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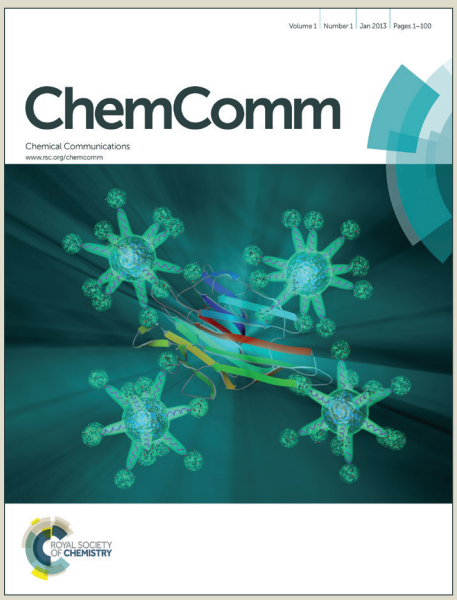

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Controlling nucleation in giant liposomes

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We introduce giant liposomes to investigate phase transformations in picoliter volumes. Precipitation of calcium carbonate in the confinement of DPPC liposomes leads to dramatic stabilization of amorphous calcium carbonate (ACC). In contrast, amorphous strontium carbonate (ASC) is a transient species, and $BaCO_3$ precipitation leads directly to the formation of crystalline witherite.

The use of metastable amorphous minerals is a widespread strategy in biological crystal growth.[1] Prominent examples are the synthesis of single crystalline spicules using an ACC precursor,[2] vesicle-borne amorphous calcium phosphate in bone mineralization,[3] and use of amorphous iron oxides.[4] It is conceptually related to sol-gel processing of ceramics,[5] may involve a fluid/fluid transition similar to those observed in protein crystallization,[6] and has generated a lot of interest as a possible model system for non-classical nucleation.[7]

At this time, there is a fascinating disconnect between experimental observations, theoretical considerations, and simulations regarding the stability of ACC in confinement. Several groups have reported an increased lifetime for ACC when confined in pores,[8] between crossed cylinders,[9] in picoliter droplets,[10] within a silica coating,[11] or in liposomes.[12] In bulk solution, crystallization of ACC particles larger than 70-100 nm was reported.[13] Navrotsky argued that there is a threshold size, below which amorphous nanoparticles are thermodynamically more stable.[5] However, others have pointed out, and simulations have confirmed, that for $CaCO_3$ this crossover should occur at a radius of 1-2 nm.[7b, 9, 14]

Noting that synthetic ACC contains up to one formula unit of water, has a long lifetime while dry, and appears to dehydrate over time even in solution, it has been suggested that biological stabilization may rely on preventing release of or contact with water.[10-11, 15] While this may be true in certain *in vitro* systems, biomineralization generally occurs in compartments delimited by phospholipid bilayer membranes that have high permeability for water.[4] In fact, ACC nanoparticles up to 200 nm in diameter can be synthesized in phospholipid vesicles (liposomes) where they remain stable against crystallization in direct contact with water.[12] Therefore, liposome reactors are ideally suited to study the dual roles of confinement and the membrane itself in biological crystallization.

Herein, we study $CaCO_3$ phase transformations in giant liposomes (d = 20-50 µm). We demonstrate that confinement results in ACC particles that are exceptionally stable despite a much increased size and exposure to water. We attribute the extended stability to the exclusion of heterogeneous nucleators and qualitatively compare the kinetics of phase transformations observed for Ca, Sr, and Ba carbonates.

