$) contained 42 mM (5.9 mg/ml) sodium phosphate (dibasic) and 8 mM (1.1 mg/ml) potassium phosphate (monobasic); no sodium chloride. The third buffer ($\mu = 0.067$) contained: 21 mM (3.0 mg/ml) sodium phosphate (dibasic) and 4 mM (0.5 mg/ml) potassium phosphate (monobasic); no sodium chloride. The phosphate salts added in the proportions listed above for each of the buffers gave a final solution pH of 7.4, without adjustment. Also, at these concentrations of phosphate buffer, the pH of the final liposome preparations (MLV and SUV) was maintained at a value of approx. pH 7.4. Prior to liposome preparation, an aliquot of each buffer was spiked with 20 mg/ml mannitol and 0.1 μCi/ml [^{14}C]mannitol.

Particle size analysis

Multilamellar vesicles were sized by a Malvern Droplet and Particle Sizer utilizing laser light scattering (model 2600c, Malvern Instruments Ltd, Malvern, U.K.). Sonicated (submicron) liposomes were sized by laser photon correlation spectroscopy using a Malvern Autosizer IIc (Malvern Instruments Ltd, Malvern, U.K.). For sizing, liposomes were diluted into the phosphate buffer originally used to hydrate and prepare each liposome.

Conductivity measurements

All conductivity measurements were performed at ambient room temperature (24°C) using a YSI (model 34, Yellow Springs, OH) conductivity meter. The electrode (YSI 3403) was housed in a pyrex body and composed of platinum coated platinum-irridium alloy. This electrode constant (k) was 100/m (SI units). Calibration of the conductivity meter was performed following instructions provided by the manufacturer. For replicate samples, conductivity measurements did not deviate by more than 3%.

Determination of ^{14}C entrapment

A 200 μl aliquot of liposome containing [^{14}C]mannitol was loaded onto the gel filtration column, and 1.4 ml fractions were collected. A 0.5 ml aliquot of each fraction was placed in a scintillation vial and combined with 10 ml of scintillation cocktail. Samples were then counted by a Beckman LS 8100 scintillation counter.

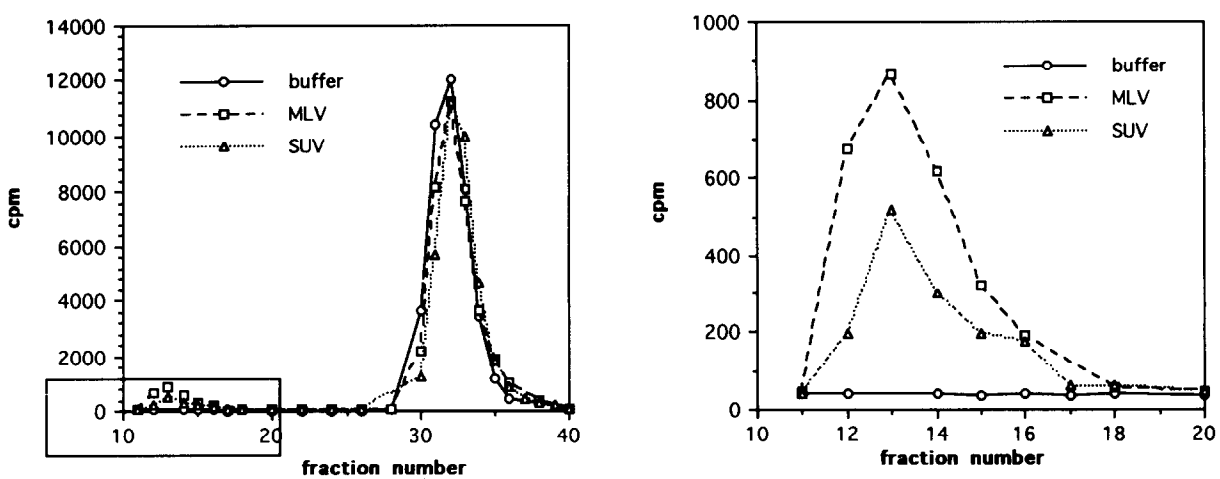

Fig. 1. (Left) (a) Gel filtration profile showing separation of free (fractions 29–38) vs liposome entrapped [^{14}C]mannitol (cpm) in 30 mg/ml DOPC/DOPG liposomes (fractions 12–16) eluted over a Sepharose 6B column at ambient room temperature; eluting buffer was as described in Materials and Methods. (Right) (b) Gel filtration profile of [^{14}C]mannitol entrapped in DOPC/DOPG liposomes (multilamellar vs small unilamellar vesicles); experimental details as described in panel (a).

