Thermally Triggered Calcium Phosphate Formation from Calcium-Loaded Liposomes

Phillip B. Messersmith*,† and Steven Starke

Departments of Restorative Dentistry and Bioengineering, University of Illinois at Chicago, Chicago, Illinois 60612

Received April 22, 1997. Revised Manuscript Received October 15, 1997[⊗]

A thermally triggered liposome-based mineralization system is described that is metastable at ambient temperature but rapidly forms calcium phosphate mineral upon warming to physiologic temperature. Mixing of a calcium-loaded lipid vesicle suspension with aqueous inorganic phosphate resulted in a stable liposome suspension whose bulk ionic concentration was highly supersaturated with respect to hydroxyapatite and other calcium phosphate minerals. The mineralization activity of metastable liposome suspensions was found to be strongly dependent both on temperature and on the composition of the phospholipids that comprise the vesicle membrane. No detectable mineral formation occurred in the metastable liposome suspensions during storage for several weeks at room temperature. However, when the liposome suspensions were heated to near the lipid chain melting transition (T_m), Ca^{2+} was released from the lipid vesicles and reacted with extravesicular phosphate to form calcium phosphate mineral. Infrared spectroscopy and powder X-ray diffraction analysis of the reaction products indicated the formation of both apatite and brushite minerals, a finding that is consistent with the changing pH conditions of thermally triggered mineralization. Mixtures of miscible phospholipids (dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine) were used to tailor T_m to physiologic temperature. This strategy was employed for the preparation of metastable liposome suspensions that were stable for long periods of time at room temperature but that mineralized rapidly when heated to 37 °C. The potential medical and dental significance of thermally triggered liposomal mineralization is discussed.

Introduction

Liposomes, spherical vesicles consisting of one or more phospholipid bilayers enclosing an aqueous phase, have been extensively investigated as pharmaceutical carrier/delivery systems for the administration of drugs, genetic material, enzymes, and contrast agents.[1,2] The major functional characteristic of liposomes that makes them useful for this purpose is their ability to isolate, protect, and deliver reagents entrapped within the enclosed volume of the vesicle. The membrane of the liposome, composed of one or more phospholipid bilayers, serves as an effective barrier to prevent drug release during transport to a targeted site. The ability to target liposomal drug delivery to a specific organ or tissue has been a major goal of liposome research over the past decade.[2]

While the concept of utilizing lipid vesicles for the synthesis of inorganic solids of controlled shape and/or size is well-established,[3−6] to the best of our knowledge the use of liposomes for delivery or in situ formation of biomaterials remains relatively unexplored. We have recently embarked on a series of studies whose aim is to take advantage of lipid vesicles to compartmentalize reactive species for the purposes of inducing the rapid in situ formation of polymer hydrogels, inorganic solids, and other materials for medical and dental applications.[7,8] In a previous report,[8] we described the preparation of Ca-loaded lipid vesicles and their use in forming calcium phosphate minerals. Our general strategy for liposome-mediated mineralization is to exploit the lipid bilayer membrane of calcium-loaded phospholipid vesicles to physically isolate concentrated calcium (intravesicular) and phosphate (extravesicular) solutions. Although highly supersaturated with respect to hydroxyapatite (HAP) and other calcium phosphate phases, the resulting liposome suspensions do not immediately mineralize due to the compartmentalization provided by the liposome membrane, which physically segregates calcium and phosphate ions. Previously

†Current address: Division of Biological Materials, Northwestern University, Chicago, IL 60611. Phone: (312)503-1789. E-mail: philm@nwu.edu.

⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1997.

(1) Ostro, M. J., Ed. *Liposomes. From Biophysics to Therapeutics*; Marcel Dekker: New York, 1987.
(2) Gregoriadis, G. *Trends Biotechnol.* **1995**, *13*, 527.
(3) Fendler, J. H. *Membrane Mimetic Chemistry*; Wiley: New York, 1982.
(4) Fendler, J. H. *Chem. Rev. (Washington, D.C.)* **1987**, *87*, 877.
(5) (a) Mann, S.; Williams, R. J. P. *J. Chem. Soc., Dalton Trans.* **1983**, *311*. (b) Mann, S.; Hannington, J. P.; Williams, R. J. P. *Nature* **1986**, *324*, 565. (c) Mann, S.; Hannington, J. P. *J. Colloid Interface Sci.* **1988**, *122*, 326.
(6) Bhandarkar, S.; Bose, A. *J. Colloid Interface Sci.* **1990**, *135*, 531. (b) Bhandarkar, S.; Bose, A. *J. Colloid Interface Sci.* **1990**, *139*, 541. (c) Yaacob, I. I.; Bhandarkar, S. *J. Mater. Res.* **1993**, *8*, 573.
(7) Cui, H.; Messersmith, P. B. *Polym. Prepr.* **1997**, *38*, 616−7.
(8) Messersmith, P. B.; Vallabhaneni, S.; Nguyen, V. *Chem. Mater.* **1998**, *10*, 107.

S0897-4756(97)00252-4 CCC: $15.00
Published on Web 01/01/1998

we utilized a surfactant-induced bilayer-to-micelle mesophase transition to release encapsulated calcium, which reacted with extravesicular phosphate to form calcium phosphate mineral.[8] In this paper we describe the preparation of thermally sensitive calcium-loaded liposomes designed to rapidly release Ca^{2+} at the lipid chain melting transition and the use of these liposomes for thermally triggered calcium phosphate mineral formation. Ultimately, our goal is to develop a liposome-containing gel, paste, or solution that is stable at or below room temperature but that deposits mineral at a controlled rate (rapid or slow as needed for a particular application) upon warming from ambient to body temperature. Such a material may be useful for the repair of skeletal tissues such as bone, enamel, and dentin.

Materials and Methods

Materials. 1,2-bis(palmitoyl)-sn-glycero-3-phosphocholine (DPPC, >99%) and 1,2-bis(myristoyl)-sn-glycero-3-phosphocholine (DMPC, >99%) were obtained from Avanti Polar Lipids and used as received. The purity of the lipids was confirmed by thin-layer chromatography, yielding a single spot on silica gel plates (Merck) developed 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.

