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Generation and characterization of iron- and barium-loaded liposomes

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Previous work (Veiro and Cullis (1990) Biochim. Biophys. Acta 1025, 109–115) has shown that Ca^{2+} can be accumulated into large unilamellar vesicles (LUVs) in the presence of a transmembrane pH gradient (inside acidic) and the Ca^{2+}-ionophore A23187. Here, the ability of A23187 to mediate the uptake of iron and barium into LUVs has been investigated. It is shown that under appropriate conditions of temperature and A23187 concentration, iron (in the form of Fe^{2+}) can be accumulated into EPC and DSPC/cholesterol (55:45; mol/mol) LUVs with an acidic interior. This uptake is dependent on the internal buffer concentration, with maximum levels of uptake in the range of 300 nmol of cation per μmol lipid. The DSPC-cholesterol LUV systems exhibit superior retention properties compared to the EPC systems. It is demonstrated that Ba^{2+} can also be loaded by similar methods. It is also shown that the maximally loaded Fe^{2+}- and Ba^{2+}-containing LUVs exhibit increased densities. This is expressed by enhanced gravimetric properties, as an increased proportion of the loaded LUVs can be pelleted by low speed centrifugation, and by enhanced electron densities, in that the Ba^{2+}-loaded systems can be directly visualized employing cryo-electron microscopy.

Introduction

Previous work from this laboratory has shown that rapid and efficient accumulation of Ca^{2+} into large unilamellar vesicles (LUVs) in response to transmembrane pH gradients (pH inside acidic) can be achieved in the presence of the Ca^{2+}-ionophore A23187 [1]. The uptake of Ca^{2+} was shown to be dependent upon the buffering capacity of the internal citrate buffer, indicating a Ca^{2+}-$2H^+$ exchange process mediated by the ionophore [1]. Among other applications this procedure offers interesting possibilities for increasing the density of LUVs by exchanging protons for cations of much higher atomic or molecular weight. In the present work we have investigated the potential of the A23187-mediated ΔpH loading technique to accumulate iron and barium into LUV systems. It is shown that loading of Fe^{2+} and Ba^{2+} to high internal concentrations can be achieved by this method. The resulting systems display properties consistent with increased densities, as evidenced by their pelleting properties after low-speed centrifugation and visualization by electron microscopy. These systems may be of utility in procedures requiring separation of LUVs from aqueous media and visualization in vitro or in vivo.

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Materials and Methods

Egg phosphatidylcholine (EPC) and distearoylphosphatidylcholine (DSPC) were obtained from Avanti Polar Lipids (Birmingham, AL). [^{14}C]Methylamine was purchased from New England Nuclear. The Ca^{2+}-ionophore A23187 was obtained from Calbiochem (Calgary). All other chemicals used were purchased from Sigma (St. Louis, MO).

To produce EPC vesicles, multilamellar vesicles (MLVs) were first produced by hydrating 50 mg EPC in 1 ml of 300 mM citrate buffer (pH 4.0). The MLVs were frozen in liquid nitrogen and thawed in warm water for five freeze-thaw cycles. This treatment increases the trapped volume of the vesicles and promotes an equilibrium solute distribution [2]. Extrusion of the frozen and thawed MLVS (FATMLVs) through two stacked polycarbonate filters (Nuclepore) (100 nm or 200 nm pore size) was performed ten times using an

extrusion device obtained from Lipex Biomembranes (Vancouver, Canada). In accordance with previous results [3] the resulting large unilamellar vesicles (LUVs) were 108 nm (100 nm pore size) or 214 nm (200 nm pore size) in diameter, as measured by quasielastic light scattering techniques employing a NICOMP particle sizer.

DSPC/cholesterol (55:45, mol/mol) vesicles were made by dissolving appropriate amounts of both compounds in chloroform. The chloroform was then removed under a stream of nitrogen and by subsequent incubation under reduced pressure. After hydration, as above, to form MLVs, the DSPC/cholesterol dispersion was extruded at 65°C.

