

Title: Microscopic Evidence for Liquid-Liquid Separation in Supersaturated CaCO₃ Solutions

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Abstract: Recent experimental observations of the onset of CaCO₃ mineralization suggest the emergence of a population of clusters that are stable rather than unstable as predicted by classical nucleation theory. This study employs molecular dynamics simulations to probe the structure, dynamics, and energetics of hydrated CaCO₃ clusters, and lattice gas simulations to explore the behavior of cluster populations prior to nucleation. Our results predict formation of a dense liquid phase through liquid-liquid separation within the concentration range where clusters are observed. Coalescence and solidification of nanoscale droplets results in formation of a solid phase whose structure is consistent with amorphous CaCO₃. The presence of a liquid-liquid binodal enables a diverse set of experimental observations to be reconciled within the context of established phase separation mechanisms.

Main Text: Calcium carbonate has been intensely studied over the past century, and its most stable polymorph at ambient conditions, calcite, is often cited as a model of classical crystal growth behavior (1). However, findings from titration, ultracentrifugation (2, 3), and cryo-TEM (3, 4) now suggest that the onset of CaCO₃ mineralization — i.e. nucleation — contradicts classical expectations. In the classical picture, nucleation is a stochastic process in which thermal fluctuations induce the formation of clusters that are unstable with respect to dissolution. Clusters increase in both size and free energy until a threshold is crossed, whereupon the energy gained by forming the bulk material overcomes the penalty for creating an interface, and growth proceeds spontaneously. Contrasting with this classical picture, recent observations of long-lived nanometer-sized clusters present prior to nucleation have led to a predominant view of calcium carbonate formation in which the nucleation pathway is “non-classical” (2, 3, 5-8), involving prenucleation clusters that are stable (or metastable) with respect to both dissolution and growth. In this model, bulk calcium carbonate forms primarily by aggregation of these clusters.

An alternative route to mineralization, liquid-liquid separation, has also been proposed based on light scattering, electron microscopy and NMR (9-11). This hypothesis is supported by experimental work documenting the formation of liquid MgSO₄ at high temperature (12), as well as the known persistence of a polymer stabilized liquid calcium carbonate phase under ambient conditions (13). Nonetheless, conclusive experimental evidence that the clusters observed at the onset of calcium carbonate mineralization are liquid does not exist. A recent study using NMR to quantify the diffusion of ions in solution following the introduction of calcium (11) concluded that a bicarbonate-rich liquid phase may exist at pH=8.5. However, as the measurements quantified the diffusion of ion species in a bulk sense only, the inhibited mobility of the bicarbonate ions could not be unambiguously assigned to

the formation of cluster species over ion-pairs; moreover the proclivity of calcium ions to structure water and influence the overall solution viscosity with increasing concentration could not be addressed. Thus, conclusive experimental evidence that the clusters observed at the onset of calcium carbonate mineralization are liquid does not exist and the relationship between the prenucleation clusters and the hypothesized liquid phase remains unclear, as does the size distribution and thermodynamic character of clusters themselves (8, 14).

Owing to the challenges faced by experimental approaches in directly quantifying the energetics of transient nanoscale cluster species that form during the nucleation process, we use molecular dynamics simulations to investigate whether initially formed clusters are stable or unstable relative to the solution and to explore their formation pathways. Earlier simulations (5, 6, 15, 16) used high ion concentrations in order to increase the frequency of ion association events and facilitate sampling of the energy landscape within timescales accessible by unbiased simulations (5, 15). After formation, the clusters generated in this manner were transferred to lower concentration environments to demonstrate their stability at more experimentally relevant conditions (5). These results show that the earliest formed clusters adopt low-density chain, ring, and branched structures. However, in the high concentration limit, growth proceeds at the diffusion limit with barriers opposing ion attachment on par with the ambient thermal energy $\sim k_B T$. While the emergence of a different type of species was observed at high concentration, the limitations of direct simulation left the nature of this transition unresolved. Application of biased sampling methods, designed to limit the amount of time the system spends exploring local features of the energy landscape (i.e. metadynamics), found that compact crystalline states could be stable at sizes as small as $\sim 2 - 4$ nm (16), though their free energy relative to ions in solution was not determined.

Neither of the aforementioned approaches shed light on the evolution of the initial hydrated

clusters to more compact dense states, which is the key to understanding the pathway to nucleation and the stability of the prenucleation clusters. Here we explore cluster stability, structure and the nucleation pathway by introducing simulation techniques that are capable of determining the nature of equilibrated calcium carbonate cluster species directly at the low concentrations used experimentally. Here the Kawska-Zahn method (17), modified to allow for solvation during all steps, was used to grow hydrated clusters into a size regime ($\sim 1.5\text{--}2$ nm in diameter) that overlaps with cryo-TEM based observations of prenucleation clusters, while temperature-based Replica-Exchange Molecular Dynamics (18, 19) was employed to hasten the exploration of the energy landscape and minimize the tendency of the system to become kinetically trapped in local energy minima during the early stages of nucleation and growth (20).

