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# Composite Phospholipid−Calcium Carbonate Microparticles: Influence of Anionic Phospholipids on the Crystallization of Calcium Carbonate

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The synthesis and characterization of calcium carbonate microparticles by reaction of calcium chloride and ammonium bicarbonate in the presence of negatively charged phospholipid mixtures of negative and zwitterionic phospholipids has been reported. Negatively charged phospholipids influence the crystal morphology of calcium carbonate and induce the formation of thermodynamically less stable veterite polymorph as opposed to calcite polymorph. The phospholipids are entrapped in the calcium carbonate microparticles during the crystallization process, with a uniform distribution of phospholipids in the interior of the microparticles. This phenomenon was exploited to encapsulate a model hydrophobic fluorophore, the tris(4,7-diphenyl-1,10-phenanthroline)-ruthenium(II) dichloride complex, to simulate encapsulation of hydrophobic drug molecules. Thermogravimetric analysis reveals that, in these microparticles, the calcium carbonate and the phospholipid exhibit strong interactions.

Calcium is the most abundant mineral in the human body. It is needed to form bones and teeth and is also required for blood clotting, transmission of signals in nerve cells, and muscle contraction. The importance of calcium for preventing osteoporosis is probably its most well-known role. Most calcium supplements contain calcium carbonate. In and of itself, calcium carbonate is also one of the principal components of many biomineralization schemes occurring in nature, primarily involving a hierarchical organization of proteins and calcium salts.[1] The control of crystal morphologies of calcium carbonate is important for biomineralization reactions. The effect of various organic additives and template mediated reactions on calcium carbonate crystal morphologies has been studied.[2−4] Biomineralization in phosphatidylcholine vesicles by a diffusion mediated process has been reported as a model system for biomineralization.[2,5] Most of the research has been centered on elucidating particle formation and understanding the kinetics and mechanisms of crystallization.[6−11] Recently, Sukhrukov et al.[13] showed that porous calcium carbonate microparticles can be synthesized by direct mixing of $Ca^{2+}$ and $CO_3^{2-}$ salts, which can be used as a template for polyelectrolyte layer-by-layer (LbL) assembly. Here, we report the synthesis of calcium carbonate microparticles of a highly porous nature, having phospholipid molecules entrapped in the porous structure, resulting in an organic/inorganic complex matrix. The microparticles can be used to load hydrophobic molecules in an inorganic core with potential applications in medicine and industry. We propose a surface mediated reaction for the preparation of the mesoporous calcium carbonate microparticles. These microparticles are amenable to LbL assembly for surface modification to make the surface of the microparticles biocompatible; the particles can also be used as templates for preparing hollow polyelectrolyte microcapsules by various approaches.[13] The presence of phospholipids in the calcium carbonate matrix also facilitates entrapment of small hydrophobic molecules in conjugation with other bioactive molecules.

For preparing calcium carbonate microparticles by incorporating phospholipids in the matrix, liposomes of DPPA (1,2-dipalmitoyl-*sn*-glycero-3-phosphate), DSPG (1,2-distearoyl-*sn*-glycero-3[phospho-rac-(1-glycerol)])(sodium salt), and DPPA 20% (w/w)−DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) mixtures were prepared in deionized water (DI water) using a standard protocol.[14] A 2 mL portion of 1 mg/mL phospholipid liposomes was first treated with (0.01−0.15 M) $Ca^{2+}$ ions ($CaCl_2$) for 10 min with slow stirring to form cochleates.[16] The cochleates were dispersed by sonication followed by immediate addition of 0.375 M $NH_4HCO_3$ with different volume ratios and in large excess of the stoichiometric reaction requirement. After 15 min of reaction, the products were centrifuged and rinsed in DI water three times followed by rinsing in a 1:1 methanol−chloroform mixture three times to remove any free ions and phospholipids. The samples were then dried under a stream of nitrogen.

Pure calcium carbonate microparticles were prepared by mixing equal volumes of $CaCl_2$ and $NH_4HCO_3$ in a constant volume reactor, under ambient conditions, with uniform stirring. Following a 15 min reaction, the reaction products were centrifuged, rinsed in DI water, and dried under a stream of dry nitrogen as well.