In order to establish a transmembrane pH gradient, the LUVs in pH 4.0 buffer were passed down a 10 cm Sephadex G-50 (50–150) column previously equilibrated with 150 mM NaCl, 20 mM Hepes (pH 7.5) (Hepes-buffered saline, HBS). The A23187 was dispensed from a stock chloroform solution of 2.5 mg/ml in chloroform/ethanol (4:1). The chloroform was subsequently removed under a stream of nitrogen and 1.1 ml of buffer (pH 7.5) containing 150 mM NaCl, 20 mM Hepes (and 10 mM ascorbate for the Fe^{2+} uptake experiments) was added. To this, 0.15 ml of either 10 mM $FeSO_4$ or 10 mM $BaSO_4$ was added. Uptake was initiated by the addition of 0.25 ml of LUVs prepared as indicated above (pH 7.5 outside, pH 4.0 inside) to this mixture. To assay uptake, 0.1-ml aliquots were removed at appropriate times by passage down 1.0 ml Sephadex G-50 columns (pre-spun). These columns were spun for one min at 2500 rpm in a clinical centrifuge to remove untrapped iron or barium. All experiments were conducted at 23°C unless stated otherwise. The final phospholipid concentrations of the reaction mixtures were generally in the range of 3–5 mM.

In order to establish which oxidation state of iron (Fe^{2+} or Fe^{3+}) was transported, conditions appropriate to a particular oxidation state were employed. Fe^{2+} solutions were maintained in the reduced state by the addition of ascorbate (10 mM) to the buffers used as indicated above and by gassing of the buffers with N_2 gas to minimize oxidation. Fe^{3+} containing solutions were obtained from $FeCl_3$.

The magnitude of the pH gradients present were measured using [^{14}C]methylamine at a concentration of 1 μCi/ml. The amount of this probe imported into the LUVs in response to ΔpH (inside acidic) was determined via liquid scintillation counting after separating untrapped material employing a spin column procedure [4]. The transmembrane pH gradient was calculated using the relationship $\Delta pH = \log[[\text{methylamine}]_{in}/[\text{methylamine}]_{out}]$, assuming a trapped volume of 1.5 l/mol for the systems extruded through the 100-nm pore size filters [5].

Centrifugation (15000 × g) of the iron or barium-loaded liposomes was performed at 4°C using an Eppendorf micro-centrifuge. Barium content of the liposomes was determined by using ICP-Mass Spectroscopy. Iron was assayed spectrophotometrically using bathophenanthroline sulfonate [6]. In this assay 0.25 ml of 3.3 M sodium acetate (pH 4.7), 0.1 ml of 5.0% Triton X-100, 0.05 ml of 0.2% (w/v) bathophenanthroline sulfonate in 1.0% thioglycolic acid and 0.55 ml of water were added to 0.05 ml of the sample to be assayed (total volume 1.0 ml). The reaction was allowed to proceed for at least 15 min before the absorbance was measured at 535 nm using a Shimadzu UV-160 spectrophotometer.

Phospholipid concentrations were calculated by a modification of the method of Fiske and SubbaRow [7]. All data are means ± S.E. ($n = 3$).

Electron dense liposomes containing Ba^{2+} were prepared using a modification of the above techniques. A dried DSPC/cholesterol (55:45, mol/mol) film was hydrated in 600 mM citrate adjusted to pH 3.0 with arginine at a concentration of 25 mg lipid/ml buffer. LUVs were then prepared as described above. A 500 μl aliquot of the resulting 100-nm vesicles was added to a Sephadex G-25 column (9 ml bed volume) and the external buffer was exchanged by elution with 150 mM NaCl, 20 mM Hepes, adjusted to pH 7.4 to result in a transmembrane pH gradient where $pH_i = 3.0$ and $pH_o = 7.4$. Barium chloride ($BaCl_2$) (24 μl of a 500 mM stock in H_2O) was added to the ionophore (12 μg of A23187) and vortexed, and 1 ml of vesicles was then added resulting in an (initial) exterior Ba^{2+} concentration of 12 mM. The suspension was vortexed vigorously and incubated at 60°C for 2.5 h. The Ba^{2+} loaded liposomes were stored at 4°C until observed by cryo-electron microscopy.