Liposome Preparation. Calcium-loaded phospholipid vesicles were prepared by the interdigitation-fusion (IF) approach,[9] as described previously.[8] A 0.2 M $CaCl_2$ solution was used for preparation of the IF vesicles (IFVs). Unless otherwise noted, the lipid concentration of the IFV suspensions was 20 mg/mL. Unencapsulated Ca^{2+} was removed from the extravesicular fluid by washing the IFV suspension with excess aqueous NaCl (300 mM), centrifuging (16000*g* for 3 min), and decanting the supernatant. This procedure was repeated a minimum of five times to ensure complete removal of unencapsulated $CaCl_2$. After the final wash, the supernatant was tested by atomic absorption spectroscopy (AAS, see below) to confirm the removal of Ca^{2+} from the extravesicular space. The amount of Ca^{2+} remaining in the liposome suspension was then determined by AAS using an Instrumentation Laboratory Video 22 spectrophotometer. Aliquots (1−10 μL) of the liposome suspensions were diluted with deionized water to within the linear range of the instrument (0−5 μg/mL), and absorbance was measured in the presence of 1% La^{3+} to eliminate phosphate interference. A calibration curve for absorbance versus [Ca^{2+}] was obtained using standard solutions of known calcium concentration. Calcium encapsulation efficiency ranged from approximately 60−75% of available Ca^{2+}.[10]

Determination of Lipid Chain Melting Temperature. The chain melting transition (T_m) of DPPC/DMPC vesicles were determined by hydrating vacuum-dried thin films of the lipid mixtures at 50 °C for 30 min. The resulting lipid suspensions (approximately 50 mg lipid/mL) were analyzed on a TA Instruments 910 dynamic scanning calorimeter (DSC) by heating at 2 °C/min. The DSC instrument was calibrated using Gallium metal (T_m = 29.91 °C).

Calcium Release Assay. Temperature-dependent release of Ca^{2+} from liposomes was detected using the Ca^{2+}-sensitive dye arsenazo III (AIII, Aldrich), which undergoes a striking change in color from red to blue upon complexation with Ca^{2+}.[11] After removal of unencapsulated Ca^{2+} from the vesicle suspension, a 10 μL aliquot was transferred to 3 mL of an AIII solution (10 μM AIII in 300 mM NaCl) buffered to pH 7.0 with HEPES (5 mM). Release of Ca^{2+} from the liposomes was followed by measuring the absorption intensity of the 650 nm band as a function of temperature. A circulating water bath and a jacketed quartz cuvette were used to heat the suspensions at a rate of 1 °C/min during measurement.

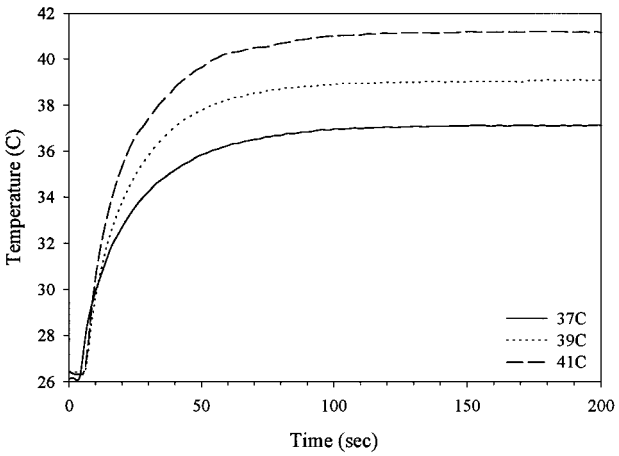

Figure 1. Temperature rise profiles of metastable liposome suspensions immersed in isothermal water baths equilibrated at 37, 39, and 41 °C. Suspensions were unstirred during measurement.

Mineralization Experiments. Temperature-activated mineral formation was detected by measuring time-dependent pH changes of phosphate-containing IFV suspensions held isothermally at various temperatures. 200 μL each of a Ca-loaded IFV suspension (20 mg of lipid/mL) and a Na_2HPO_4 solution (67 mM; pH 8.9) were combined in a glass vial at 25 °C. Initial pH values of the metastable suspensions (prior to heating) ranged from 8.4 to 8.6. After equilibrating the phosphate-containing suspensions at 25 °C for 30 min, they were immersed in an isothermal water bath at temperatures between 33 and 45 °C. Measurement of the sample temperature after immersion yielded temperature rise profiles as shown in Figure 1; approximately 1−2 min elapsed before equilibration at the bath temperature.

Sample temperature, bath temperature, and the voltage output from a calomel pH electrode were simultaneously recorded versus time on a computer data acquisition system. Since the sample temperature was changing during the course of the experiment it was necessary to calculate temperature-corrected pH values, which was accomplished using the following form of the Nernst equation:

$$E = E_x - 0.186T_k(\text{pH}) \quad (1)$$

where E is the electrode output in mV, E_x is an electrode constant (determined at pH 7.0), and T_k is the sample temperature in kelvin. The slope of the equation (−0.186) was determined by two-point calibration using standardized buffer solutions.

Mineral Recovery/Characterization. Mineral was recovered from the liposome suspensions by centrifuging at 16000*g* for 5 min, after which the supernatant was poured off, and the pellet washed twice with NaCl (0.3 M) and twice with distilled water. Following this, the pellet was dispersed in ethanol and centrifuged at 16000*g* for 5 min, and the supernatant poured off. This process was repeated once more with ethanol and twice with chloroform before drying the pellet in vacuo. Infrared spectra of the recovered minerals were obtained as KBr pellets using a Perkin-Elmer Paragon 1000 FT-IR spectrometer. Wide-angle powder X-ray diffraction (XRD) was performed using a Siemens D5000 diffractometer with Cu Kα radiation (λ = 1.54 Å).

Results

The thermotropic phase behavior of liposomes constructed from mixtures of DPPC and DMPC were

(9) Ahl, P. L.; et al. *Biochim. Biophys. Acta* **1994**, *1195*, 237.

(10) Some batch-to-batch variation in Ca^{2+} encapsulation efficiency was observed for liposome preparations; typical calcium concentrations of the liposome suspensions ranged from 135 to 150 mM.

(11) (a) Sokolove, P. M.; Kester, M. B. *Anal. Biochem.* **1989**, *177*, 402. (b) Weissmann, G.; Collins, T.; Evers, A.; Dunham, P. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 510.