In agreement with previous studies (5, 15), our simulations show that low-density configurations are observed for small clusters; however, such arrangements give way to more condensed states very rapidly with further ion additions (**Fig. 1A**). The dynamic character of the clusters is quantified through determination of the constituent ion diffusivities (20). For all cluster sizes investigated, we obtain diffusive characteristics that are inconsistent with the solid state. Indeed, plotted as a function of cluster size, ion diffusivities fall largely within the range of self-diffusivities expressed by several common solvents (21) (**Fig. 1B**) and are significantly higher than in bulk amorphous calcium carbonate (ACC) and calcite, indicating that the clusters are droplets of a dense ion-rich liquid phase of $\text{CaCO}_3(n\text{H}_2\text{O})$. The ion diffusivities decrease as the dense liquid phase grows; however, the rate of decline gradually abates and approaches a constant value characteristic of the “bulk” liquid phase. The leveling off of the ion diffusivity is accompanied by a gradual increase in the average coordination of calcium by carbonate ions, and a smooth crossover in the most probable coordination number from 2 to 3 that culminates at ~ 26 ions (**Fig. S1**). However, as evidenced by a plot of the average Ca-C coordination number against the ion diffusivity (**Fig. S2**), the structural and dynamical properties of the clusters are correlated and smoothly trending, suggesting that the formation of the

dense liquid is not marked by an abrupt transition in cluster character (supplementary online text).

A plot of the free energy versus cluster size (**Fig. 1C**) displays an entirely downhill free energy landscape, except for a possible slight increase at 26 ions that is correlated with the gradual evolution of the Ca-C coordination number from 2 to 3 (20). However, the upturn in free energy is within the statistical confidence of our analysis. Moreover, if real, it represents a thermodynamic impedance to the formation of the dense liquid phase of less than $\sim 8 k_B T$ (20 kJ / mol at 300K) at the simulated conditions ($[Ca^{2+}] = [CO_3^{2-}] = 0.015$ mol / L), which is not sufficient to halt the growth of the clusters. Therefore, the most salient result of the free energy analysis is the observation that the free energy decreases monotonically with cluster size.

The observed free energy landscape, combined with the absence of a significant repulsive barrier opposing cluster association (6), is characteristic of a solution that has exceeded its stability limit and is undergoing a spontaneous phase separation by spinodal decomposition (23). The accessibility of the spinodal region at relatively modest concentrations has significant consequences for the mineralization process. Thermodynamically, it indicates that a liquid-liquid coexistence line exists between the dense liquid phase and the ion-poor solution phase (**Fig. 2**), such that the two liquids are in a state of metastable equilibrium with respect to solid $CaCO_3$ phases over a wide range of solution conditions.

The existence of this dense liquid phase enables both conventional ion-by-ion and cluster-mediated crystallization pathways to be described in terms of established phase separation models, without invoking non-classical constructs. The schematic representation of the presumptive phase relationships within the system (**Fig. 2**) displays regions in composition and temperature space where both direct and indirect crystallization mechanisms are possible. The exact relationships are a complex function of chemical factors (pH, ionic strength, concentration, composition, temperature etc.) and may shift significantly depending on environmental conditions. Based on the retrograde solubility exhibited

by solid CaCO_3 phases, the known behavior of MgSO_4 at high temperatures (12), and the interpretations of Faatz et al. (9), the binodal orientation is likely concave up. A solution with a composition falling within the ion-poor solution field of the phase diagram is thermodynamically favored to nucleate one of the crystalline phases of CaCO_3 . However, in practice the thermodynamic barriers opposing nucleation in this regime are predicted to be well in excess of $100 k_B T$ (24), preventing homogeneous nucleation from occurring on observable timescales.

As the ion activity product increases (at constant temperature), the liquid-liquid coexistence line is encountered and homogeneous nucleation of the dense liquid phase becomes possible. Dense liquid formation on short timescales is more likely than direct crystallization because the excess free energy of the solution-liquid interface is significantly reduced relative to the solution-crystal interface, resulting in a lower thermodynamic barrier to liquid-liquid separation than to crystallization. As the ion-activity product is increased even further, the spinodal line is crossed. This point marks the limit of solution stability and the point where the barrier opposing nucleation becomes comparable to the ambient thermal energy; infinitesimal fluctuations in the density of ions in solution give rise to clusters that are thermodynamically unstable with respect to growth and a macroscopic quantity of the dense liquid phase emerges.

