Preparation of Calcium-Loaded Liposomes and Their Use in Calcium Phosphate Formation

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Liposome encapsulation technology has been used to entrap aqueous calcium salts within dipalmitoylphosphatidylcholine lipid vesicles, which were then used to form calcium phosphate minerals. The calcium encapsulation efficiency was found to depend upon a number of factors that included calcium salt concentration, vesicle size, lipid concentration, and method of vesicle preparation. Removal of unencapsulated calcium by ion exchange resulted in calcium-loaded liposome suspensions with calcium concentration as high as 85 mM. Addition of inorganic phosphate to the calcium-loaded liposomes resulted in liposome suspensions that, although highly supersaturated with respect to calcium phosphates, exhibited metastability as a result of the physical separation of calcium (intravesicular) and phosphate (extravesicular) ions. Calcium ion release and mineral formation was activated by a bilayer-to-micelle mesophase transformation induced by the addition of lipid surfactant. Characterization of the mineral product by infrared spectroscopy, X-ray diffraction, and electron microscopy revealed the formation of apatite and brushite mineral under basic and acidic conditions, respectively. The potential use of calcium-loaded liposomes for in situ formation of mineral for medical and dental applications is discussed.

Introduction

Phospholipids are one of the major building blocks of biological membranes which, together with membrane proteins and cholesterol, control cell shape, compartmentalize reactions, and mediate many cell functions such as the storage of compounds, ion transport, cell fusion, and metabolism. In the presence of water, phospholipids spontaneously aggregate into ordered lyotropic liquid-crystalline phases, the form of which depends upon intrinsic factors such as the nature of the lipid headgroup, the length and degree of unsaturation of the acyl chains, and extrinsic factors such as temperature, pH, concentration, and the presence of solutes and other lipids.[1] A common and biologically important lipid mesophase is the lamellar bilayer,[2] exemplified by the ubiquitous biological cell membrane. Over 30 years ago Bangham discovered that phospholipids spontaneously form closed spherical or oval structures in aqueous solutions where one or more phospholipid bilayers entrap solvent within the interior.[3] Vesicles composed of naturally occurring or synthetic phospholipids are often referred to as liposomes; because of their similarity to biological membranes, liposomes have been used extensively in basic research to model cell membranes and to study cell fusion, membrane properties, and transport.[4]

Liposomes have also been extensively investigated as pharmaceutical carrier/delivery systems, an application in which the protective nature of the phospholipid bilayer is exploited to deliver a molecule or therapeutic agent to a specific site (e.g., an organ or tumor) in vivo.[5] Liposomes are ideal for this purpose because they can entrap a wide range of solutes including drugs, contrast agents, genetic material, enzymes, and other biomolecules.[6] Liposomal formulations of antimicrobial agents (amphotericin B), anticancer agents (doxorubicin), contrast agents for diagnostic imaging (Mn, Fe, Gd, In complexes), and blood substitutes (hemoglobin) have been extensively investigated, are in clinical trials, or are on the market in some countries.[5]

The lipid bilayer membrane of liposomes serves to define two discontinuous and isolated aqueous phases, namely, the interior volume of the vesicle and the extravesicular space. The barrier and selective transport properties of the phospholipid bilayer control the flow of reactive species from one phase into the other and can result in significant compositional (i.e., chemical) differences between the two phases of the same aqueous suspension. The internal volume of a lipid vesicle can therefore be considered a microreactor within which controlled chemical reactions can be performed.[7]

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The defined reaction space within lipid vesicles has been used by a number of investigators to study the formation of clusters, nanometer-sized inorganic particles, and biominerals.[8−12] Typically, inorganic ions encapsulated within the vesicles are allowed to react with suitable ions of opposite charge permeating at a controlled rate through the vesicle membrane. In these studies, the vesicles have been used to provide nucleation sites, control reaction kinetics and the size, geometry, and aggregation properties of the products. Nanoscale particles of oxides,[8,9c] hydroxides,[9a,b] and semiconductor particles[10] have been synthesized using this technique.

Eanes and co-workers[11,12] utilized liposomes to model the mineral precipitation sequence believed to occur during matrix vesicle calcification. Matrix vesicles are extracellular lipid membrane-bound bodies reported to be the initial site of mineral deposition in certain tissues.[13] As models of matrix vesicles, Eanes and co-workers prepared phosphate-loaded liposomes by encapsulating inorganic phosphate solutions within the aqueous cores of liposomes constructed from phosphatidylcholine, dicetyl phosphate, and cholesterol.[11] These liposomes were then made permeable to external Ca^{2+} using a calcium ionophore. Apatite mineral readily formed inside liposomes made permeable to Ca^{2+}, and also outside of vesicles when intraliposomally formed HA crystals penetrate the surrounding lipid bilayer membrane and seed crystallization in the external medium.

The primary functional characteristic of liposomes that render them useful for drug delivery technology (i.e., their ability to encapsulate, protect, and deliver reagents) can potentially be exploited to deliver inorganic minerals such as calcium phosphates for use in medicine and dentistry. Our strategy for accomplishing this is to take advantage of liposomes to encapsulate aqueous $CaCl_2$ and to physically isolate this reagent from an extravesicular inorganic phosphate solution. The physical barrier imposed by the vesicle membrane prevents the reaction of Ca^{2+} and phosphate ions, even at "global" supersaturation levels normally sufficient to give immediate mineral formation of aqueous calcium phosphate solutions. Our goal is to control, through alteration of membrane barrier properties, the mixing and subsequent reaction of Ca^{2+} and inorganic phosphate to form calcium phosphate minerals. Ultimately, we seek to design Ca-loaded lipid vesicles that rapidly release Ca^{2+} in response to an applied stimulus (light, heat, etc.) for the formation of calcium phosphate minerals in situ.[14] Here we describe the preparation of Ca-loaded liposomes and the processing parameters that maximize Ca^{2+} encapsulation efficiency. We also demonstrate, through the use of a lipid surfactant, how disruption of the lipid bilayer of Ca-loaded vesicles can be used to rapidly form calcium phosphate minerals. The type of mineral formed by this reaction can be modified by controlling the pH of the reaction medium.