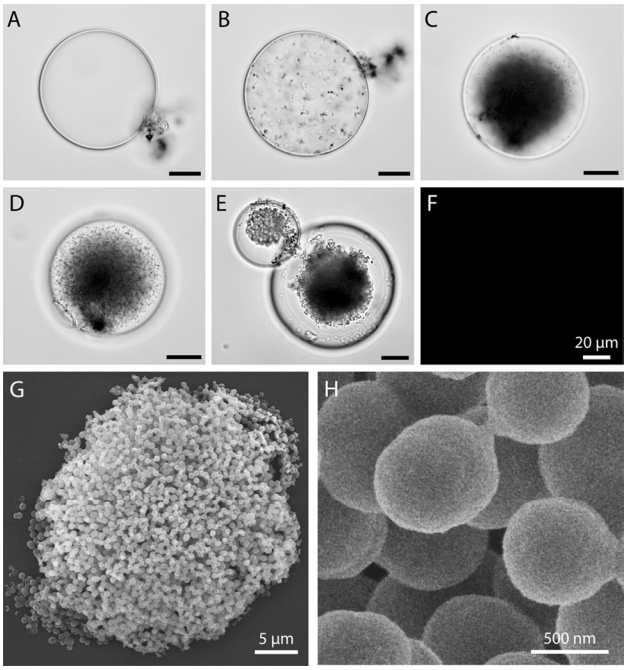


Figure 1. (A-F) Light microscopy images of Ca^{2+}-loaded giant PC liposomes (A) before the addition of $(NH_4)_2CO_3$; (B) within 5 min after addition, a great number of particles precipitate; (C-D) within 2 h, particles aggregate and sink to the bottom (two different focal planes shown); (E-F) within 24 h, the aggregate condenses and exhibits a rough surface, but under polarized light is not

birefringent (F). Scalebars represent 20 µm. (G-H) SEM images of an isolated ACC aggregate (G), consisting of ACC nanoparticles 650 ± 5 nm in diameter (H)

Giant liposomes were synthesized using the method of Horger and coworkers.[16] Briefly, a dry film of phosphatidylcholine (PC) supported on agarose was gently hydrated with 1 M aqueous $CaCl_2$. The resulting liposomes are 20-50 µm in diameter (Fig. 1A), with varying number of lamellae. Ca^{2+} remaining in solution outside the liposomes was exchanged against an isosmotic solution of NaCl. Precipitation of $CaCO_3$ in the lumen, i.e. the aqueous interior of the liposome, was initiated by addition of aqueous $(NH_4)_2CO_3$.[12] Within 4-5 min, a large number of precipitates appear all at once throughout the lumen (Fig 1B), consistent with homogenous nucleation, nucleation on suspended nucleators, or spinodal decomposition, but not heterogeneous nucleation on the membrane.[6b, 17]

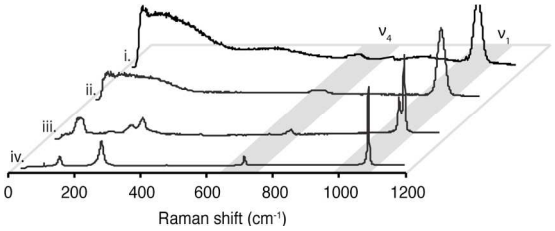


Figure 2. Raman spectra of (i) a liposome-encapsulated ACC aggregate recorded *in situ*, and of bulk synthetic (ii) ACC, (iii) vaterite, and (iv) calcite powders.

Particles rapidly aggregate and sink to the bottom of the liposome (Fig. 1C/D). Over the course of 24 h, further aggregation and coarsening leads to formation of a roughly textured mass that has a diameter of 10-40 µm. Precipitates are not birefringent and remain so for up to 8 days. Confocal Raman spectra recorded *in situ* show the typical features of bulk synthetic ACC (Fig. 2 and SI).[1] SEM reveals that aggregates consist of spherical particles ~ 650 nm in diameter (Fig 1G/H). In combination, this is strong evidence that liposome-encapsulated ACC is identical to hydrated ACC precipitated in bulk. For bulk ACC, however, moisture in the ambient air is sufficient to cause crystallization.[18]

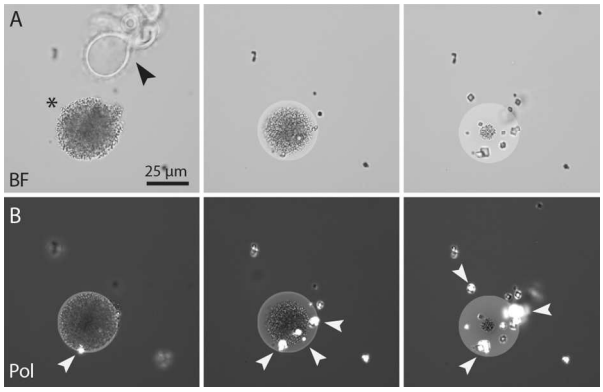


Figure 3. Lysis of liposomes (black arrowhead) with 3% Triton-X results in dissolution of encapsulated ACC (asterisk), and re-precipitation of calcite in the immediate vicinity (white arrowheads). (A) Bright field (BF) images taken over ~ 5 min, and (B) polarized light (Pol) images taken immediately after images in A. The white circle indicates the size of the original ACC precipitate.