For cryo-electron microscopy, a thin film was formed by applying a drop of the vesicle suspension to a 700 mesh gold grid with a pasteur pipet followed by blotting from behind with Whatman No. 50 filter paper. The thin films were then vitrified by plunging the grids into liquid ethane cooled with liquid nitrogen in a Reichart Jung Universal Cryo Fixation system. The grid was removed from the ethane in a liquid nitrogen-chilled environment and blotted from the edge with filter paper to remove any excess ethane which may have been left on the grid. The grid was kept in liquid nitrogen within the fixation unit until the cryotransfer device was in place. The grid was immediately transferred to a specimen holder within the cryotransfer unit. At no time was the grid exposed to ambient temperatures and care was taken to dry and cool all tools before handling the grid [14].

The grid was transferred to a Zeiss 10C STEM electron microscope equipped with a Gatan 126 cold stage. The stage and anticontaminator were kept at 120

K and 110–119 K, respectively, with liquid nitrogen. Regions of thin vitreous ice were observed with an acceleration voltage of 60–80 kV. Care was taken not to damage the sample with high beam intensities. The vesicles were readily observed in the vitreous layer [14].

Results

The first set of experiments was designed to demonstrate the ability of A23187 to mediate the uptake of iron into EPC LUVs in response to a pH gradient (inside acidic) and to determine the specificity for the Fe^{2+} or Fe^{3+} species. As shown in Fig. 1, iron presented as $FeSO_4$, and maintained in the ferrous state by the presence of ascorbate (10 mM), was readily accumulated by the LUVs to achieve high interior concentrations of approx. 250 nmol/μmol phospholipid under the experimental conditions employed. Assuming an interior trapped volume of 1.5 l/mol phospholipid, this corresponds to an interior Fe^{2+} concentration of 167 mM, as compared to the (initial) exterior Fe^{2+} concentration of 2 mM. As also shown in Fig. 1, Fe^{3+} (presented as $FeCl_3$) was not accumulated, indicating that A23187 was effectively unable to transport the ferric form.

In order to determine the appropriate concentration of A23187 for Fe^{2+} transport, a variety of A23187 concentrations were employed and uptake monitored as shown in Fig. 2. A convenient time course for uptake was observed at 2.5 μg/ml A23187, corresponding to an initial rate of Fe^{2+} transport of 30 nmol/min per μmol phospholipid. This may be compared to a rate of approx. 4 nmol Ca^{2+}/min per μmol phospholipid in the presence of 0.1 mg/ml A23187 observed under the same experimental conditions [1], and reflects the less efficient transport of Fe^{2+} by the Ca^{2+} ionophore.

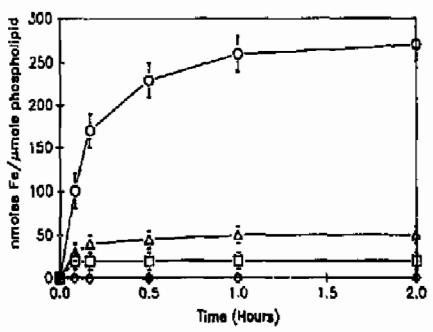

Fig. 1. Time-course of the accumulation of Fe^{2+} (○) and Fe^{3+} (◇) into EPC LUVs (100 nm diameter) experiencing a transmembrane pH gradient, inside acidic ($pH_i = 4.0$; $pH_o = 7.5$) in the presence of A23187 (10 μg/ml). The initial external iron concentration was 2 mM. The EPC LUVs (3 mM) were prepared in 300 mM citrate buffer (pH 4.0) and the untrapped (exterior) buffer exchanged for 150 mM NaCl, 20 mM Hepes (pH 7.5). The ferrous (Fe^{2+}) species was provided as $FeSO_4$ and was maintained in the reduced state by the presence of ascorbate (10 mM). Control vesicles exhibit no pH gradient $pH_i = pH_o = 7.5$ (△) and $pH_i = pH_o = 4.0$ (□).