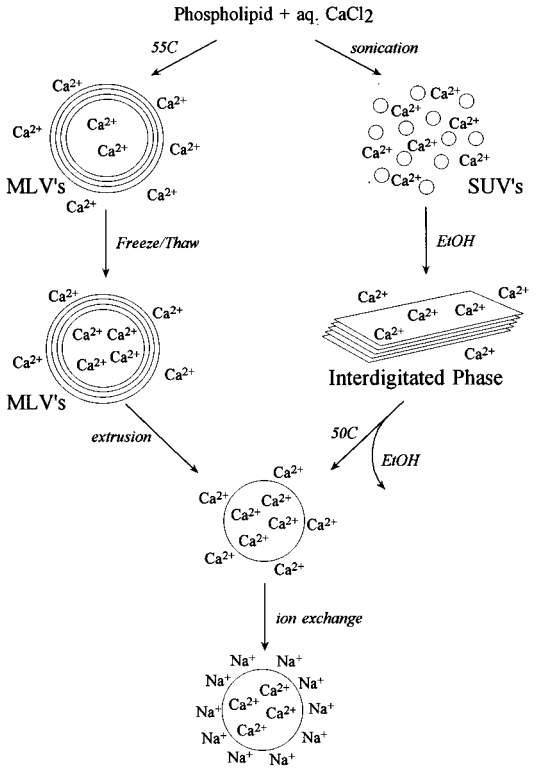

Figure 1. Schematic illustration of the processing steps utilized in the formation of Ca-loaded liposomes by conventional (left) and interdigitation-fusion (right) approaches. Both methods yield Ca-loaded liposomes suspended in aqueous NaCl.

Materials and Methods

Materials. 1,2-Bis(palmitoyl)-*sn*-glycero-3-phosphocholine (DPPC, >99%) was obtained from Avanti Polar Lipids and used as received. The purity of the lipid was confirmed by thin-layer chromatography, yielding a single spot on silica gel plates (Merck) using a mobile phase of $CHCl_3$:CH_3OH:H_2O (65:25:4). Reagent grade $CaCl_2 \cdot 2H_2O$ and Na_2HPO_4 were obtained from Fisher Scientific and used as received. All other materials were of reagent grade or better and used as received.

Liposome Preparation and Characterization. Ca-loaded phospholipid vesicles were prepared using two different approaches as illustrated in Figure 1. In the conventional approach,[15] multilamellar vesicles (MLVs) were first formed by hydrating a dry thin film of DPPC in a round-bottom flask with unbuffered aqueous calcium chloride at 55 °C for 30−60 min. Gentle vortexing during hydration yielded a milky-white lipid suspension. Following vesicle formation, multiple freeze/thaw cycles from −80 to 55 °C were used to maximize encapsulation efficiency and equilibrate the intra- and extraliposomal solution concentrations.[16] Typically, the MLV suspensions were then passed through a liposome extruder (Liposofast Basic, Avestin Inc.) to yield large unilamellar vesicles (LUVs) of variable size. Vesicle diameters were controlled using nuclear track membranes (Nucleopore) with pore diameters ranging from 100 to 400 nm. Lipid concentrations ranged from 1 to 200 mg/mL, whereas the concentration of the aqueous $CaCl_2$ varied from 0 to 200 mM.

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Alternatively, Ca-loaded vesicles were made by the interdigitation-fusion (IF) approach (Figure 1).[17] Briefly, a dry thin film of DPPC was hydrated with aqueous $CaCl_2$ for 30 min at 50−55 °C. The resulting MLV suspension was sonicated at 55 °C until optically clear using a 2 mm diameter probe-type ultrasonicator (Heat Systems, frequency 25 kHz), forming small unilamellar vesicles (SUVs). The SUV suspension was cooled to room temperature and centrifuged to remove metal particles released from the probe tip, and then an isothermal amount of absolute ethanol (4 M) was added with stirring. Upon EtOH addition the suspension became white and highly viscous, indicating formation of a lamellar interdigitated phase.[17] After incubation at room temperature for 15 min, the suspension was heated to 50 °C and then bubbled with dry N_2 for 30 min to remove EtOH, yielding Ca-encapsulated interdigitation-fusion vesicles (IFVs).

Unencapsulated Ca^{2+} was removed from the extravesicular fluid by exposing the LUV/IFV suspensions to an ion-exchange resin (Amberlight IR-120+, Na form) at room temperature for 30 min. The amount of Ca^{2+} remaining in the liposome suspensions after ion exchange was determined by atomic absorption spectroscopy (AAS) using an Instrumentation Laboratory Video 22 spectrophotometer. 1−10 μL aliquots of the liposome suspensions were diluted with deionized water to within the linear range of the instrument (0−5 μg/mL), and absorbance measured in the presence of 1% La^{3+} to eliminate phosphate interference. Two or three readings were taken for each sample; standard deviations of such measurements were determined to be within 5% of the mean. A calibration curve for absorbance versus [Ca^{2+}] was obtained using standard solutions of known calcium concentration. Vesicle size distributions were determined by dynamic light scattering using an argon ion laser and Brookhaven Instruments BI-9000 correlator. The turbidities of LUV/IFV suspensions were determined at λ = 400 nm on a Perkin-Elmer 55B spectrophotometer.