Liposome integrity during gel filtration–osmotic shock

The buffers used to hydrate liposomes, as described above, vary in tonicity; liposomes prepared with the lower ionic strength buffers (μ = 0.067, 0.155) are hypotonic relative to the buffer used as mobile phase during gel filtration. The question then arises as to whether or not liposome integrity is compromised during gel filtration. To address this issue, a batch of 30 mg/ml liposomes was hydrated with low ionic strength buffer ($\mu = 0.067$) containing a trace amount of [^{14}C]mannitol and 2% unlabeled mannitol, as described above. One aliquot of these liposomes was eluted over the Sepharose 6B gel column with the relatively hypertonic eluting mobile phase; this was compared to the same liposome dispersion eluted from the same column using an eluting buffer having equal tonicity to the interior of the liposome. Recovery of [^{14}C]mannitol (percent entrapment) within the liposome was compared for the two experiments. The results showed no difference in the amount of [^{14}C]mannitol entrapped within the liposomes. The hypotonic liposomes were not adversely affected by osmotic shock when eluted from the gel filtration column using the relatively hypertonic mobile phase.

Results

Gel filtration was used to determine the amount of [^{14}C]mannitol entrapped within each liposome preparation. Fig. 1a and b shows the elution profiles of free [^{14}C]mannitol in hydrating buffer ($\mu = 0.273$); a 30 mg/ml multilamellar DOPC/DOPG vesicle; and a 30 mg/ml small unilamellar DOPC/DOPG vesicle (both hydrated with $\mu = 0.273$ buffer containing [^{14}C]-mannitol). Free, or un-entrapped mannitol eluted between fractions 29 and 38; mannitol entrapped inside liposome vesicles eluted between fractions 12 and 16.

The amount of liposome entrapped mannitol, as determined by gel filtration, increased with increasing lipid concentration. Fig. 2 shows mannitol entrapment in DOPC/DOPG liposomes over a broad range of lipid concentration. At or

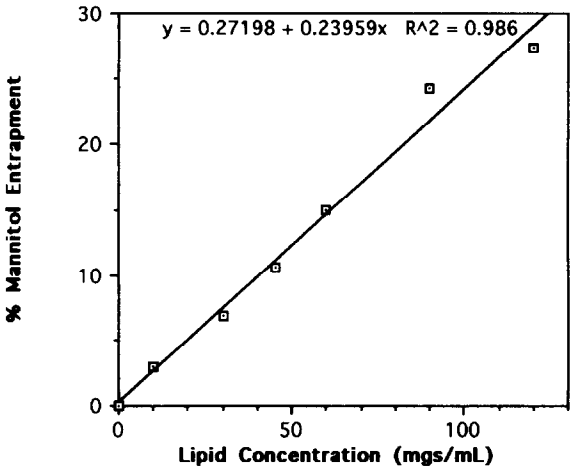

Fig. 2. Percent mannitol entrapment in DOPC/DOPG multilamellar liposome vesicles shown as a function of lipid concentration. The percent mannitol entrapment was determined by gel filtration; experimental details as described in Fig. 1a.

below a total lipid concentration of 60 mg/ml, recovery of total radioactivity from the gel filtration column was close to 100%. At concentrations above 60 mg/ml, efficiency of gel filtration decreased significantly as manifested by decreased recovery of total radioactivity. Measured mannitol entrapment values at 90 and 120 mg/ml reflected greater error due to this drop in column efficiency. Therefore, these values deviate slightly from the line shown in Fig. 2. Due to the decreased gel filtration efficiency at higher lipid concentrations, all described experiments were performed at or below a lipid concentration of 60 mg/ml.