Confocal fluorescence microscopy reveals that ACC does not form on or coat the membrane, suggesting that the interaction is weak (SI). As Langmuir monolayers of PC do not prevent nucleation of vaterite and calcite,[19] we can rule out that the membrane itself stabilizes ACC. To further confirm that ACC precipitates are stabilized exclusively by confinement, liposomes were lysed with detergent (Fig. 3). As soon as liposomes break open, we observe rapid precipitation of calcite, and concurrent dissolution of ACC. Calcite crystals preferentially form on the glass substrate in the vicinity of the dissolving precipitate. While we cannot exclude that some calcite crystals nucleate on the surface of ACC particles, it is clear that this does not occur inside the liposome before lysis. This indicates that ACC, the liposome membrane, or any impurities present do not act as heterogeneous nucleators.

We estimate that the total volume of the ACC aggregate in a giant liposome with d = 50 µm to be of 2-3x10^3 µm^3. This aggregate consists of ACC nanoparticles that are each ~1,000 times larger in volume than current estimates for the threshold of stability, and they are in direct contact with water. It is thus unlikely that a) there is a threshold size for the stability of the particles, and b) that limiting the ability to release water, or limiting the contact with water stabilizes ACC in this system. However, ACC could be kinetically stable in the absence of suitable heterogeneous nucleators for one of the crystalline polymorphs. This is analogous to the classical observation that small liquid metal droplets display large supercooling on account of a low number of heterogeneous nucleators.[20] For liposomes, it is well documented, if poorly understood, that large molecules and particles are encapsulated at rather low efficiency.[21] Thus, it is conceivable that the number of heterogeneous nucleators in the liposome is lower than in bulk.

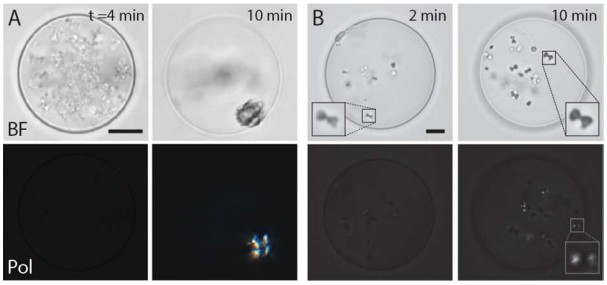
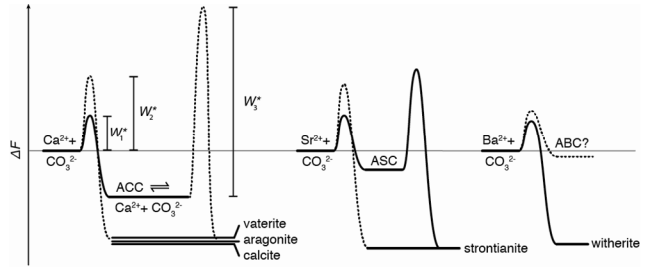


Figure 4. Precipitation of $SrCO_3$ (A) and $BaCO_3$ (B) in giant PC liposomes. Absence of birefringence in (A) at 4 min indicates formation of ASC. Birefringence in A at 10 min, and in B at 2 min and 10 min indicates rapid nucleation and growth of crystalline strontianite and witherite, respectively. Scale bar represents 10 µm.