Mineral Formation from Ca-Loaded Vesicles. For mineralization experiments, inorganic phosphate was introduced into the extravesicular space following removal of unencapsulated Ca^{2+} and then allowed to react with encapsulated Ca^{2+} by dissolving the vesicle membrane using a lipid surfactant, Triton X-100. Following determination of Ca^{2+} concentration of the liposome suspension by AAS, solid Na_2HPO_4 was added to the liposome suspension to achieve a Ca/P molar ratio of 1.5. The pH of the liposome suspension during mineralization reactions was controlled by adding Tris-HCl (100 mM, pH 7.4) or PIPES (100 mM, pH 6.5) prior to surfactant addition, or was unbuffered. At room temperature and with stirring, Triton X-100 (25% solution in H_2O) was then added to achieve a surfactant/lipid mole ratio of 3. The suspension cleared considerably within 30 min of surfactant addition, resulting in a slightly cloudy suspension. This suspension was left unstirred for 24 h at room temperature and then centrifuged at 3000 rpm and the clear supernatant decanted. The white pellet was washed twice with distilled water, and extracted twice in ethanol and twice in $CHCl_3$ before drying in vacuo at room temperature.

Fourier transform infrared (FT-IR) spectra of the mineral products were obtained on KBr pellets using a Perkin-Elmer Paragon 1000 FT-IR instrument, at resolution of 4 cm^{-1}. Powder X-ray diffraction (XRD) was performed using a Siemens D5000 diffractometer with Cu Kα radiation (λ = 1.54 Å). Transmission electron microscopy (TEM) was performed on a JEOL 100S microscope operating at 80 kV. Mineral powder was ultrasonically dispersed in acetone and pipeted onto a Formvar-coated copper grid and air-dried prior to imaging.

Results

The first step in the formation of Ca-loaded liposomes by the conventional approach involves the incubation of DPPC in aqueous $CaCl_2$ (Figure 1) at temperatures above the gel (L_β) to liquid-crystal (L_α) phase transition temperature. During incubation, spontaneous formation of MLVs occurs, encapsulating a portion of the hydrating solution. Maximum encapsulation efficiency was obtained only after subjecting the MLV suspension to multiple freeze−thaw cycles, which serves to equilibrate intra- and extravesicular calcium salt concentration and maximize encapsulated volume.[16] Liposomes resulting from this process have a broad size distribution but are typically large, with significant fractions greater than 500 nm in diameter. When desired, LUVs of smaller diameter were obtained by extrusion through nuclear track membranes of controlled pore size (Table 1). Shown in Figure 2 are vesicle size distributions for LUVs obtained by extrusion through 100 and 400 nm membranes. Mean vesicle diameters were found to be slightly smaller than the diameter of the membrane used for extrusion.

Table 1. Relationship between Membrane Pore Diameter and Mean Vesicle Diameter[a] of Conventionally Prepared (Extruded) Ca-Loaded LUVs

extrusion pore diam (nm)	mean vesicle diam[a] (nm)
400	351
200	163
100	80

[a] Determined by dynamic light scattering.

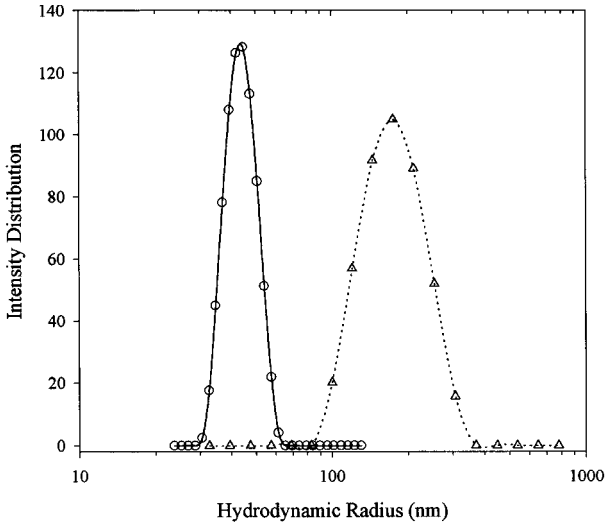

Figure 2. Dynamic light-scattering vesicle size distributions of liposomes prepared by the conventional method and extruded through Nucleopore membranes with pore diameters of 100 nm (○) and 400 nm (△).

To illustrate the removal of unencapsulated Ca^{2+} from the extravesicular space, aliquots of the liposome suspensions were taken at various times during ion exchange and Ca^{2+} concentration determined. As shown in Figure 3, in the absence of lipid (aqueous $CaCl_2$ only) the ion-exchange resin removes most of the available Ca^{2+} from solution during the first 5 min, and nearly quantitatively during the 30 min exchange period. While the Ca^{2+} concentration in liposome-containing suspensions was also found to decrease during the first 5 min, a plateau in Ca^{2+} concentration was reached that was vesicle size dependent. The Ca^{2+} remaining in the liposome suspensions after ion exchange corresponds to that which is encapsulated and therefore protected from the ion-exchange reaction by the barrier properties of