Conductivity is a measure of the electrical current carried by positive and negative ions present in solution. The electrical conductivity of a solution containing electrolytes is the reciprocal of the resistance of the solution, with units of measure being μSi/cm. Conductivity measurements were taken for all liposome preparations made with the various buffers (ionic strengths of 0.273, 0.155, 0.067). As the lipid concentration (and concomitant liposome level) increased, the measured conductivity of each liposome preparation decreased; this finding was found to be true for all three buffers tested. Conductivity of the liposome dispersions decreased as the lipid concen-

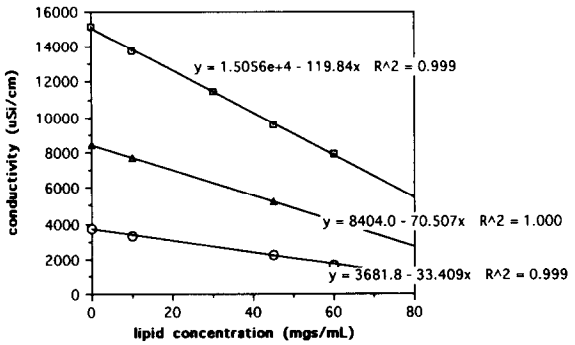

Fig. 3. Conductivity measurements of DOPC/DOPG multilamellar liposome dispersions as a function of lipid concentration. Liposome dispersions were prepared with pH 7.4 phosphate buffer at three different ionic strengths ($\mu = 0.273$ (⊡), 0.155 (△), 0.067 (□)).

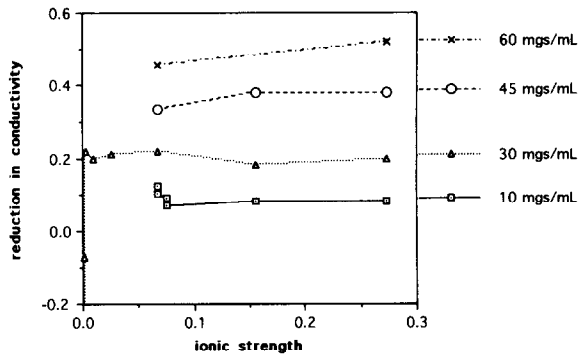

Fig. 4. Reduction in conductivity ($1 - k_L / k_B$, where k_L denotes the conductivity of the DOPC/DOPG multilamellar liposome dispersion, and k_B is that of the liposome hydrating buffer) of liposome dispersions (10, 30, 45 and 60 mg/ml) as a function of ionic strength (μ) of the liposome hydrating buffer.

tration increased. Figure 3 clearly demonstrates this phenomenon for DOPC/DOPG multilamellar liposomes. This trend suggests that ions were effectively removed from the buffer media; larger amounts of ions were removed as liposome capture volume increased with increasing lipid concentration. Once inside the vesicles, buffer ions were shielded and experienced reduced mobility such that their contribution to conductivity was no longer apparent.

To evaluate the extent of conductivity contributed by the DOPC/DOPG phospholipid head groups, the data from Fig. 3 were re-analyzed. Fig. 4 shows reduction in conductivity (or, the difference between measured conductivity of the liposome vs measured conductivity of lipid hydrating buffer) relative to buffer ionic strength. The data show that reduction in conductivity remained fairly constant for each liposome lipid concentration over a broad ionic strength range. Only at very low ionic strength ($\mu = 0.003$) did the charge associated with liposome surfaces contribute to net conductivity. Each line on the graph corresponds to specific liposome lipid concentrations; the reduction in conductivity, or the effective removal of ions from the liposome dispersions, was greater for greater lipid concentrations, again suggesting a connection to liposome capture volume.