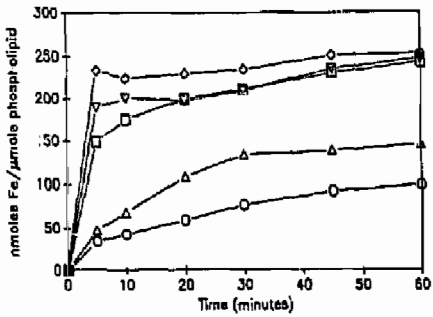

Fig. 2. Influence of the A23187 concentration on the accumulation of Fe^{2+} into EPC LUVs (100 nm diameter) experiencing a transmembrane pH gradient, inside acidic ($pH_i = 4.0$; $pH_o = 7.5$). The Fe^{2+} was kept in the reduced state by the presence of ascorbate (10 mM). For other conditions see Fig. 1, and Methods. The A23187 concentrations employed were 0.5 μg/ml (○); 1.0 μg/ml (△); 2.5 μg/ml (□); 5 μg/ml (▽); 10 μ/ml (◇).

As indicated in the Introduction one of the objectives of this investigation was to achieve high encapsulation levels of a cation such as iron, resulting in LUV systems which, due to their higher gravimetric density could be readily pelleted by centrifugation, or due to their higher electron density could be more readily visualized by electron microscopy techniques. It is likely that Fe^{2+} uptake proceeds via a Fe^{2+}-$2H^+$ exchange, and thus higher internal buffer concentrations should result in higher levels of accumulated Fe^{2+}. Entrapment levels were therefore monitored for internal citrate concentrations up to 1 M for a variety of external Fe^{2+} concentrations. As shown in Fig. 3A, the amount of accumulated Fe^{2+} increases as the concentration of entrapped citrate is increased until a plateau region is reached, which is dependent on the (initial) exterior Fe^{2+} concentration. As indicated in Fig. 3B, the point at which the plateauing effect is observed coincides with trapping efficiencies of 80% or more, and thus reflects the lack of available exterior Fe^{2+} to import. The maximum uptake levels achieved (250 nmol/μmol phospholipid) were observed for 1 M entrapped citrate and 10 mM (initial) exterior Fe^{2+} concentrations.

As shown elsewhere [3] the trapped volume of LUVs generated by extrusion increases significantly as the vesicle size increases. Increased uptake (on an iron/phospholipid basis) could therefore be expected for larger systems, due to an increased buffering capacity. This effect is shown in Table I. The small increase in accumulated Fe^{2+} per μmol lipid observed for di-

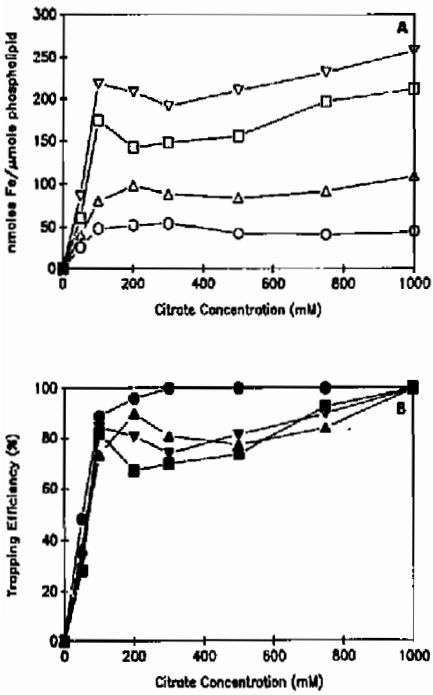

Fig. 3. (A) Effect of varying the external iron concentration and internal citrate concentration on the amount of Fe^{2+} accumulated into 100 nm EPC vesicles with an acidic interior ($pH_i = 4.0$; $pH_o = 7.5$). Iron concentrations of 1 (○), 2 (△), 5 (□) and 10 (▽) mM were used. The concentration of A23187 used was 5 μg/ml and the temperature was 23°C. (B) Effect of varying external iron concentrations and internal citrate concentrations on the trapping efficiency of Fe^{2+} uptake into 100 nm EPC vesicles.