To establish how general this phenomenon is, we investigated the precipitation of $SrCO_3$ and $BaCO_3$ at approximately equal initial supersaturation ($\sigma \approx 12$, see SI). Precipitation of $SrCO_3$ is similar to $CaCO_3$, with many small precipitates formed within the first 3-4 minutes that do not exhibit birefringence (Fig. 4). However, within 5-10 min, one birefringent particle appears that grows rapidly while the first-formed precipitates dissolve. In the case of $BaCO_3$, there was no intermediate precipitate and one or more spherulitic sheaves were observed as early as one minute into the precipitation. Birefringent precipitates were identified as strontianite ($SrCO_3$) and witherite ($BaCO_3$) by in situ Raman microscopy (SI). While we were unable to record spectra for the transient $SrCO_3$ precipitate, we interpret the absence of birefringence and the rapid dissolution-

reprecipitation as evidence that it is metastable amorphous strontium carbonate (ASC). We find no indication for the formation of amorphous barium carbonate (ABC). Tremel and Gower previously reported evidence for transient ASC and ABC.[22]

Within the time resolution of our experiments, there is no difference in the rate by which ACC and ASC are formed. However, the lifetimes of the amorphous metal carbonates decrease dramatically, from at least $7{\cdot}10^5$ s for ACC to $2\text{-}3{\cdot}10^2$ s for ASC to infinitesimal, for (hypothetical) ABC. This is to be expected if the lifetime is determined by the relative height of the barrier to nucleation of the amorphous vs. the crystalline carbonate (Scheme 1). Based on its rapid formation the barrier to ACC formation (W_1*) is low (or absent entirely if a case of spinodal decomposition[6b, 17]). In the absence of a suitable heterogeneous nucleator, we expect a very high barrier W_2^* for the crystalline polymorphs.[23] Under these conditions, ACC forms rapidly, decreasing the supersaturation σ for the crystalline polymorphs because the activities of Ca^{2+} and CO_3^{2-} are now set by the solubility of ACC. As $W^* \propto \sigma^{-2}$, the resulting increase in barrier height (W_3^*) – by a factor of up to ~5 in our system – kinetically traps metastable ACC. In the case of $SrCO_3$, the barrier to nucleation of ASC is likely similar in magnitude to that of ACC. The barrier for strontianite formation, however, is significantly reduced. Even though ASC precipitates first, lowering supersaturation, the resulting delay is brief. As soon as strontianite nucleates and grows, the resulting drop in ion activities leads to rapid dissolution of ASC. Finally, the barrier to witherite nucleation appears to be close to or below that of ABC.



Scheme 1: Free energy levels of the initial, metastable, supersaturated solutions, metastable amorphous $Ca^{2+}/Sr^{2+}/Ba^{2+}$-carbonates in their mother liquor, and the crystalline polymorphs in equilibrium with their mother liquors are drawn to scale based on equilibrium solubility data. The kinetics of formation of the amorphous carbonates are proposed to be controlled controlled by the relative height of the barriers W_1* and W_2*, the lifetime by W_3*. The height of these barrier scales with the reciprocal of the square of the vertical distance of starting material and products in this diagram, which is a measure of the supersaturation. Solid lines correspond to processes observed in giant liposomes, dashed lines to those not observed.

The acceleration of the rate of crystallization from calcite to witherite could be a consequence of a lower barrier to homogeneous nucleation (SI). As heterogeneous nucleation in most systems remains far more likely, relative rates are likely also affected by the interfacial energy between nuclei of the crystalline polymorphs and the liposome membrane, the amorphous precursor, or random impurities. Obviously, organisms that choose confinement to control biomineralization must carefully engineer the surface properties of the confining structure and of any surfaces within.

From these experiments, we draw two important conclusions with implications for biological and bio-inspired crystal growth in confinement: I) nucleation kinetics of the crystalline Ca/Sr/Ba-carbonate can control the lifetime of the amorphous carbonates; II) exclusion of heterogeneous nucleators for ACC present in bulk may be an intrinsic feature of mineralization in intracellular compartments. Given the diverse chemistry of lipids, it may be possible to tune liposome composition to control the relative barriers to nucleation of specific polymorphs.[24] We envisage that giant liposomes will emerge as a powerful platform to study the role of confinement also of proteins and pharmaceuticals. Clearly, however, $CaCO_3$ is special in that ACC can be stabilized with minimal effort. Whether this is fortuitous, or a consequence of evolutionary optimization of lipid structure or biomineral use, is another fascinating topic.

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Notes and references

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