To better define the influence of ionic strength on the conductivity of liposome dispersions and the effect of phospholipid head group, multilamellar and small unilamellar DOPC/DOPG liposomes containing 30 mg/ml lipid were prepared using ionic strengths ranging from 0 (water) to 0.273. The results showed that for DOPC/DOPG liposomes prepared in low ionic strength buffers, conductivity of the liposome dispersion was influenced by the exposed charge of the phospholipid. Table 1 and Fig. 5 show that for MLV liposomes (with an average size of 3 μm), reduction in conductivity quickly reached a steady state value; at buffer ionic strengths greater than 0.003 any contribution to conductivity by the phospholipid had been overwhelmed by the conductivity of buffer. Small liposomes (with an average size of 76 nm) most notably influenced con-

TABLE 1

Effect of ionic strength on reduction in conductivity for 30 mg / ml DOPC / DOPG MLV and SUV liposomes

Ionic strength	Reduction in conductivity	
	MLV liposomes	SUV liposomes
0.000	−8.750	−95.375
0.001	−0.007	−2.776
0.003	0.220	−1.059
0.010	0.200	−0.357
0.025	0.210	0.008
0.067	0.220	−0.099
0.155	0.180	0.106
0.273	0.204	0.099

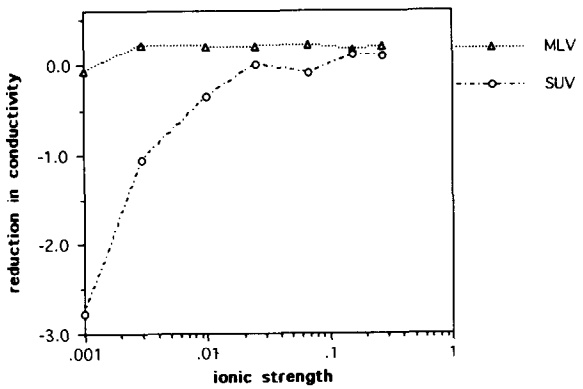

Fig. 5. Reduction in conductivity $(1-k_L/k_B)$ is shown as a function of buffer ionic strength; this figure compares DOPC/DOPG (30 mg/ml) multilamellar liposomes to small vesicles of identical composition.

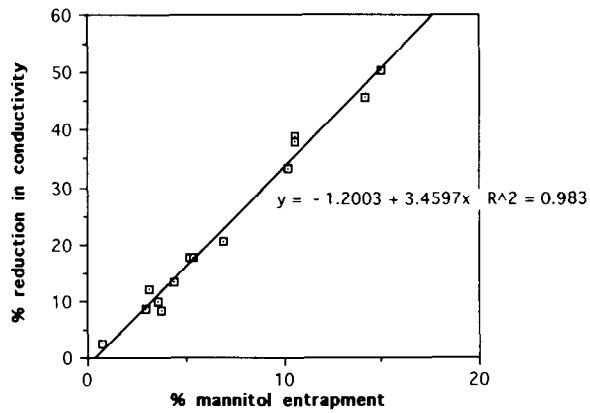

Fig. 6. Correlation between percent reduction in conductivity and percent mannitol entrapment for DOPC/DOPG multilamellar and small unilamellar vesicles (prepared over a total lipid concentration of 10–60 mg/ml, with three ionic strength buffers, $\mu = 0.273$, 0.155, 0.067).

ductivity of the liposome dispersion. Greater surface area exposing a greater percentage of phospholipid head groups and surface charge contributed more effectively to overall conductivity of the dispersion. The liposome's contribution to conductivity was manifested by a negative value for reduction in conductivity; i.e., measured conductivity for the liposome suspension was greater than the measured conductivity for corresponding hydrating buffer. As a result, greater buffer ionic strengths were required before an equilibrium in reduction of conductivity was reached for SUVs.

From the data obtained for DOPC/DOPG liposomes, a clear correlation between reduction in conductivity and liposome capture volume (as measured by [^{14}C]mannitol entrapment) was established, as shown in Fig. 6. This graph demonstrates that measured conductivity of these liposome dispersions decreased by 0.35% per μl of aqueous solution captured. Calculated * capture volumes (μl/mg lipid) for both MLV and SUV liposomes are given in Table 2. Table 2 shows that MLV liposomes consistently captured approx. 2.5 μl aqueous solution per mg of lipid used in the liposome preparation; and SUV liposomes captured approx. 0.96 μl per mg of lipid.