To study the morphology and microstructure of the $CaCO_3$ microparticles, the samples obtained were visualized using scanning electron microscopy (SEM, Gemini, Leo 1550). The

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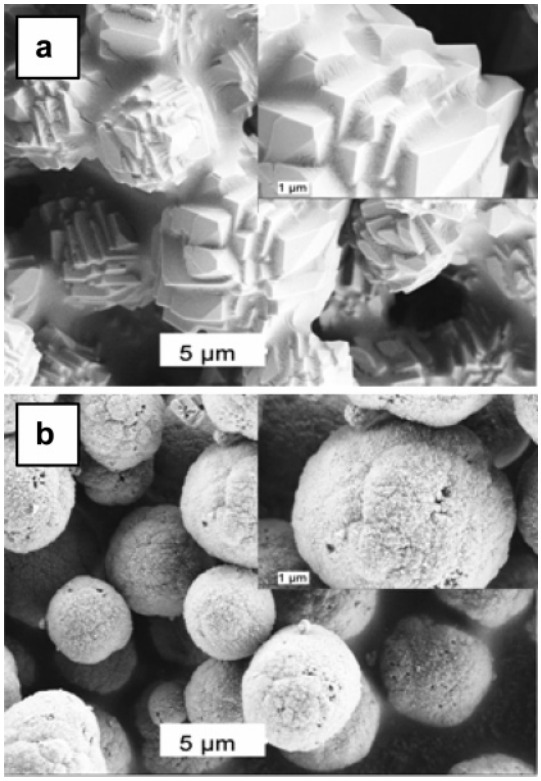


**Figure 1.** Representative SEM images of calcite microcrystals prepared by reaction of 1 mL of 0.01 M $CaCl_2$ + 1 mL of 0.375 M $NH_4HCO_3$ + 2 mL of DI water (a). Porous $CaCO_3$ microparticles made by first reacting 2 mL of 1 mg/mL DPPA + 1 mL of 0.01 M $CaCl_2$ + (after 10 min) 1 mL of $NH_4HCO_3$ (b); these microparticles contain 3% (w/w) phospholipids in the matrix.

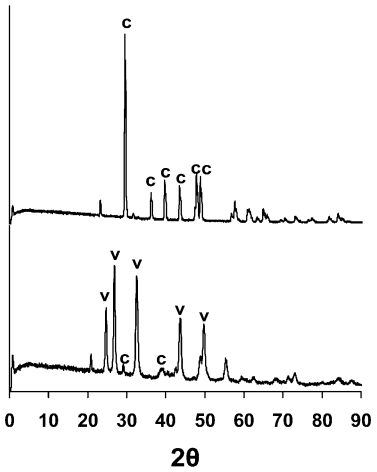


**Figure 2.** X-ray diffraction pattern of calcium carbonate microparticles. The top graph shows the X-ray diffraction pattern of calcite with the principal peaks highlighted.[4] The bottom graph shows the X-ray diffraction pattern of calcium carbonate microparticles synthesized in the presence of DPPA (V-veterite, C-calcite).

crystal structures of the $CaCO_3$ microparticles synthesized in the presence and absence of anionic phospholipids were also characterized using X-ray diffraction. In the absence of any additives, the reaction of $Ca^{2+}$ and $HCO_3^-$ yields particles with diameters in the range 10 ± 5 $\mu$m. The SEM images in Figure 1a along with the powder X-ray diffraction pattern in Figure 2 indicate calcite polymorph as the predominant form resulting from the direct reaction of $Ca^{2+}$ and $HCO_3^-$.

The shape of the calcite crystals depends on the rate of mixing of lipids during reaction. No stirring results in wide size

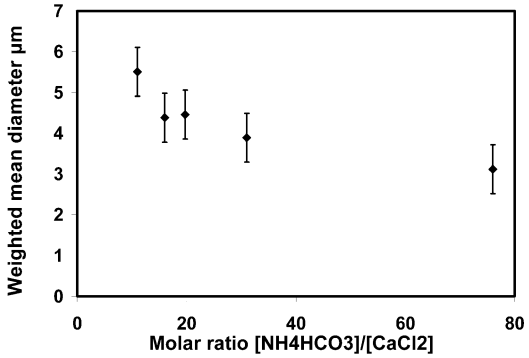


**Figure 3.** Effect of the ratio of $Ca^{2+}$ to $HCO_3^-$ ions on the size of calcium carbonate microparticles based on a 0.375 M concentration of $NH_4HCO_3$ in total volume, with the DPPA concentration held constant at 0.2 mg/mL.

distributions of particles with fewer crystal faces, whereas vigorous stirring yields narrower size distributions with multifaceted particles (Figure 1a). Under identical conditions to those used for the preparation of calcite microparticles described above, the presence of phospholipids in the reaction mixture results in particles that are porous in nature and without any crystalline edges (Figure 1b). The size distributions of these microparticles are in a range of 5 ± 2 $\mu$m.