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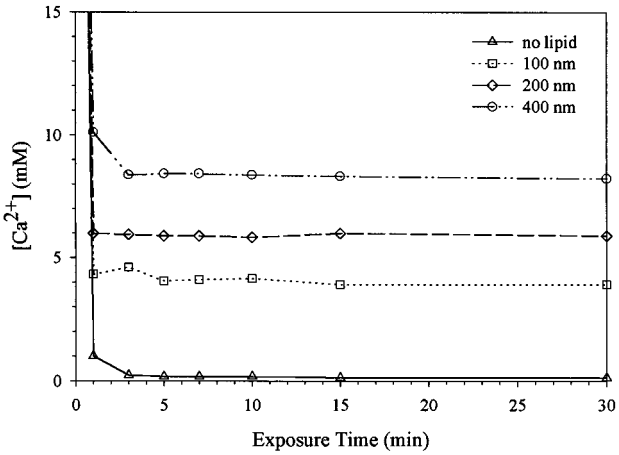

Figure 3. Calcium concentration during ion exchange of 50 mM aqueous $CaCl_2$ (△) and conventionally prepared liposome suspensions ([DPPC] = 25 mg/mL; [$CaCl_2$] = 50 mM) extruded through 400 (○), 200 (◊), and 100 nm (□) membranes.

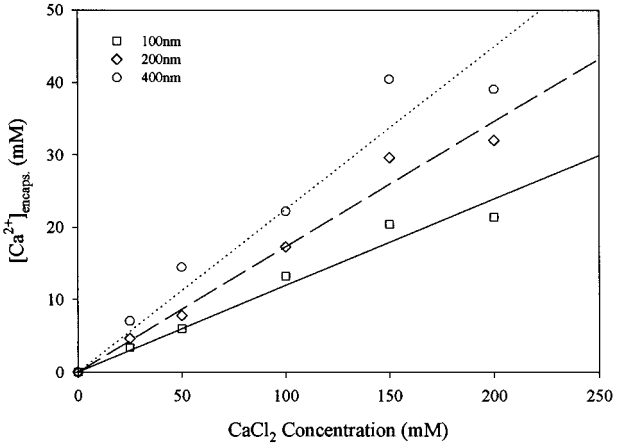

Figure 4. $[Ca^{2+}]_{encaps}$ versus concentration of $CaCl_2$ hydrating solution for conventionally prepared ([DPPC] = 50 mg/mL) liposomes extruded through 400 (○), 200 (◊), and 100 nm (□) membranes. Samples were ion exchanged for 30 min prior to calcium determination.

the vesicle membrane. The Ca^{2+} concentration of the liposome suspension after ion exchange, hereafter designated $[Ca^{2+}]_{encaps}$, was found to increase with increasing vesicle size. For unilamellar vesicles this is an expected result due to the larger encapsulated volume (per mole of lipid) of larger vesicles.[18]

The relationship between $[Ca^{2+}]_{encaps}$ and concentration of the aqueous $CaCl_2$ solution used to form the liposomes is shown in Figure 4. $[Ca^{2+}]_{encaps}$ was found to increase in an essentially linear fashion with $CaCl_2$ concentration between 0 and 200 mM. The dependence of $[Ca^{2+}]_{encaps}$ on vesicle size can also be seen in Figure 4, with larger vesicles yielding higher $[Ca^{2+}]_{encaps}$ regardless of $CaCl_2$ concentration. With conventional liposome processing, calcium encapsulation efficiency was found to decrease significantly above [$CaCl_2$] = 200 mM.

The dependence of $[Ca^{2+}]_{encaps}$ on DPPC concentration was demonstrated by preparing Ca-loaded vesicles by the conventional approach at DPPC concentrations of 0−200 mg/mL and then measuring $[Ca^{2+}]_{encaps}$ after 30 min of ion exchange. $[Ca^{2+}]_{encaps}$ was found to increase linearly with DPPC concentration as shown in Figure 5. An increase in the viscosity of the liposome suspension with increasing DPPC concentration was observed; suspensions with DPPC concentrations greater than 100 mg/mL were highly viscous. Liposome suspensions with DPPC concentrations greater than 250 mg/mL could not be prepared by the conventional method due to ion-exchange difficulties.

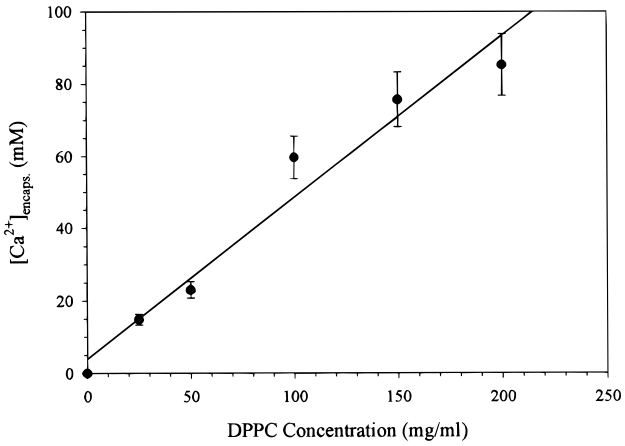

Figure 5. $[Ca^{2+}]_{encaps}$ versus DPPC concentration for conventionally prepared liposomes ([$CaCl_2$] = 200 mM) extruded through a 200 nm Nucleopore membrane. Samples were ion exchanged for 30 min prior to calcium determination.