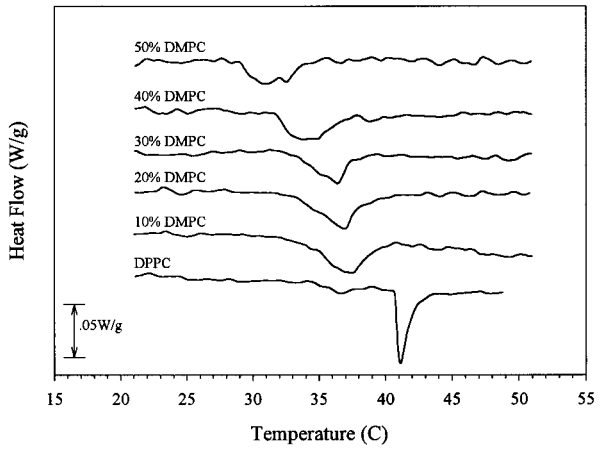

Figure 2. DSC scans of liposome suspensions prepared from mixtures of DMPC and DPPC. Spectra are displaced vertically for clarity.

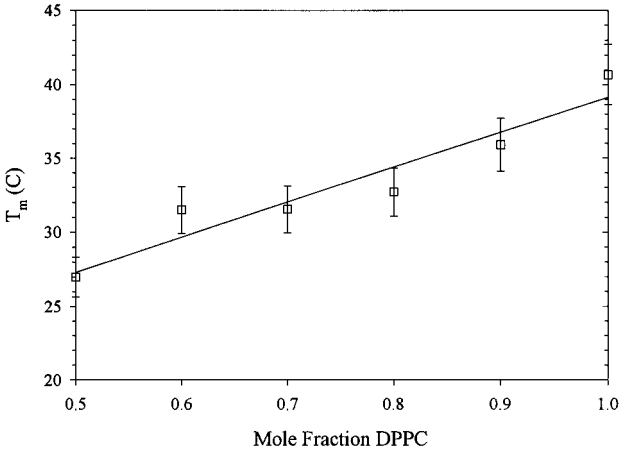

Figure 3. Lipid chain melting temperature (T_m) versus mole fraction DPPC of DPPC/DMPC liposome suspensions.

investigated by DSC, and scans of DPPC-rich vesicle suspensions (>50% DPPC) are shown in Figure 2. Although considerably broadened in DMPC-containing mixtures, a single endothermic event corresponding to the phase transition from gel ($< T_m$) to liquid crystalline ($> T_m$) was observed, suggestive of lipid miscibility.[12] The T_m onset temperatures extrapolated from the DSC scans are plotted versus mole fraction DPPC in Figure 3. As expected, the addition of DMPC to DPPC resulted in lowering of T_m from that of pure DPPC (41 °C), with a nearly linear relationship between DMPC content and T_m. Since our main goal was to design vesicle systems that release calcium for mineral formation at or near body temperature (37 °C), only DPPC/DMPC mixtures containing 0−20 mol % DMPC were selected for further study.

Ca^{2+} release from the liposomes was detected using AIII, which in the absence of Ca^{2+} was found to have a broad absorbance between 450 and 610 nm with a peak maximum of 543 nm, along with a small shoulder peak centered at 630 nm (Figure 4). At room temperature, a dilute suspension of Ca-loaded DPPC liposomes in AIII solution exhibited an absorbance spectrum similar to that of AIII. However, when this suspension was

(12) Mabrey, S.; Sturtevant, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3862.

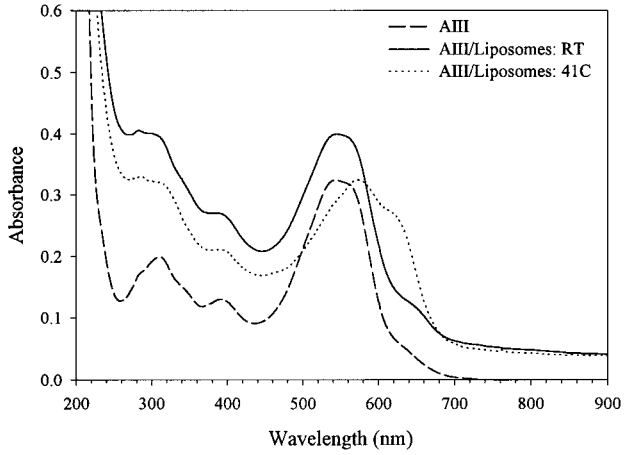

Figure 4. Room-temperature UV−vis spectra of AIII, an AIII/calcium-loaded DPPC liposome suspension, and an AIII/calcium-loaded DPPC liposome suspension after heating to 41 °C for 10 min.

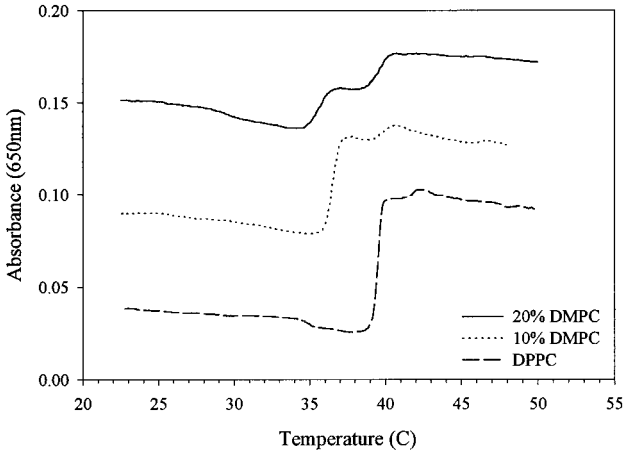

Figure 5. Absorbance at 650 nm of AIII suspensions containing DPPC, 10 mol % DMPC, and 20 mol % DMPC liposomes during heating from room temperature to 50 °C. Suspensions were unstirred during measurement. Plots are displaced vertically for clarity.

heated at the T_m of DPPC (41 °C) for 10 min a substantial change in the absorbance spectrum was noted. The AIII absorbance at 543 nm was significantly attenuated, while new peaks centered at 572 and 611 nm emerged, corresponding to the formation of Ca^{2+}−AIII complex.[11] This result shows that Ca^{2+} is released from DPPC vesicles when heated at the lipid chain melting temperature.