The powder X-ray diffraction pattern of calcium carbonate microparticles synthesized in the presence of DPPA (Figure 2) shows that the microparticles consist of the less stable but more soluble vaterite polymorph. However, traces of calcite peaks are also seen (less than 2 vol %), which can be attributed to the phase transformation of the microparticles or some nucleation occurring in the aqueous phase as opposed to the phospholipid surface.

The size of the particles depends on the concentration of reactants. The molar ratio of calcium and bicarbonate influences the average size of microparticles, as shown in Figure 3. The mean diameter of the microparticles decreases with decreasing $CaCl_2$ concentration. Higher molar concentrations of $CaCl_2$, relative to phospholipids, result in a non-homogeneous distribution of phospholipids in the $CaCO_3$ matrix, occasionally yielding rombohedral $CaCO_3$ calcite in the final product. This observation can also be correlated to the fact that, with lower phospholipid concentrations or nonuniform stirring of the reaction mixture, nucleation may begin in the aqueous phase as opposed to on the phospholipid surface and yields a mixture of porous $CaCO_3$ microparticles as well as calcite crystals.

We hypothesize that a higher concentration of phospholipids to $Ca^{2+}$ results in a narrower size distribution of the resulting microparticles. In the presence of excess phospholipids, most of the calcium ions are bound to the phospholipid surfaces, which results in fewer reaction centers in the media. The nucleation occurs on phospholipid surfaces or begins in solution and propagates on the phospholipid surface, resulting in a predominantly phospholipid surface mediated reaction. To verify this hypothesis, 5% (w/w) 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(carboxyfluorescein) (FITC-EA) was admixed with DPPA and the resulting microparticles were visualized using a confocal laser scanning microscope (CLSM, DMI-RE2). The distribution of fluorescence was uniform in the calcium carbonate matrixes that were prepared in the presence of excess phospholipids (Figure 4a) compared to microparticles prepared in a phospholipid deficient reaction mixture (Figure 4a, inset). The images also illustrate that the distribution of phospholipids

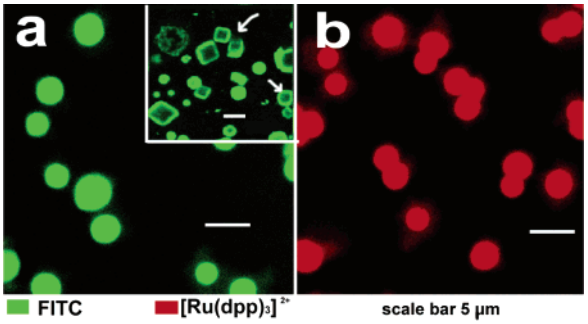


**Figure 4.** CLSM image of a cross section of (a) calcium carbonate microparticles incorporating FITC-EA; in a phospholipid deficient medium, the nonuniform distribution of phospholipids in the matrix is observed (inset) and (b) prepared by reaction of $CaCl_2$ and $NH_4HCO_3$ in the presence of a DPPA 20% (w/w)−DPPC mixture containing $[Ru(dpp)_3]^{2+}$ which is a hydrophobic molecule localized in the hydrophobic region of a fatty acid side chain.

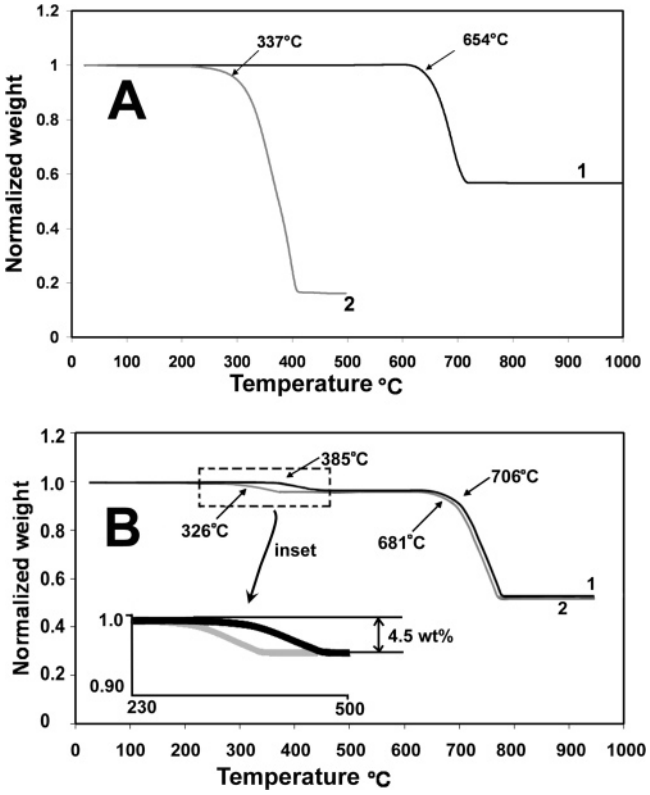


**Figure 5.** Thermogravimetric analysis of (A) calcite and DPPA and (B) calcium carbonate microparticles prepared in the presence of DPPA; the change in weight is shown as a percentage of the total weight (inset) (1) and a mixture of calcite and DPPA (2).