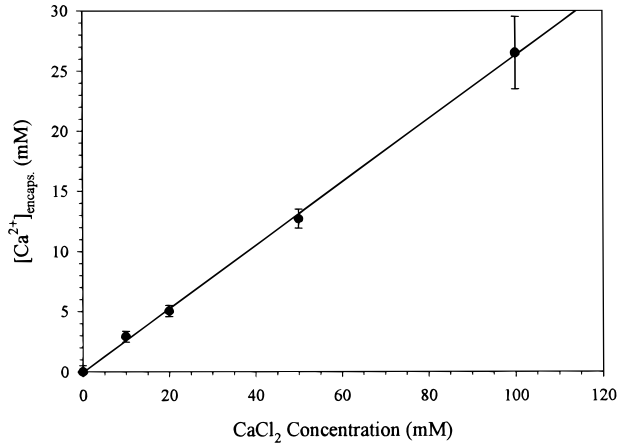

Figure 6. $[Ca^{2+}]_{encaps}$ versus concentration of $CaCl_2$ hydrating solution for liposomes prepared by the interdigitation-fusion method ([DPPC] = 20 mg/mL). Samples were ion exchanged for 30 min prior to calcium determination.

Ca-loaded vesicles prepared using the IF approach were more efficient in encapsulating calcium than those prepared by the conventional method. This is illustrated in Figure 6, which shows $[Ca^{2+}]_{encaps}$ as a function of the concentration of the $CaCl_2$ solution used to form the IFVs. For example, when the data in Figure 6 are compared to the encapsulation data for conventionally prepared liposomes (Figure 4), it can be seen that similar values of $[Ca^{2+}]_{encaps}$ could be obtained by the IF technique at DPPC concentrations only 40% of that required by conventional processing. This result may be due to the large mean diameter (1−6 μm) of vesicles produced by IF processing.[17]

Release of Ca^{2+} from the liposome interior upon surfactant addition can be seen in Figure 7, which shows the time-dependent Ca^{2+} concentration of a convention-

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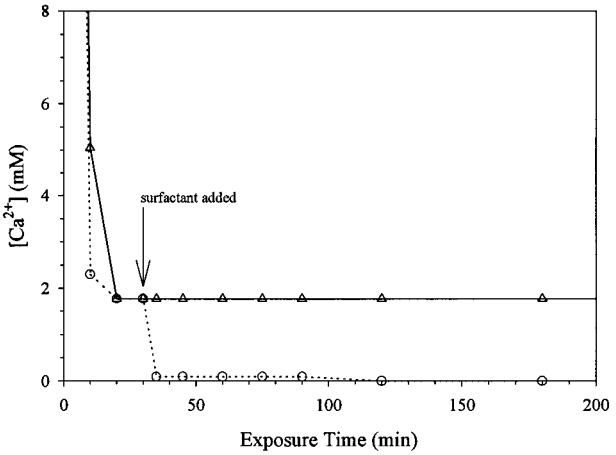

Figure 7. Calcium concentration versus time of exposure to ion-exchange resin. A conventionally prepared liposome suspension ([DPPC] = 25 mg/mL; [$CaCl_2$] = 50 mM) was divided into two portions and exposed to ion-exchange resin continuously for over 3 h. After 30 min at room temperature, surfactant was added to one sample (○) at a surfactant/lipid molar ratio of 3. Both suspensions remained in contact with the ion-exchange resin for the entire experiment.

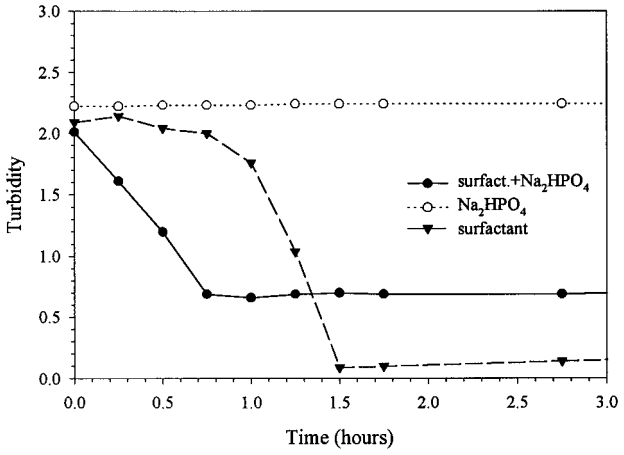

Figure 8. Optical turbidity (λ = 400 nm) of Ca^{2+}-loaded liposome suspension containing extravesicular PO_4^{3-}. Lipid surfactant (Triton X-100) was added at time $T = 0$ in sufficient quantity to cause dissolution of the lipid bilayer, release of entrapped calcium, and mineral formation (●). Identical suspensions to which only surfactant (▼) or Na_2HPO_4 (○) was added became clear or remained unchanged, respectively. [DPPC] = 100 mg/mL; hydrating solution = 200 mM $CaCl_2$; $[Ca^{2+}]_{encaps}$ = 72 mM; $[PO_4^{3-}]$ = 48 mM.

ally prepared liposome suspension in contact with ion-exchange resin. As described above, an initial rapid decrease in Ca^{2+} concentration occurred, corresponding to the removal of unencapsulated Ca^{2+} from the extravesicular space. Following the initial drop in Ca^{2+} concentration, a nonzero value of $[Ca^{2+}]$ was reached, which corresponded to encapsulated Ca^{2+}. When this suspension is left in contact with the ion-exchange resin, little change in $[Ca^{2+}]$ occurred over a period of several hours, indicating the protective nature of the liposome membrane. However, when lipid surfactant was added to the suspension Ca^{2+} concentration rapidly fell to near zero, indicating the release of Ca^{2+} from the liposomes and subsequent removal of Ca^{2+} from solution due to ion exchange.