Neutral (DOPC) and cationic (DOPC/SA) multilamellar liposomes, at several lipid concentrations, were evaluated for reduction in conductivity and percent mannitol entrapment. Data for these latter liposome dispersions are presented in Fig. 7. Capture volume of multilamellar DOPC/SA liposomes was similar to that of DOPC/DOPG liposomes with an average capture vol-

TABLE 2

Capture volume measured for DOPC/DOPG liposome dispersion

Lipid concentration (mg/ml)	MLV capture volume (μl/mg)	SUV capture volume (μl/mg)
10	2.94	0.76
30	2.30	1.20
45	2.35	0.97
60	2.49	0.89
90	2.69	–
120	2.28	–
	$\bar{x} = 2.51$	$\bar{x} = 0.96$
	SD = 0.26	SD = 0.18

* Fig. 6 shows the following relationship between % reduction in conductivity (y) and % mannitol entrapment (x): $y = -1.2003 + 3.4597x$. Capture volume (cv, in μl) can be calculated from % mannitol entrapment values by:

cv = % mannitol entrapment/1 ml · 1000 μl/1 ml · 1/100%

cv = % mannitol entrapment · 10.

Therefore, % reduction in conductivity $(y) \approx 3.4597x = 3.4597\text{cv}/10 = 0.34597\text{cv}$. So, a 0.35% reduction in conductivity occurs per μl of volume captured.

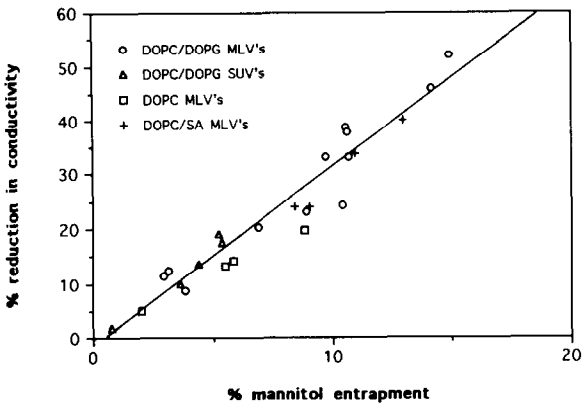

Fig. 7. Correlation between percent reduction in conductivity and percent mannitol entrapment. Lipid vesicles studied: negative [(○) DOPC/DOPG multilamellar and (△) small unilamellar vesicles], neutral [(□) DOPC multilamellar vesicles] and positive [(+) DOPC/SA multilamellar] liposomes. This figure combines data from Fig. 6 with results for neutral and cationic liposomes to give an overall linear relationship of: $y = -1.6027 + 3.2562x$ with a correlation coefficient of 0.924.

ume of approx. 2.63 μl per mg of lipid. The neutral multilamellar DOPC liposomes exhibited a reduced capture volume of approx. 1.78 μl per mg of lipid.

Discussion

Understanding the physical parameters associated with a liposome vehicle will provide a more rational approach to development of liposomes as drug delivery systems. By characterizing the conductivity of prepared liposomes, the observation was made that a net loss of conductivity (or a removal of the conducting species from solution) occurred upon formation of liposome vesicles. This loss was found to be a convenient measure of liposome capture volume. Reductions in conductivity were found to increase as capture volume or lipid concentration increased. Multilamellar vesicles captured greater volumes and showed greater reductions in conductivity, when compared to smaller vesicles. The experimental method described in this paper therefore allows convenient and accurate determination of the volume fraction of solution entrapped by either MLV or SUV liposomes. Either by manufacture or subsequent processing, liposomes can have a variety of sizes (Pick, 1981; Oku et al., 1983; Hope et al., 1985; Shew et al., 1985; Talsma et al., 1987); with the proper controls, measuring reduction in conductivity should also be able to serve as a means of determining relative vesicle size.

Conductivity, as correlated to volume of entrapment, was established using DOPC/DOPG vesicles, alternate liposome compositions resulted in similar reductions in conductivity. Limited evaluation of neutral (DOPC) and cationic (DOPC/SA) multilamellar liposomes also exhibited a trend of conductivity reduction with increased lipid concentration. Calculation of capture volume showed that positively charged vesicles contained a similar aqueous volume to negatively charged vesicles. But, neutral liposomes captured less aqueous volume. This finding is expected since charge repulsion provided by either DOPG or stearylamine would tend to expand the interlamellar regions and thereby allow for greater capture volume.