To more fully investigate the correlation between lipid composition, T_m, and Ca^{2+} release, Ca^{2+}-loaded liposome suspensions (0, 10, or 20 mol % DMPC) containing extravesicular AIII were heated from room temperature to 50 °C at 1 °C/min to determine the temperature of Ca^{2+} release. Release of encapsulated Ca^{2+} was detected by an increase in the absorbance at 650 nm corresponding to formation of the Ca^{2+}−AIII complex. As shown in Figure 5, the absorbance of the Ca^{2+}-loaded DPPC liposome suspension remained relatively constant above room temperature, increased dramatically at approximately 39 °C, and remained relatively constant above 40 °C.[13] Several changes in Ca^{2+} release behavior were noted for liposomes constructed from DPPC/DMPC mixtures. For example, the onset of Ca^{2+} release (as

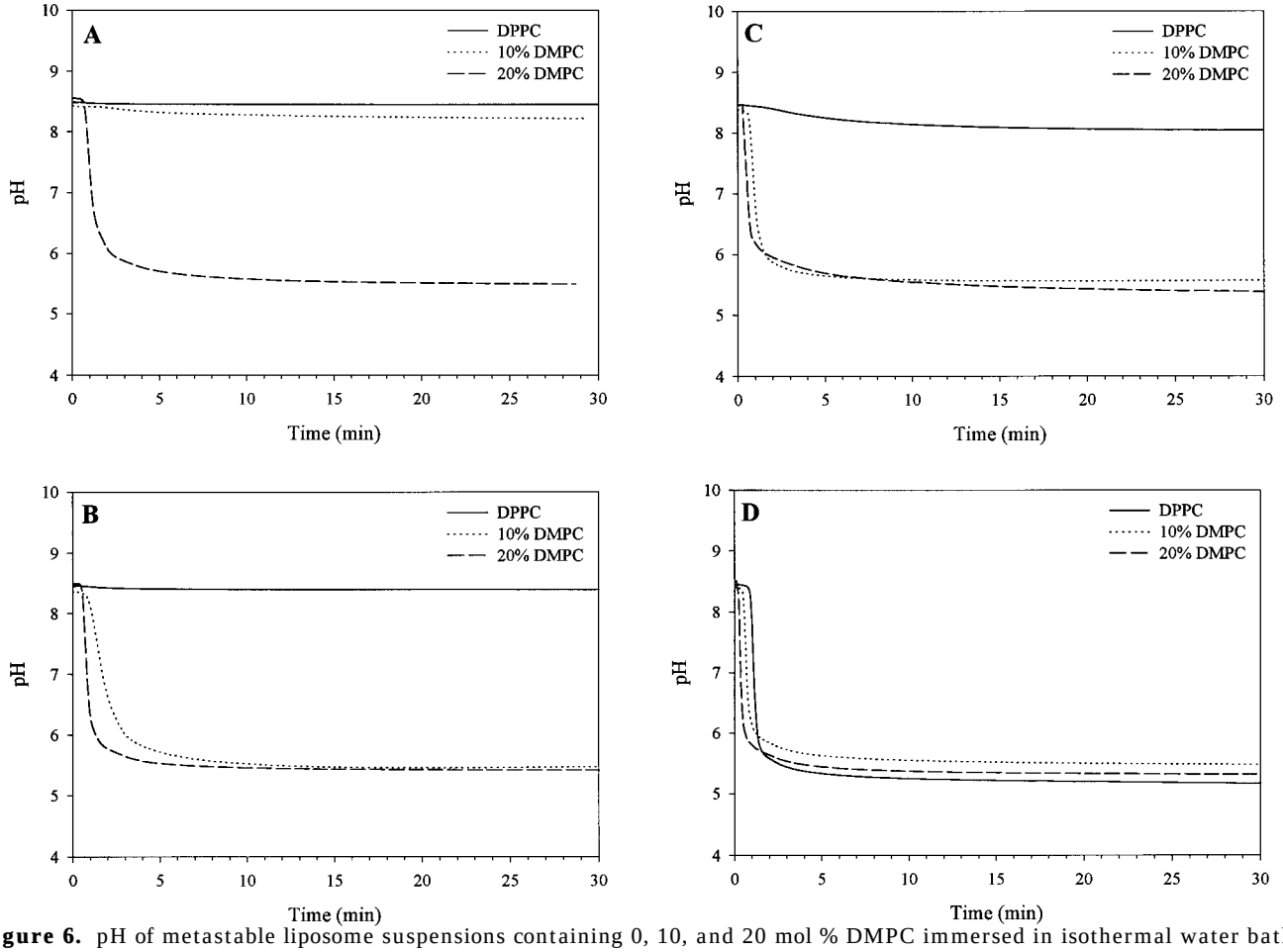

Figure 6. pH of metastable liposome suspensions containing 0, 10, and 20 mol % DMPC immersed in isothermal water baths equilibrated at (A) 35 °C, (B) 37 °C, (C) 39 °C, and (D) 41 °C. Suspensions were equilibrated at 25 °C for 30 min and then immersed in water bath at time = 0. Samples were unstirred during measurement.

detected by the temperature at which the absorbance increases dramatically) was found to be 36 and 34.5 °C for liposomes containing 10 and 20 mol % DMPC, respectively. Furthermore, the DMPC-containing liposomes exhibited a secondary absorbance increase at 38−39 °C. The secondary absorbance increase was of smaller magnitude than the primary increase, but was found to increase in intensity with increasing DMPC content.

In the presence of extravesicular phosphate (and provided the calcium concentration within the vesicles is high enough), release of Ca^{2+} from vesicles will culminate in the formation of hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$) and/or brushite ($CaHPO_4 \cdot 2H_2O$) according to the following idealized equations:

$$10CaCl_2 + 6Na_2HPO_4 + 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 12NaCl + 8HCl \quad (2)$$

$$CaCl_2 + NaH_2PO_4 + 2H_2O \rightarrow CaHPO_4 \cdot 2H_2O + NaCl + HCl \quad (3)$$

Formation of either HAP (eq 2) or brushite (eq 3) will result in liberation of acid and a drop in pH, which we used as an indicator of mineral formation in phosphate-containing liposome suspensions. Equal volumes of a Ca-loaded liposome suspension[10] and a sodium phosphate solution (67 mM) were combined, with the resulting mixtures having initial pH values of 8.4−8.6, which is nearly that of the measured pH of the sodium phosphate solution. Although highly supersaturated with respect to apatite and other calcium phosphate phases,[14,15] the pH of the Ca-loaded vesicle/phosphate suspensions changed only very slowly with time. After 30 min of equilibration at room temperature, the liposome suspensions were immersed (time = 0) in an isothermal water bath equilibrated at various temperatures, and the pH recorded for 30 min. Measurement of the sample temperature during the experiment yielded temperature rise profiles such as those shown in Figure 1. Approximately 2 min was required to fully heat the sample to the bath temperature.