Ca-loaded vesicles were used to form calcium phosphate minerals by adding inorganic phosphate to the extravesicular space of an ion-exchanged liposome suspension and then mixing this suspension with a lipid surfactant (Triton X-100) to dissolve the vesicle membrane and allow mixing of Ca^{2+} and phosphate ions. The formation of mineral particles was followed by monitoring changes in the turbidity of vesicle suspensions containing extravesicular phosphate (Ca/P = 1.5); the formation of mineral particles in the suspension are expected to give rise to increased suspension turbidity. Time-dependent turbidity changes of a Ca-loaded liposome suspension of initial $[Ca^{2+}]_{encaps}$ = 72 mM were monitored following the addition of Na_2HPO_4 and surfactant to the extravesicular space (Figure 8). Control experiments in which either Na_2HPO_4 or surfactant alone was added to the Ca-loaded liposomes were used to demonstrate the stability of the phosphate-containing liposomes and the dissolution of vesicle membranes, respectively.

In the absence of extravesicular phosphate, addition of lipid surfactant to the Ca-loaded liposome suspension resulted in dissolution of the liposomes and formation of a low turbidity suspension containing mixed lipid/surfactant micelles (dashed line in Figure 8).[19] When Na_2HPO_4 was added to the extravesicular space but not surfactant (dotted line in Figure 8), minimal changes in turbidity were observed for up to 24 h, even though the resulting suspension was highly supersaturated with respect to apatite and brushite ($[Ca^{2+}]$ = 72 mM; $[PO_4^{3-}]$ = 48 mM). Provided the phosphate-containing liposome suspension was kept at room temperature, no mineralization occurred within at least the first 24 h after phosphate addition; this was determined by centrifugation followed by decantation of the supernatant (no pellet was obtained).

When surfactant was added to the liposome suspension containing Na_2HPO_4 in sufficient quantity to dissolve all lipid, turbidity decreased during the first 45 min but the suspension did not become optically clear (solid line in Figure 8). Dissolution of the vesicle membrane in this case resulted in release of entrapped Ca^{2+}, which further reacted with phosphate ions to form mineral; it is the mineral particles that account for the residual turbidity of the surfactant-treated liposome suspension. Centrifugation of the surfactant-treated liposome suspension yielded a solid white pellet, which after washing and lipid extraction was characterized by infrared spectroscopy, X-ray diffraction, and electron microscopy.

In unbuffered suspensions, the pH of the suspension decreased following surfactant addition, indicating liberation of acid as a result of mineral formation. In buffered suspensions, the pH of the liposome suspensions remained within 0.1 pH unit of the original value (7.4 for Tris-HCl and 6.5 for PIPES). Control of the pH during surfactant addition provided a means for controlling the type of mineral formed. IR spectra of the mineral products of liposome mineralization reactions were obtained, and peak absorption frequencies of major infrared absorption bands of mineral obtained at pH 7.4 and 6.5 are listed in Table 2. The spectrum of mineral formed at basic pH had major absorption bands char-

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Table 2. Infrared Absorption Frequencies and Assignments of Mineral Products

pH of reaction medium	band freq (cm^{-1})	assignment[a]
7.4	3417.9	O−H stretch
	1030.5	P−O stretch
	602.7	O−P−O bend
	565.5	O−P−O bend
6.5	3540.2	(H−)O−H stretch
	3489.1	(H−)O−H stretch
	3289.3	(H−)O−H stretch
	3164.5	(H−)O−H stretch
	1649.3	H−O−H bend
	1215.9	P−O−H bend
	1135.6	P−O stretch
	1065.9	P−O stretch
	990.3	P−O stretch
	871.5	P−O(H) stretch
	793.6	P−O−H bend
	575.8	O−P−O bend
	529.9	O−P−O bend

[a] Assignments according to ref 20.

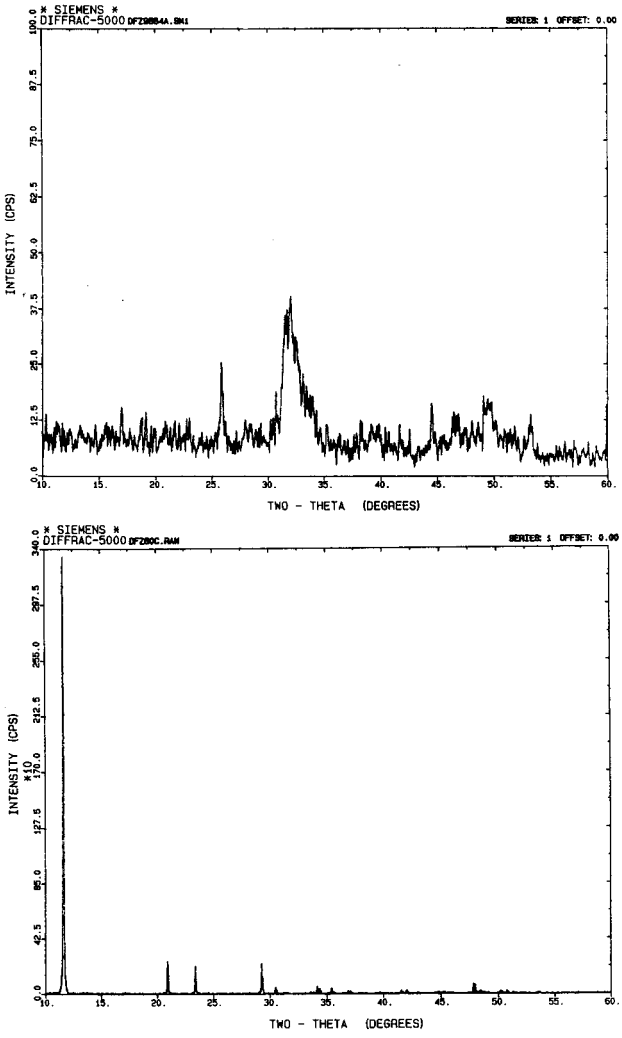

Figure 9. Powder XRD patterns of mineral obtained by surfactant addition to metastable liposome suspension buffered to pH 7.4 (top) and 6.5 (bottom).