Entrapped polar drugs do not typically interact with the hydrophobic regions (phospholipid bilayers) of liposomes, and are found within the aqueous core and interlamellar spaces of the vesicles (Juliano et al., 1979). The amount of drug entrapped within the aqueous regions of the liposomes will be limited by capture volume. Because capture volume can be readily determined by reduction in conductivity, the amount of a hydrophilic, neutral drug or marker can therefore also be quickly calculated.

With appropriate liposome characterization, this new method for determining capture volume may be used as a measure of liposome stability. Storage of liposomes in the form of a solution or dispersion may lead to: (a) drug leakage from the vesicle; and/or (b) vesicle fusion. Storage of liposomes as a freeze dried product also poses stability concerns (Crowe et al., 1985; Fransen et al., 1986; Womersley et al., 1986): (a) liposome size and capture volume upon reconstitution; and (b) liposome integrity during freeze drying and subsequent storage in the dried form. Routinely measuring conductivity of these stored vesicles can conveniently provide accurate information relating to size and integrity of the liposomes. Pre-

sumably, changes in conductivity will indicate whether liposomes have leaked (increase in conductivity) or fused (decrease in conductivity).

In conclusion, the reduction in conductivity seen with a liposome dispersion upon vesicle formation is directly related to the volume (and amount of solute) captured by the liposome. The convenience of this measurement can facilitate liposome characterization and possibly stability assessment.

References

Anzai, K., Yoshida, M. and Kirino, Y., Change in intravesicular volume of liposomes by freeze-thaw treatment as studied by the ESR stopped-flow technique. *Biochim. Biophys. Acta,* 1021 (1990) 21–26.

Crowe, L.M., Crowe, J.H., Rudolph, A., Womersley, C. and Appel, L., Preservation of freeze-dried liposomes by trehalose. *Arch. Biochem. Biophys.,* 242 (1985) 240–247.

Fransen, G.J., Salemink, P.J.M. and Crommelin, D.J.A., Critical parameters in freezing liposomes. *Int. J. Pharm.,* 33 (1986) 27–35.

Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R., Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta,* 812 (1985) 55–65.

Juliano, R.L. and Stamp, D., Interactions of drugs with lipid membranes. characterization of liposomes containing polar of non-polar antitumor drugs. *Biochim. Biophys. Acta,* 586 (1979) 137–145.

Nozaki, Y., Lasic, D., Tanford, C. and Reynolds, J.A., Size analysis of phospholipid vesicle preparations. *Science,* 217 (1982) 366–367.

Oku, N. and MacDonald, R.C., Differential effects of alkali metal chlorides on formation of giant liposomes by freezing and thawing dialysis. *Biochemistry,* 22 (1983) 855–863.

Pick, U., Liposomes with a large trapping capacity prepared by freezing and thawing of sonicated phospholipid mixtures. *Arch. Biochem. Biophys.,* 212 (1981) 186–194.

Reynolds, J., Nozaki, Y. and Tanford, C., Gel-exclusion chromatography on S 1000 Sephacryl: Application to phospholipid vesicles. *Anal. Biochem.,* 130 (1983) 471–474.

Rogers, J.A. and Cheuk T., Partial specific volumes of liposome systems. *Chem. Phys. Lipids,* 53 (1990) 211–217.

Shew, R.L. and Deamer, D.W., A novel method for encapsulation of macromolecules in liposomes. *Biochim. Biophys. Acta,* 816 (1985) 1–8.

Talsma, H., Jousma, H., Nicolay, K. and Crommelin, D.J.A., Multilamellar or multivesicular vesicles? *Int. J. Pharm.,* 37 (1987) 171–173.

Weiner, N., Martin, F. and Riaz, M., Liposomes as a drug delivery system. *Drug Dev. Ind. Pharm.,*, 15 (1989) 1523–1554.

Womersley, C., Uster, P.S., Rudolph, A.S. and Crowe, J., Inhibition of dehydration-induced fusion between liposomal membranes by carbohydrates as measured by fluorescence energy transfer. *Cryobiology,* 23 (1986) 245–255.