Shown in Figure 6 are plots of pH versus time at 35, 37, 39, and 41 °C for Ca-loaded vesicles made from 0, 10, and 20 mol % DMPC. At 35 °C, the pH of the 20% DMPC suspension dropped nearly 3 pH units within the

(13) Some minor changes in the absorbance at 650 nm were detected in DPPC suspensions at approximately 34 and 43 °C, which are believed to be due to temperature-induced changes in DPPC vesicles. The former is believed to be related to structural changes occurring at the DPPC pretransition, while the origin of the latter is unknown. These minor changes in absorbance are believed to be independent of Ca^{2+} release, as they were observed in control experiments of Ca-loaded DPPC suspensions performed in the absence of AIII (data not shown).

(14) Driessens, F. C. M.; Verbeeck, R. M. H. *Biominerals*; CRC Press: Boca Raton, FL, 1990; pp 37−59.

(15) Nancollas, G. H. In *Biomineralization: Chemical and Biochemical Perspectives*; Mann, S., Webb, J., Williams, R. J. P., Eds.; VCH Publishers: New York, 1989; pp 157−187.

Table 1. Experimental Values of ΔpH_{30} and $t_{80\%}$ for Ca^{2+}-Loaded Liposome/Na_2HPO_4 Suspensions Reacted for 30 min at Various Temperatures[a]

	DPPC		10% DMPC		20% DMPC	
temp (°C)	ΔpH_{30}	$t_{80\%}$ (s)	ΔpH_{30}	$t_{80\%}$ (s)	ΔpH_{30}	$t_{80\%}$ (s)
35	0.03	NA[b]	0.21	780	3.04	118
37	0.06	NA[b]	2.88	172	3.08	76
39	0.43	670	2.81	84	3.09	98
41	3.22	84	2.91	66	3.17	40

[a] ΔpH_{30} = total pH change after 30 min of reaction at the temperature indicated. $t_{80\%}$ = elapsed time at which 80% of the pH change occurred. [b] Calculation of $t_{80\%}$ was not possible due to the small total pH change.

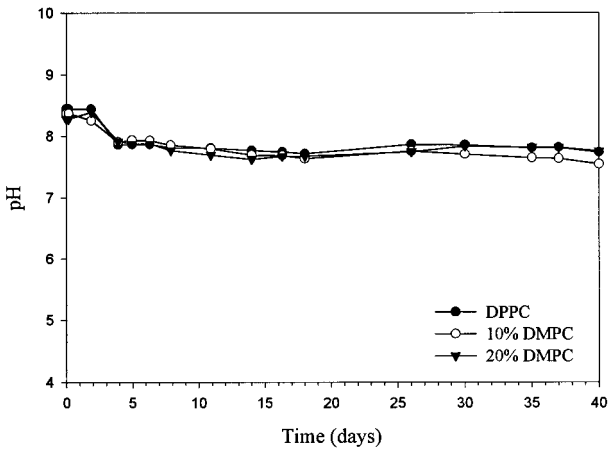

Figure 7. pH of metastable liposome suspensions containing 0, 10, and 20 mol % DMPC during storage at room temperature. Samples were left unstirred during storage but were gently vortexed just prior to pH measurement.

first 2 min, whereas the 10% DMPC suspension fell only 0.2 pH units during the 30 min of the experiment. In contrast, the pH of the DPPC suspension remained virtually unchanged during the same time period. At 37 °C the pH of both the DMPC-containing suspensions dropped nearly 3 pH units during the first 5 min, although the pH of the 20% DMPC suspension fell at a noticeably faster rate than the 10% DMPC suspension. Again however, the pH of the DPPC suspension changed only slightly during the experiment. At 39 °C the pH of both DMPC-containing suspensions dropped rapidly within the first 2 min, whereas the pH of the DPPC suspension dropped approximately 0.4 pH units in 30 min. Finally, at 41 °C the pH of all liposome suspensions studied dropped to less than 6.0 within 2 min of immersion. For comparison purposes, the elapsed time required to reach 80% of the total pH change ($t_{80\%}$) was calculated from each of the curves shown in Figure 6. These values are listed in Table 1.

Long-term pH experiments were also employed to assess the room-temperature stability of the metastable liposome suspensions. Suspensions were prepared as described above and the pH monitored for up to 40 days at room temperature without stirring (Figure 7). During the first 2 days at room temperature there were only minor changes in pH, although between day 2 and day 4 there was a drop in pH of about 0.5 units for all suspensions. Following this initial drop in pH, the suspensions remained relatively stable over a period of several weeks, dropping by an average of only 0.1−0.2 pH units more during this time. No major differences were observed in the behavior of liposome suspensions

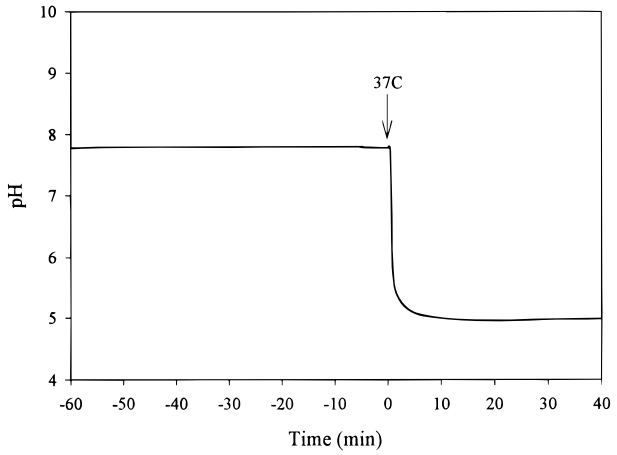

Figure 8. pH of a metastable DPPC/DMPC liposome suspension containing 10 mol % DMPC. Suspension was stored at room temperature for 2 days and then immersed in a 37 °C water bath at time = 0. Sample was unstirred during measurement.