ameters above 200 nm is primarily due to the increasingly multilamellar character of such liposomes [3]. The gravimetric properties of the 100-nm and 200-nm diameter systems, as reflected by pelleting properties on centrifugation (15000 × g, 20 min) are summarized in

TABLE I

Effect of liposome size on iron entrapment

The mean vesicle diameter was determined after loading vesicles containing 300 mM citrate with Fe^{2+} (2 mM initial external concentration) for 2 h at 23°C in the presence of 10 μg/ml A23187 ($n = 3$).

Filter pore size (nm)	Liposome diameter (nm)	Iron uptake (nmol Fe^{2+}/ μmol phospholipid)
50	73 ± 23	176 ± 4
100	93 ± 31	181 ± 2
200	164 ± 72	347 ± 15
400	244 ± 97	387 ± 2

TABLE II

Gravimetric properties of EPC LUVs loaded with iron

Uptake conditions: initial interior citrate concentration 1.0 M.; initial external iron concentration 10 mM; accumulation time 2 h at 23°C; A23187 concentration 10 μg/ml. The results presented are the means of three separate experiments. SPN indicates supernatant; PLT indicates pellet formed following centrifugation at 15 000 × g for 20 min ($n = 3$). n.d., not determined.

	Filter pore size (nm)	Lipid (%)	Iron (%)	Iron to lipid ratio (nmol Fe^{2+}/ μmol lipid)
SPN	100	97.3 ± 3.0	98.2 ± 3.5	271 ± 50
PLT	100	2.7 ± 1.0	1.8 ± 0.6	487 ± 80
SPN (control)	100	100	n.d.	-
PLT (control)	100	0	n.d.	-
SPN	200	53.5 ± 5.9	34.6 ± 3.6	630 ± 30
PLT	200	46.5 ± 5.3	65.4 ± 7.3	1370 ± 85
SPN (control)	200	85.0 ± 3.6	n.d.	-
PLT (control)	200	15.0 ± 2.6	n.d.	-

Table II. It may be observed that the increased densities of the 100-nm Fe^{2+}-loaded systems are not sufficient to result in significantly increased pelleting on low speed centrifugation, however, the 200-nm diameter Fe^{2+}-loaded systems do exhibit significantly increased proportions in the pellet following centrifugation. It may also be noted that the iron-to-lipid ratios in the pelleted fraction are significantly greater than for the LUVs remaining in the supernatant, indicating a heterogeneity in Fe^{2+} accumulation.

The 200-nm diameter EPC LUVs loaded with Fe^{2+}identified in Table II constitute a significant step towards satisfying one of the initial aims of this investigation, namely to prepare LUV systems which can be readily isolated by low-speed centrifugation. It is of interest, however, to extend these studies to elements of higher atomic weight, which could exhibit improved density characteristics. In this regard, it was found that A23187-mediated, pH gradient dependent loading of barium into EPC LUVs could be readily achieved (results not shown). The larger atomic weight of barium (137.3) compared to iron (55.8) would be expected to result in more dense LUVs, assuming comparable levels of loading. This was observed, as shown in Table III for the 100-nm diameter EPC system, as Ba^{2+}-loaded LUVs exhibit improved pelleting properties upon low speed centrifugation in comparison to the 100 nm diameter Fe^{2+}-loaded systems (Table II).

A final area of investigation concerned the influence of acummulated Fe^{2+} or Ba^{2+} on the electron density of LUV systems as visualized by cryo-electron mircoscopy. The stability of the LUVs containing Fe^{2+} or Ba^{2+} was of concern in these experiments given possible lytic events that could occur prior to or during the vitrification process. In this regard, the presence of

TABLE III

Gravimetric properties of EPC LUVs loaded with barium (100-nm pore size)

Ba^{2+} uptake conditions: 2 h at 60°C with 20 mM external barium, 50 μg/ml A23187 ($n = 3$).