acteristic of poorly crystalline apatite.[20] However, mineral formed from liposomes under acidic conditions yielded an infrared spectrum similar to brushite ($CaHPO_4 \cdot 2H_2O$).[20] Powder X-ray diffraction (Figure 9) confirmed the identity of the mineral phases formed under basic and acidic conditions as apatite and brushite, respectively. Transmission electron images of the apatite mineral revealed platelike crystals typical of apatite crystals synthesized from aqueous solutions at low temperatures (Figure 10) and are in many respects similar in size and shape to apatite crystals extracted from bone.[21] TEM images of the brushite mineral could not be obtained at 80 kV due to in situ dehydration of the crystals and formation of monetite ($CaHPO_4$). Scanning electron micrographs of the brushite mineral revealed large platelike crystals (Figure 11).

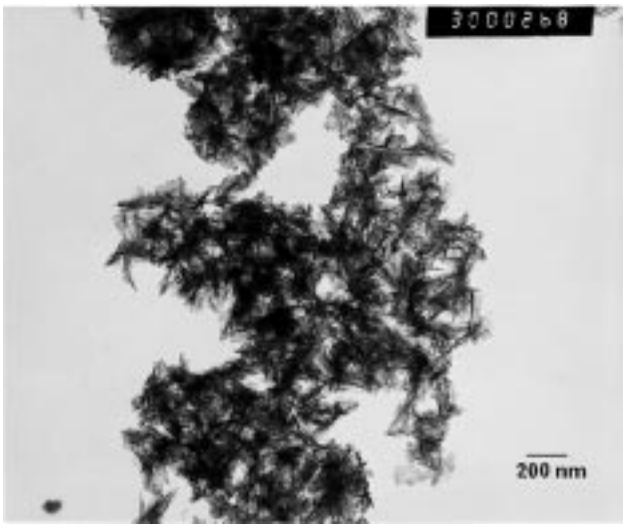

Figure 10. Transmission electron micrograph of mineral obtained by surfactant addition to a metastable liposome suspension buffered to pH 7.4.

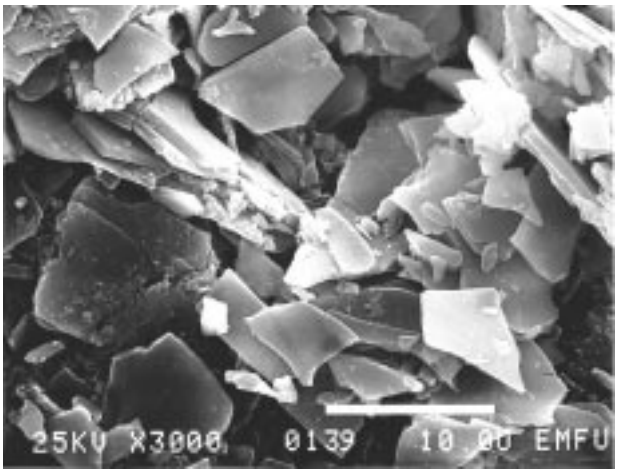

Figure 11. Scanning electron micrograph of mineral obtained by surfactant addition to a metastable liposome suspension buffered to pH 6.5.

Discussion

The precursors chosen for mineral formation were simple water-soluble calcium and phosphate salts, which are well suited for use with established liposome processing approaches.[6] As shown schematically in Figure 1, both conventional and IFV approaches were used to sequester aqueous $CaCl_2$ within large unilamellar vesicles. The effect of liposome preparation conditions on Ca^{2+} encapsulation efficiency is of particular

(21) Posner, A. S. *Bone Miner. Res.* **1987**, *5*, 65.

importance, since the amount of mineral formed by this process will ultimately depend upon the total amount of Ca^{2+} encapsulated within the liposomes. As shown in Figures 3−6, the amount of Ca^{2+} encapsulated within DPPC liposomes depends upon a number of factors, including the concentration of the $CaCl_2$ hydrating solution, DPPC concentration, vesicle size, and method of preparation. Although the highest value of Ca^{2+} concentration (85 mM) was obtained in liposomes prepared by the conventional method, liposomes prepared using the IF approach were much more efficient in encapsulating Ca^{2+}. As shown in Figure 6, liposome suspensions processed by the IF approach required 60% less lipid than the conventional approach to achieve similar values of $[Ca^{2+}]_{encaps}$. Although we did not determine the size distribution of vesicles processed by the IF approach, others have reported the average diameters of IFVs to be greater than 1 μm.[17] Therefore, it is likely that the increased Ca^{2+} encapsulation efficiency of IF vesicles is due to larger vesicle size, compared to the conventionally prepared (extruded) vesicles. Ongoing experiments are aimed at determining the optimum IFV processing conditions for high efficiency Ca^{2+} encapsulation.

The removal of unencapsulated Ca^{2+} from the extravesicular space by ion exchange (Figure 3) is an important step in the processing of Ca-loaded vesicles. Without this step, the introduction of inorganic phosphate to the liposome suspension would result in high supersaturation with respect to many calcium phosphate minerals and would result in immediate mineral precipitation in the extravesicular space. After removal of unencapsulated Ca^{2+}, addition of sodium phosphate to liposome suspensions having $[Ca^{2+}]_{encaps}$ equal to or greater than 80 mM results in highly supersaturated conditions with respect to apatite, brushite, and other calcium phosphates.[22] However, due to the sequestering of Ca^{2+} ions within the liposomes reaction of Ca^{2+} and phosphate is prevented, resulting in metastability.