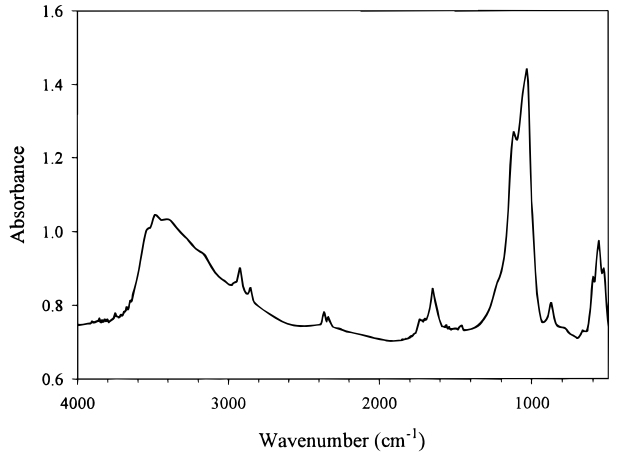

Figure 9. Infrared spectrum of mineral recovered from a metastable liposome suspension (20 mol % DMPC) after isothermal reaction at 37 °C for 30 min.

with respect to lipid composition. Furthermore, when liposome suspensions were aged for long periods of time at room temperature and then heated to T_m, they reacted in a manner identical with that of freshly prepared suspensions in that the pH dropped rapidly within the first few minutes of heating (Figure 8).

Mineral recovered from liposome suspensions after reaction at T_m for 30 min was characterized using infrared spectroscopy (Figure 9) and powder XRD (Figure 10). The infrared spectrum exhibited features characteristic of both apatite and brushite minerals.[16] The spectrum contained the absorption bands typical of a poorly crystalline apatite (absorption bands at 3418, 1032, 600, and 562 cm^{-1}), upon which were superimposed a number of weaker bands characteristic of brushite (absorption bands at 3484, 1651, 875, and 530 cm^{-1}). The presence of both brushite and poorly crystalline apatite was also apparent in the XRD pattern (Figure 10). Although dominated by the crystalline peaks of brushite, the XRD pattern contains a pair of broad, low intensity reflections at approximately 26 and 32° 2θ corresponding to the 002 and 211 reflections of

(16) Bailey, R. T.; Holt, C. In *Calcified Tissue*; Hukins, D. W. L., Ed.; CRC Press: Boca Raton, FL, 1989; pp 93−120.

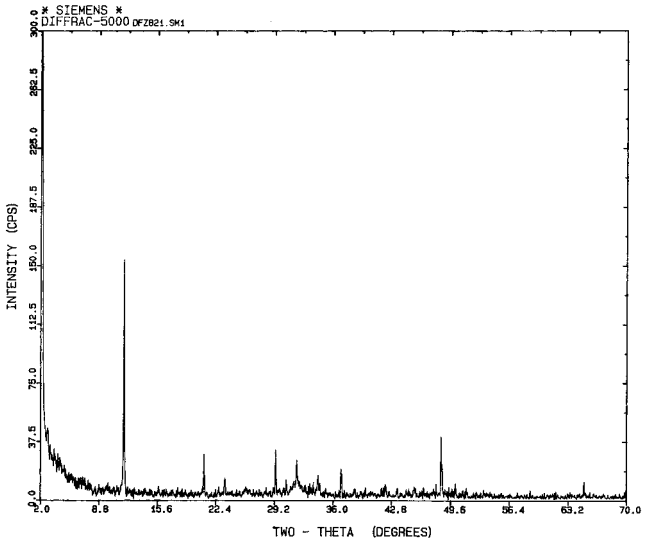

Figure 10. Powder XRD scan of mineral recovered from a metastable liposome suspension (20 mol % DMPC) after isothermal reaction at 37 °C for 30 min.

apatite, respectively. Thus, it is concluded that thermal treatment of metastable liposome suspensions for 30 min at T_m resulted in formation of a mixture of poorly crystalline apatite and brushite.

Discussion

Numerous strategies have been devised to trigger release of encapsulated reagents from liposomes upon exposure to an applied physical or chemical stimulus.[17] Stimuli-sensitive liposomes have been reported in the literature that utilize temperature, pH, or light to effect release of entrapped reagents.[18−22] Many of these systems exploit an increase in membrane permeability associated with a physical or chemical change in the phospholipids as a result of the applied stimulus. An example of this approach is the release of entrapped substances from liposomes at temperatures near the lipid chain melting transition (T_m), a temperature at which bilayer permeability is known to be significantly enhanced.[21,22] This characteristic of lipid bilayers allows the design of drug-filled liposomes that are relatively impermeable to encapsulated molecules at one temperature but highly permeable at another temperature. Liposomes formulated to selectively release their contents a few degrees above physiologic temperature have been used in conjunction with local hyperthermia for targeted drug delivery to tumors.[22]

Our temperature-activated liposome mineralization system (Figure 11) is a variation of this approach that exploits two well-known properties of phospholipid bilayers: (1) the low permeability of lipid bilayers to multivalent ions in the gel state (i.e., $< T_m$),[23−25] and (2) the high permeability of lipid bilayers to small molecules and ions that occurs at T_m.[26−28] These two properties of phospholipid bilayers made it possible for us to construct liposome suspensions that were highly supersaturated with respect to HAP and exhibited two distinct reactivity regimes: a low-temperature ($T < T_m$) "metastable" state in which little or no mineral formation occurs, and a high-temperature ($T = T_m$) reactive state in which mineralization occurs rapidly.

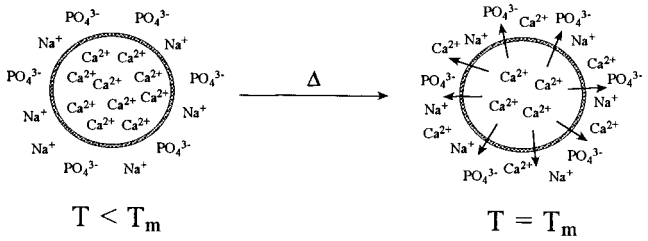

Figure 11. Schematic illustration of thermally triggered liposome mineralization. At temperatures below the lipid chain melting temperature (T_m), the liposome membrane effectively isolates encapsulated calcium from extravesicular phosphate ions. At T_m ion transport permits mixing of calcium and phosphate, which ultimately react to form calcium phosphate mineral.