	Supernatant	Pellet
Lipid–(controls) [b]	93.8 ± 2.2%	6.2 ± 0.6%
Lipid	79.4 ± 5.7%	20.6 ± 3.8%
Barium	61.1 ± 5.1%	38.9 ± 13.7%
nmol Ba/μmol lipid	462 ± 60	984 ± 344
Vesicle diameter (nm)	110 ± 30	130 ± 36

[b] 100-nm vesicles that contained no barium.

long chain saturated phospholipids and cholesterol is well known to increase the stability and decrease leakage from liposomal systems. This is illustrated in Fig. 4 for entrapped Fe^{2+} and Ba^{2+}. As shown in Fig. 4A over a 24 h time course (23°C), considerable leakage of entrapped Fe^{2+}, following the rapid initial uptake, is observed for EPC LUVs. In contrast, little leakage is observed over a 24 h time course at 65°C following uptake of Fe^{2+} into DSPC/cholesterol (55 : 45, mol/mol) LUVs (Fig 4B). Similar stability is also observed for DSPC/cholesterol LUVs loaded with Ba^{2+}, as illustrated in Fig 4C. Cryo-electron microscopy studies on the Fe^{2+}-loaded systems revealed slightly improved contrast resulting from a uniform darkening of the LUV interior (results not shown). However, the Ba^{2+}-loaded systems exhibited remarkably improved contrast as illustrated in Fig. 5. It is likely that the electron dense particles observed entrapped within the LUVs correspond to crystalline barium citrate precipitates.

Discussion

The studies reported here establish the ability of A23187 to mediate ΔpH dependent loading of Fe^{2+} and Ba^{2+} into LUVs with an acidic interior. Features of interest concern the mechanism of the A23187 loading process, the physical state of the loaded Fe^{2+} and Ba^{2+} and the utility of the dense liposomes that can be thus generated. With respect to the ability of A23187 to mediate the transbilaver transport of iron, it was first reported by Young et al. [8] that A23187 could facilitate the transbilayer transport of iron in model (liposome) membranes and red blood cells. The results presented here show that A23187 transports the ferrous form and that extremely high levels of Fe^{2+} entrapment (170 mM) can be achieved employing the pH gradient approach. The ability of A23187 to bind Ba^{2+} has been reported previously, where the affinity of A23187 for Ba^{2+} is at least 100-fold less than that observed for Ca^{2+} [13]. This corresponds to the reduced ability of A23187 to transport Ba^{2+} as compared to Fe^{2+} (Fig. 4). Even though the affinity of A23187 for Ba^{2+} is small, substantial amounts can be loaded into liposomes (330 mM interior concentration) with a transmembrane pH gradient (inside acidic).

With regard to mechanism, it is likely that the A23187 mediated process involves the exchange of Fe^{2+} and Ba^{2+} for two H^+ ions, as observed for Ca^{2+}