The use of surfactant to induce Ca^{2+} release was intended only as a model system to demonstrate the potential for triggering mineralization in the metastable liposome suspension. Surfactant partitions to and dissolves the phospholipid bilayer, forming mixed micelles of surfactant and lipid.[19] Typically, the bilayer-to-micelle transition induced by surfactant addition is accompanied by a large decrease in optical density (dotted line in Figure 8), which is due to the small size of micelles in comparison to the wavelength of visible light.[19] When surfactant was added to a Ca-loaded liposome suspension containing inorganic phosphate, the net effect of liposome membrane dissolution was the mixing of calcium and phosphate coupled with mineral formation as a result of high supersaturation. The mineral particles formed in this process were responsible for the residual turbidity observed in the suspension after surfactant addition (solid line in Figure 8).

The measured changes in turbidity of a liposome suspension following surfactant addition are the result of a multistep kinetic process which involves (1) dissolution of surfactant into the aqueous phase, (2) partitioning of surfactant to the lipid bilayer, (3) saturation of the lipid bilayer with surfactant, and (4) transformation of the mixed surfactant/lipid bilayer to mixed micelles.[23] Further inspection of the data shown in Figure 8 reveals an accelerated bilayer-to-micelle transition in the presence of inorganic phosphate. Although the source of this effect is at the present time unknown, the accelerated vesicle dissolution may be due to a cooperative effect between phosphate ion and surfactant, or it could possibly be due to interactions between mineral, surfactant, and lipid.

It is also worth noting that significant differences may exist between the kinetics of Ca^{2+} release (and consequently mineral formation) and the bilayer-to-micelle transition as measured by turbidity changes. Evidence for this is apparent in Figure 7, in which it can be seen that release of Ca^{2+} from liposomes is nearly complete within 5 min after surfactant addition. The apparent discrepancy between rapid Ca^{2+} release (Figure 7) and relatively slow liposome dissolution (Figure 8) can be explained by considering that encapsulated solutes can escape from holes or gaps in the lipid bilayer long before full dissolution of the bilayer is complete. For example, holes in otherwise intact liposomes could form in small areas of surfactant-rich bilayer that have transformed to micelles, leaving behind gaps in the vesicle membrane from which Ca^{2+} can escape. Thus, in the surfactant-induced mineralization experiments, it is likely that Ca^{2+} release and mineral formation began long before the process of bilayer-to-micelle transformation was complete.

By controlling the pH of the reaction medium during surfactant addition, we were able to produce either poorly crystalline apatite (pH > 7.4) or brushite (pH < 7.0) by buffering to the desired pH prior to surfactant addition. For suspensions that were initially basic but were unbuffered, a mixture of both apatite and brushite phases were obtained (data not shown), which we attribute to a drop in pH as a consequence of acid liberated during mineral formation. The ability to control the type of mineral formed, as well as its formation kinetics, may be important in future applications of this technology medicine and dentistry.

The use of liposomes to form calcium phosphate minerals in situ for medical/dental use would constitute a significant departure from the traditional use of liposomes as delivery vehicles for organic pharmaceutical agents, biomolecules, and imaging contrast agents.[4−6] Previous investigations utilizing liposomes to study calcium phosphate mineralization were primarily intended to model in vivo conditions present during mineralized tissue formation and focused mainly on the role of acidic phospholipids in matrix vesicle mineralization.[11,12] An important distinction between this study and previous liposome mineralization studies is the use of Ca-encapsulated liposomes as opposed to phosphate-encapsulated liposomes. Furthermore, our primary goal is not to duplicate in vivo mineralization conditions or to study the sequence of events occurring during vesicle-mediated mineralization but rather to determine the potential utility of liposomes as delivery vehicles for in situ formation of calcium phosphate minerals. Calcium phosphate minerals, in particular hydroxyapatite (HAP,

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(23) Lichtenberg, D.; Robson, R. J.; Dennis, E. A. *Biochim. Biophys. Acta* **1983**, *737*, 285.

$Ca_{10}(PO_4)_6(OH)_2$), brushite ($CaHPO_4 \cdot 2H_2O$), octacalcium phosphate ($Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$), and amorphous calcium phosphate, are of interest because of their presence in mineralized skeletal tissue and due to their successful history as medical and dental implant materials.[24]

The significant results of this study are that stable, highly supersaturated calcium phosphate suspensions can be prepared using liposome encapsulation technology and that the relatively impermeable liposome membrane prevents reaction of sequestered Ca^{2+} with extravesicular phosphate until a significant change in bilayer permeability or integrity occurs. In this paper we have utilized a model approach for inducing mineral formation from metastable liposome suspensions, namely, that of surfactant-induced bilayer-to-micelle transition. In addition to this approach, we are also actively pursuing other mechanisms for inducing Ca^{2+} release and mineral formation. One strategy that may be more useful in a clinical setting is to utilize liposomes designed to release their contents under the influence of an applied stimulus, such as light, heat, or a change in pH. In a companion paper, we report on a thermally activated liposomal mineralization system in which calcium release and mineral formation are activated by a temperature change (e.g., from ambient to body temperature).[14] We believe such a system could be used to rapidly form calcium phosphates in situ for remineralization of dentin, enamel, or bone.

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