Metastable State at Temperatures Below T_m. When Ca-loaded liposomes are combined with extravesicular phosphate the high Ca^{2+} and phosphate ion concentrations render the suspension supersaturated with respect to HAP, brushite, and other calcium phosphates.[14,15] Because they do not immediately mineralize, we consider the room-temperature liposome suspensions to be functionally metastable, since release of Ca^{2+} from the vesicles will result in mineral formation according to eqs 2 and 3. The stability exhibited by the room-temperature suspensions against calcium phosphate formation can be understood by considering the nature of the ionic species present, their concentrations both inside and outside of the vesicles, and the permeabilities of the individual ionic species across the lipid bilayer.[29] In the present case the relevant ions include Ca^{2+}, Na^+, H^+, Cl^-, OH^-, and the various orthophosphate species PO_4^{3-}, HPO_4^{2-}, and $H_2PO_4^-$. When the Ca-loaded liposome suspension is initially combined with the Na_2HPO_4 solution due to the high pH (>8), HPO_4^{2-} is the predominant phosphate species, and concentration gradients are established for each species across the liposome membrane (e.g., $[Ca^{2+}]^{in} > [Ca^{2+}]^{out}$; $[HPO_4^{2-}]^{out} > [HPO_4^{2-}]^{in}$, etc.).

Under conditions of constant temperature and pressure the electrochemical potential difference for a spe-

(17) For a recent review, see: Gerasimov, O. V.; Rui, Y.; Thompson, D. H. In *Vesicles*; Rosoff, M. Ed.; Marcell Dekker: New York, 1996; pp 679−746.
(18) Thomas, J. L.; Tou, H.; Tirrell, D. A. *J. Am. Chem. Soc.* **1995**, *117*, 2949.
(19) Frankel, D. A.; Lamparski, H.; Liman, U.; O'Brien, D. F. *J. Am. Chem. Soc.* **1989**, *111*, 9262.
(20) (a) Anderson, V. C.; Thompson, D. H. *Biochim. Biophys. Acta* **1992**, *1109*, 33. (b) Thompson, D. H.; et al. *Biochim. Biophys. Acta* **1996**, *1279*, 25.
(21) Papahadjopoulos, D.; Jacobsen, K.; Nir, S.; Isac, T. *Biochim. Biophys. Acta* **1973**, *311*, 330.
(22) Yatvin, M. B.; Weinstein, J. N.; Dennis, W. H.; Blumenthal, R. *Science* **1978**, *202*, 1290.
(23) Cevc, G. In *Phospholipids Handbook*; Cevc, G., Ed.; Marcel Dekker: New York, 1993; pp 639−661.
(24) Bangham, A. D.; Standish, M. M.; Watkins, J. C.; Weissmann, G. *Protoplasma* **1967**, *63*, 183.
(25) Deamer, D. W.; Bramhall, J. *J. Chem. Phys. Lipids* **1986**, *40*, 167.
(26) Marsh, D.; Watts, A.; Knowles, P. F. *Biochim. Biophys. Acta* **1976**, *858*, 161.
(27) (a) El-Mashok, E. M.; Tsong, T. Y. *Biochemistry* **1985**, *24*, 2884. (b) Ueno, M.; Yoshida, S.; Horikoshi, I. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 1588.
(28) Clerc, S. G.; Thompson, T. E. *Biophys. J.* **1995**, 68, 2333.
(29) Mann, S.; Kime, M. J.; Ratcliffe, R. G.; Williams, R. J. P. *J. Chem. Soc., Dalton Trans.* **1983**, 771.

cies i ($\Delta\tilde{\mu}_i$) existing across a membrane separating two compartments which have a common solvent, is given by

$$\Delta\tilde{\mu}_i = z_i F(\Delta\psi) + RT \ln(c_i^{\text{out}}/c_i^{\text{in}}) \quad (4)$$

where F = the Faraday constant, z_i = the valence of species i, T = temperature, R = the universal gas constant, $\Delta\psi$ is the electrical potential difference across the membrane, and c_i^{in} and c_i^{out} are the concentrations of species i inside and outside of the membrane, respectively.[30]

To a first approximation, Ca^{2+} and HPO_4^{2-} ions can be considered relatively impermeable compared to the other species.[23−25,29] However, diffusion of the more permeable ions such as H^+, Na^+, OH^-, and Cl^- can occur over time, resulting in compositional changes in both the intra- and extravesicular solution. For example, to equalize the difference between intravesicular pH (initially that of 0.2 M $CaCl_2$, approximately 6.3) and extravesicular pH (initially 8.4−8.6) OH^- ions can diffuse down the concentration gradient (i.e., $[OH^-]^{\text{out}} > [OH^-]^{\text{in}}$) and into the vesicle. However, to preserve charge neutrality this movement must be balanced by counter-diffusion of anions, presumably Cl^-. Alternatively, a similar type of exchange between H^+ ($[H^+]^{\text{in}} > [H^+]^{\text{out}}$) and Na^+ ($[Na^+]^{\text{out}} > [Na^+]^{\text{in}}$) ions could occur. Either mechanism will give rise to a reduction in pH of the extravesicular suspension, which is observed during the early stages of room-temperature storage of the liposome suspensions (Figure 7). Eventually an equilibrium will be reached when the electrical potential difference balances the chemical potential difference (i.e., concentration gradient) existing across the membrane so that the total driving force is zero and net diffusion of species across the membrane ceases. In reality, however, Ca^{2+} and HPO_4^{2-} species slowly permeate through the liposome membrane over time, contributing to long-term compositional changes in the intra- and extravesicular compartments. Ultimately, this could lead to supersaturation of the intra- or extravesicular space and mineral formation during storage at room temperature. Other mechanisms that may contribute to mineral formation during storage include liposome rupture as a result of osmotic pressure gradients, hydrolysis of the phospholipids, and room-temperature fluctuations that may increase the permeability of the liposome membrane.