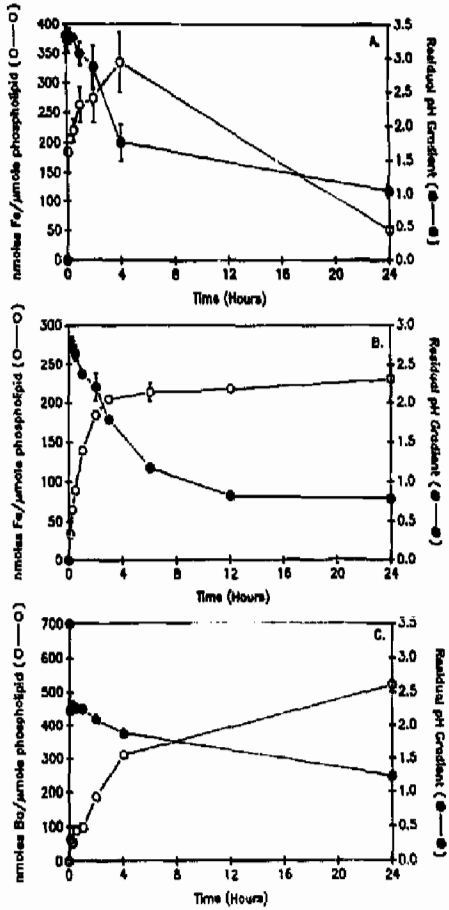

Fig. 4. Loading and retention of Fe^{2+} in (A) EPC LUVs and (B) DSPC/cholesterol (55:45, mol/mol) LUVs. The LUVs were prepared by extrusion through filters with 100 nm pore size and exhibited a pH gradient ($pH_i = 4.0$; $pH_o = 7.5$). The internal buffer was 300 mM citrate and the initial external iron (Fe^{2+}) concentration was 2.0 mM. The experimental temperature was 65°C for the DSPC/cholesterol LUVs and 23°C for the EPC LUVs. Part (C) illustrates the retention of Ba^{2+} following loading into 100 nm DSPC/cholesterol (55:45, mol/mol) vesicles (65°C). The internal buffer concentration was 1.0 M citrate and the external barium concentration was 10 mM.

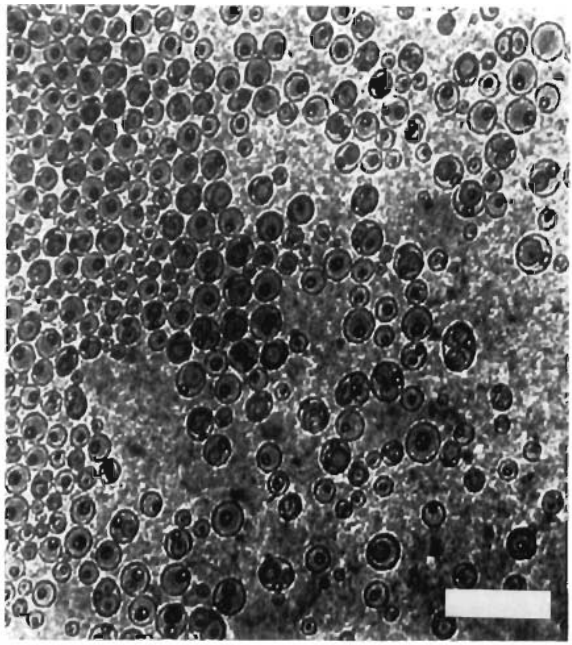

Fig. 5. Cryo-electron micrograph of 100 nm DSPC/cholesterol (55:45; mol/mol) vesicles containing barium which was loaded using A23187 and a transmembrane pH gradient (pH_i = 5.0; pH_o = 7.4) at 60°C for 2.5 h (see Methods). The internal buffer was 600 mM citrate and the (initial) external barium concentration was 12 mM. The barium was visualized by intensity contrast, while the membranes were visualized by phase contrast. The scale bar represents 500 nm. The entrapped barium concentration was 212 nmol Ba^{2+} per μmol phospholipid.

[1]. What is less clear is the form of the accumulated material. The observation of electron dense regions in the Ba^{2+}-loaded systems suggests the formulation of barium citrate precipitates.

LUVs with enhanced densities as evidenced by gravimetric properties and higher electron densities giving rise to greater contrast by electron microscopy have a variety of important potential uses. The ability to isolate LUVs from serum components in vitro, for example, would be of considerable utility in identifying serum proteins which associate strongly with liposomes and which may play a role in clearance processes in vivo [12]. Alternatively, enhanced EM contrast could allow the direct detection of extracellular and intracellular LUVs in vitro and in vivo. Such an ability would be of considerable utility in assaying the targeting of liposomes, as well as determining their integrity and metabolic fate.

In summary, the results of this work establish the A23187-mediated loading procedure into LUVs with an acidic interior as a relatively general method of exchanging protons entrapped in LUVs for cations of considerably higher atomic weight. The enhanced gravimetric densities of these systems result in potential applications in LUV isolation from complex media, whereas the enhanced electron densities can result in direct visualization of LUVs employing electron microscopy.

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