The predicted liquid-liquid separation also provides a mechanism for generating clusters of various sizes, as a generic consequence of the spatial correlations that result from particle interactions (25). We demonstrate this mechanism within the Ising lattice gas, a canonical model of phase change that enables exploration of the general dynamics of solutions driven out of equilibrium independent of system-specific chemical details (26, 27). The results exhibit a number of features consistent with experimental observations.

First, in the spinodal regime, hierarchical cluster-cluster association generates a population of large clusters. These clusters form on a monotonically decreasing free energy landscape (**Figs. 3A, S3**)

akin to the results of the atomistic simulations (**Fig. 1C**) and therefore have no special thermodynamic status. At the ion concentrations characteristic of the experiments in (2,3,9), the mean radius $R(t)$ of this population of clusters should evolve rapidly as $R(t) \sim 100 \text{ nm } (t/s)^{1/3}$ (28). Although the initial microscopic phase observed by Faatz et al. (9) is not clearly identifiable as either liquid or solid, the evolution of the particle size distribution as displayed in their figures 1b and 2 is consistent with this expectation.

Second, an additional population of small clusters also emerges alongside the larger rapidly growing products of spinodal decomposition described above (**Fig. 3B**) (25). These clusters form rapidly and persist throughout the spinodal process. Following phase change the small cluster size distribution is stable in a statistical sense, though the lifetime of an individual species is finite. Near the critical temperature the width of the distribution also broadens (**Fig. 3B**) due to a significant reduction in the interfacial tension (25, 29) (Faatz et al. (9) estimated that the critical temperature is close to room temperature on the basis that the number density of large clusters tended toward zero at $\sim 10^\circ\text{C}$). These results are consistent with those of Pouget et al. (3) who observed large particles coexisting with a persistent population of much smaller clusters (0.7-1.1 nm). Though Pouget et al. attributed the larger size fraction to amorphous calcium carbonate, the low-dose electron diffraction technique employed may not be able to distinguish between a cryogenically frozen liquid phase and a bonafide amorphous solid. Therefore, a conventional liquid-liquid phase separation mechanism can account for a richly-structured fluid of clusters of sizes qualitatively similar to those seen experimentally, both small (2,3) and large (3,9).

As per the discussion above, it is clear that the presence of the binodal and its associated critical temperature exerts considerable influence over the small cluster distribution that emerges after phase change. However, the region of the phase diagram below the binodal is similarly affected. Cluster size distributions obtained from the single phase region are qualitatively similar to the post phase-separated small cluster distributions displayed in **Fig. 3B**, and likewise broaden with increased proximity to the critical temperature. Therefore, when a binodal exists, observed cluster size distributions may differ

substantially from those predicted by classical nucleation theory, although the phase separation mechanism remains conventional.

To discern whether the liquid clusters identified in this study are reasonable models for the nanoscopic species observed at the onset of phase separation, a model of solid ACC was constructed by randomly aggregating clusters of the dense liquid phase and thereafter reducing the water content, by a simulated dehydration process, until it reaches the point where it is commensurate with that of amorphous hydrated calcium carbonate (20). The local order within the model ACC structure was quantified through calculation of the total pair distribution function and compared with the results of X-ray scattering measurements for validation. This approach is distinct from the previous effort to model the structure with reverse Monte Carlo methods (30) because the structure is derived from an assembly and dehydration process that mimics a plausible growth mechanism.

The model pair distribution function is distinct from those for the crystalline CaCO_3 polymorphs, which are ordered indefinitely beyond the 15 Å radius that comprises the coherent X-ray scattering length in amorphous calcium carbonate, and is also in general registry with the experimentally measured distribution function (**Fig. 4**). The most significant differences between the model and experimental structures arise at small radial distances (O-H and C-O distances) where the experimental signal is most subject to interference from Fourier transformation. Additionally, the Ca-O peak centered at ~ 2.4 Å is slightly split in the model structure, showing the presence of two peaks arising from nearest neighbor oxygen contacts in water molecules and carbonate ions. However, the major features of the experimental pair distribution function are reproduced. While the model structure is by no means unique or exact, the general correspondence of the model and experimental structures supports the notion that amorphous calcium carbonate forms by ion-rich liquid cluster aggregation (2, 3), followed by dehydration and solidification.

Recent experimental results on CaCO_3 mineralization have shifted the focus of research towards exploration of prenucleation clusters as the crucial precursor species during the mineral formation process (2, 3, 5–8). Both the present simulations and previous work (5) support the experimental measurements that show the initial association of calcium carbonate species in solution is exothermic, leading to a significant reduction in the free calcium ion concentration (2). However, while sedimentation coefficients obtained from seminal analytical ultracentrifugation (AUC) experiments (2,3) were initially believed to provide evidence that the prenucleation cluster population was long-lived and narrowly distributed, more recent interpretations (8, 14) suggest they represent an average over an unknown distribution of clusters detected over the course of many hours. This latter view is supported by sample-to-sample variations that are larger than the apparent cluster size distributions themselves (3), as well as cryo-TEM results (3) revealing both small ($\sim 0.7 - 1.1$ nm) and large clusters (30 – 250 nm) and cluster coalescence.