Reactive State at T_m. Lipid bilayer permeability has been observed to be several orders of magnitude higher at T_m than even a few degrees above or below, a phenomenon that has been attributed to the presence of highly permeable interfacial regions between coexisting "solid" ($< T_m$) and "fluid" ($> T_m$) bilayer regions.[26] Although permeability at T_m apparently depends on solute characteristics such as molecular weight and charge,[27] rapid release kinetics (1−2 min to reach equilibrium) have been observed at T_m for small molecular weight solutes.[21,27]

The data shown in Figures 4 and 5 demonstrate that heating Ca-loaded liposomes to T_m results in release of encapsulated Ca^{2+}. For DPPC liposomes, at temperatures well below T_m (for DPPC, T_m = 41 °C) Ca^{2+} leakage was minimal, reflecting the relative impermeability of lipid bilayers to Ca^{2+} ions below T_m. The rate of Ca^{2+} release became significant a few degrees below T_m (39 °C), peaked at approximately 40 °C, and then decreased again above 42 °C. Temperature-dependent Ca^{2+} release was qualitatively similar for DMPC-containing liposomes except that the onset of Ca^{2+} release was lower than pure DPPC, with the magnitude of the drop being related to the mol % DMPC in the lipid mixture. For example, liposomes containing 10 mol % DMPC began releasing Ca^{2+} at 36 °C, or 3 °C less than that of pure DPPC. Likewise, for liposomes containing 20 mol % DMPC the onset of Ca^{2+} release was further reduced to 34.5 °C.

Like Ca^{2+} release, mineral formation in metastable liposome suspensions was found to be strongly dependent on both lipid composition and reaction temperature. For the isothermal mineralization experiments we selected liposomes containing 0, 10, and 20% DMPC because these compositions yielded Ca^{2+} release temperatures near 37 °C. Comparison of the DSC thermal transition data (Figures 2 and 3) with the mineralization data (Figure 6 and Table 1) reveals that, in general, mineral formation occurred to a significant extent only in suspensions whose lipid T_m was at or below that of the reaction temperature. For example, at 35 °C the 20% DMPC suspension rapidly mineralized, whereas the 10% DMPC and pure DPPC suspensions exhibited little evidence of mineralization. However, at 37 and 39 °C rapid mineralization of both DMPC-containing suspensions occurred. Rapid mineralization of the DPPC suspension did not occur until 41 °C, the highest temperature studied. The data suggest that a temperature-induced increase in permeability of the phospholipid bilayer near T_m leads to release of Ca^{2+}, which reacts with extravesicular phosphate to form mineral. Good agreement between the onset of Ca^{2+} release (Figure 5), rapid drop in pH (Figure 6), and the T_m of each lipid composition (Figures 2 and 3) supports this interpretation.

The formation of a mixture of apatite and brushite minerals upon heating of liposome suspensions to T_m is in agreement with the known pH dependence of calcium phosphate crystallization[14,15] and with our earlier findings on the effect of pH on surfactant-induced mineral formation from metastable liposome suspensions.[8] During the initial stages of mineral formation from metastable liposome suspensions, the high pH value (approximately 8.5 initially) and high concentration of calcium and phosphate ions are most likely to lead to the formation of apatite mineral, probably through a precursor phase such as amorphous calcium phosphate (ACP) and possibly octacalcium phosphate (OCP). However, as Ca^{2+} release and mineral formation progresses, acid is liberated as a byproduct of the reaction between calcium chloride and sodium phosphate (eqs 2 and 3), eventually reducing the pH below 7. Under these acidic conditions, the prevalence of the $H_2PO_4^-$ species in solution increases, favoring formation of more acidic calcium phosphates such as brushite.[14,15] Thus, it is likely that under the changing pH conditions of the liposome mineralization experiment, formation of apatite mineral occurs during the initial (alkaline)

(30) Schultz, S. G. *Basic Principles of Membrane Transport*; Cambridge University Press: New York, 1980.

stages, followed by brushite formation during the later (acidic) stages. Although these experiments were not buffered, we have shown in our earlier experiments that the use of buffer can provide more control over the type of mineral formed from metastable liposome suspensions.[8] More detailed experiments are being planned to fully investigate the type, quantity, and sequence of mineral formation occurring during thermally triggered liposome mineralization.

It is apparent from inspection of the data in Figure 6 and Table 1 that Ca^{2+} release and mineral formation from pure DPPC suspensions becomes significant only above approximately 39 °C; consequently, vesicles composed of pure DPPC may not be useful for rapid mineralization at body temperature (37 °C). However, tailoring the composition of the lipid vesicles (by addition of DMPC) to adjust T_m could be adopted as a strategy to trigger mineralization at body temperature. Furthermore, control of lipid composition could be utilized to adjust the mineralization rate, which is likely to depend on the rate of ion transport through the lipid bilayer, which in turn should depend on the proximity of the reaction temperature to T_m. For lipid compositions with T_m at or near the reaction temperature, rapid mineralization can be achieved due to the high permeability of lipid bilayers at T_m. For example, examination of the data in Figure 6 and Table 1 reveals that the mineralization reaction (as indicated by $t_{80\%}$) was nearly complete in just over 1 min for the liposomes containing 20 mol % DMPC. In contrast, it should also be possible to produce a slowly mineralizing system by adjusting the lipid composition so that T_m is a few degrees above body temperature.

An important outcome of this study is the demonstration that metastable liposome suspensions can be prepared, stored at room temperature for long periods of time, and rapidly mineralized when subsequently heated to body temperature. From a clinical perspective, implementing thermally triggered mineralization could be as simple as allowing a liposome-containing paste, gel, or solution to warm to body temperature after being applied (e.g., via syringe). Such an approach has many potential applications in medicine and dentistry. For example, a liposome-based remineralizing solution applied topically or as a mouth rinse might be useful for depositing apatite mineral as a treatment for dentin hypersensitivity or for remineralizing incipient caries. For orthopedic and maxillofacial applications, thermally triggered in situ mineral formation could be used to deposit a mineral or mineral/polymer composite bone substitute material to repair diseased or traumatized bone defects. If the amount of mineral formed could be increased or if the liposome suspension were to be used in combination with a polymer to form a mineral/polymer composite, in situ mineralization might provide the mechanical properties necessary for these applications.

Acknowledgment. The authors are grateful for financial support provided by the Whitaker Foundation and the University of Illinois at Chicago.

CM9702528