in the matrix is uniform and lends support to the postulated phospholipid surface mediated reaction of calcium and carbonate ions.

Thermogravimetric analysis of the calcium carbonate microparticles prepared in the presence of DPPA indicated that the DPPA in the calcium carbonate matrix desorbed at higher temperatures (385 ± 6 °C), compared to either pure DPPA (337 ± 6 °C) or a physical mixture of phospholipid with calcite microparticles (326 ± 6 °C) (Figure 5). The desorption of carbon dioxide from calcium carbonate also occurs at a higher temperature (706 ± 6 °C) compared to pure calcite (654 ± 6 °C) or a physical mixture of calcite and DPPA (681 ± 6 °C). The higher temperature of desorption can be attributed to a strong calcium carbonate phospholipid interaction. The microparticles contain ∼2.5−5% by weight of phospholipids in the matrix. It is known that $Ca^{2+}$ ions interact with anionic phospholipids (phosphatidic acid, phosphatidyl serine) to form a coordinate complex, such that each $Ca^{2+}$ ion demonstrates four, and in some cases six, coordination valencies that bond with phosphate groups of phosphilipids or with other molecules such as water.[16] Lamellar amphiphile−calciumhydroxyapatite structures have been extensively studied, which has also led researchers to suggest the existence of such calcium ion−phospholipid coordination.[17,18] We believe this theory could be extended to the carbonated calcium ions in our system. Another contributing factor to the delayed desorption could be the strong van der Waals and hydrophobic interactions between the phospholipid vesicles and the mesoporous matrix of the calcium carbonate. The negatively charged phospholipid vesicle structure prevents $HCO_3^-$ ion penetration into the interior of the vesicle, thus limiting the calcification occurring only on the surface of that structure; this could also explain the porous nature of the calcium carbonate microparticles synthesized.

The stability of porous calcium carbonate microparticles in DI water was studied using particles incorporating phospholipids (FITC-EA, DPPA, and DPPC mixture) with confocal microscopy (Leica DMI-RE2). Over a period of 1−3 weeks, the veterite microparticles recrystallized to calcite. The first signs of recrystallization were observed after 1 week of storage in an aqueous solution and progressed for 3−4 weeks, after which nearly 90% of the microparticles had recrystallized and the phospholipids were excluded from the matrix. However, when dried, the particles, following preparation using a methanol−chloroform mixture, showed no change in morphology over 3−4 months.

To study the possibility of incorporating hydrophobic molecules, a model fluorophore, the tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) dichloride complex ($[Ru(dpp)_3]Cl_2$), was incorporated in the phospholipid vesicles and the resulting microparticles were studied using a CLSM (Figure 4b). $[Ru(dpp)_3]^{2+}$ is insoluble in DI water but readily localizes in the hydrophobic region of the phospholipid bilayer. The distribution of the dye is uniform in the matrix of the calcium carbonate. Comparing parts a and b of Figure 4, we can see that the distribution of the hydrophobic molecule $[Ru(dpp)_3]^{2+}$ is as uniform as the distribution of the amphiphile itself. This result is important from a pharmaceutical standpoint, since it demonstrates the possibility that these microparticles can be used as carriers for hydrophobic drug molecules. This phenomenon has been used to entrap the drugs Nifidapine and dexamethasone in the calcium carbonate matrix and will be reported elsewhere.

In conclusion, anionic phospholipid (phosphatidic acid, phosphor glycerol) mediated synthesis of calcium carbonate microparticles from $Ca^{2+}$ and $HCO_3^-$ salts yields a composite organic/inorganic matrix. The inorganic matrix is predominantly the veterite polymorph of calcium carbonate. The microparticles contain ∼2.5−5% by weight of phospholipids in the matrix. The phospholipids show stable entrapment in the calcium carbonate matrix with strong interactions with the matrix. Such an architecture is conducive to the entrapment of small hydrophobic molecules and other bioactive substances in an inorganic matrix.

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