The findings reported here demonstrate that, if the atomic potentials widely used to simulate the behavior of the calcium carbonate system are accurate, then the system should exhibit a liquid-liquid binodal and this phase behavior will result in cluster dynamics that are consistent with the above ultracentrifugation and TEM observations, as well as those based on light scattering and NMR (9-11). Although the phase separation mechanism depends upon where the system lies relative to the binodal, our molecular dynamics simulations suggest that the spinodal line is easily accessible in experiments at room temperature. Further, on the basis of our Ising model simulations, we would also expect a population of small clusters to coexist with large clusters produced by spinodal decomposition. Although we cannot make quantitative predictions for experimental cluster size distributions, we predict that this distribution of small clusters broadens on approaching the critical temperature (9). Upon crossing the spinodal, growth and coalescence of the dense liquid clusters is predicted and their dehydration produces an amorphous solid exhibiting a structure consistent with that determined

experimentally for ACC.

Based on these findings, we argue that liquid-liquid phase separation can explain the behavior of calcium and carbonate-bearing solutions within the context of established mechanisms without negating long-standing physical concepts (8). Thus, obtaining experimental data that can distinguish stable prenucleation clusters from those produced through liquid-liquid separation is of the utmost importance.

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Fig. 1: Structural, Dynamical, and Energetic Properties of CaCO₃ Clusters. (A) Snapshots taken

from replica-exchange molecular dynamics simulations showing the evolution of polymeric cluster configurations towards denser structures at larger sizes. (B) Plot showing the diffusivity of calcium ions within the cluster species at various stages of growth compared to two solid phases of calcium carbonate, calcite and ACC (from simulation) and the self-diffusivities (experimental) of several common solvents. The error bars represent the mean \pm SEM for $N=6$ simulation trajectories at each cluster size (C) The free energy of the solvated ions as a function of cluster size determined at $[\text{Ca}^{2+}] = [\text{CO}_3^{2-}] = 0.015 \text{ mol / L}$ using the method of Lin and co-workers (22). The symbols represent the mean \pm SEM for $N=240$ free energy calculations at each cluster size.

Fig. 2: Schematic Representation of the Phase Relationships in the $\text{CaCO}_3\text{-H}_2\text{O}$ System. The green horizontal line represents a constant temperature slice through the stability fields as the solution ion activity product is increased. The solubility of all polymorphs is represented by a single solubility line (SL), which bounds the blue undersaturated solution field. This simplification highlights that the solid phases of CaCO_3 (calcite, aragonite, vaterite, presumably ACC) all display the same general retrograde solubility behavior. Indirect nucleation of the solid phases proceeds to the high concentration side of the dashed black liquid-liquid coexistence line (L-L). The bright yellow phase field bounded by the L-L line and the dashed red spinodal line (SP) indicates the conditions where nucleation of the dense liquid phase is possible. In the region bounded by the spinodal line the solution is unstable to fluctuations and liquid-liquid separation proceeds spontaneously.

Fig. 3: Classical liquid-liquid phase separation can generate coexisting populations of small and large clusters. (A) Left: Temperature-density phase diagram for the 2d Ising lattice gas (see also **Fig. S3, & movies S1—S3**). Center: the free energy barrier to phase change diminishes with supersaturation. Right: in the small-barrier (spinodal) regime, an evolving cluster population is generated (see inset

snapshot). The distribution of cluster sizes (colored according to how much of the system's mass the clusters contain) versus time (t_D is the characteristic time for a monomer to diffuse a length equal to its diameter) is shown at each point. (B) Left: Phase diagram for the 3d Ising lattice gas. Right: equilibrated cluster size distributions following the completion of phase separation carried out at a range of densities near (top) and far from (bottom) the critical temperature. ρ_0 is the binodal density. Near the critical temperature, a broad distribution of small clusters coexists with the largest products of phase change. Small clusters are seen even in undersaturated solution. Because of lattice artifacts, cluster size distributions from the Ising model can only be compared to experimental distributions on a qualitative level (25) (supplementary online text).

Fig. 4: **Local Order in the CaCO₃ Polymorphs.** The total X-ray pair distribution functions of crystalline and amorphous polymorphs of calcium carbonate are compared with that of the model ACC structure produced through aggregation of the cluster species identified in this study.

Supplementary Materials:

Materials and Methods

Supplementary Text

Figures S1-S3

Movies S1-S3

References (31-60)