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Review Paper

Pre-nucleation Clusters and Complex Nucleation in Soft Matter and the Potential Roles of Space Experiments

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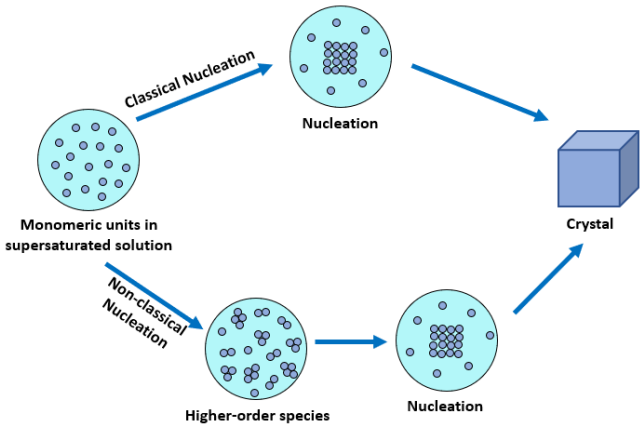
Abstract: For more than a century, Classical Nucleation Theory (CNT) has been used to explain the process of crystallization in supersaturated solutions. According to CNT, nucleation is a single-step process that occurs via monomer-by-monomer addition.
However, recent findings from experiments and numerical simulations have shown that nucleation is a multi-step process that occurs via more complex pathways that involve intermediate species such as ion complexes, dense liquid precursors, or even nanocrystals.



Such non-classical pathways observed in protein solutions, colloidal suspensions and electrolytes are reviewed in this paper. The formation of stable Pre-nucleation Clusters (PNCs) in the crystallization of biominerals is also discussed. In spite of the mounting evidence for non-classical nucleation behaviors, the knowledge about the structural evolution of the intermediate phases and their role in polymorph selection is still limited. It has also been observed that gravitational force interferes with the crystallization behavior of materials thereby posing limitation to ground-based experiments. Microgravity conditions, coupled with containerless processing techniques provide a suitable alternative to overcome these limitations.

Keywords: Classical nucleation theory, Non-classical nucleation, Pre-nucleation clusters, Microgravity, Containerless processing
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1. Introduction

Crystallization is the process of formation of an ordered solid phase from a highly disordered solution. It is one of the most widespread phenomena occurring in nature. The formation of snowflakes[1,2], deposition of minerals in their ores[3], development of skeletal systems of living organisms[4–6], and volcanic activity[7,8] are some examples of consequences of crystallization. Apart from its importance in such natural occurrences, crystallization is also a fundamental step in numerous industrial applications. One of the primary objectives of industrial crystallization is to produce crystals of the desired polymorph, shape, size, and crystal size distribution as they can determine the chemical and physical properties of the final product. The early stages of crystallization are very critical in deciding these aforementioned characteristics. In order to obtain the desired characteristics, it is highly essential to have control over the crystallization process. For many decades, the pharmaceutical industry has utilized crystallization for separation and purification steps in the synthesis of drugs[9,10]. Polymorphism especially plays a decisive role in the bioavailability of drugs[11]. Crystallization of salts such as sodium sulfate and magnesium sulfate in the micropores of bricks generates pressure that could

be large enough to cause damage to buildings and historical monuments[12,13]. In this case too, polymorphism plays a critical role – the pressure induced due to the crystallizing salt depends on the polymorph and researchers are finding ways to inhibit such damage[14–16]. Despite its importance in many natural and industrial processes, and findings from large-scale research for more than a century, the mechanism of crystallization has not been understood completely and several important questions remain unanswered.

Crystallization involves nucleation and growth. Nucleation is a first-order phase transition by which clusters are formed in a supersaturated solution. These clusters, called nuclei, play a central role for crystallization. Classical Nucleation Theory (CNT) has been widely used to describe nucleation and growth for over a century[17]. According to CNT, atoms, ions, or molecules in a solution associate stochastically and structure themselves to form nuclei. These nuclei become stable once the free energy barrier is overcome and begin to grow in size. Despite the fact that CNT has been able to successfully demonstrate process of crystallization, the recent revelations on multiple intermediate phases involved during nucleation seem to lie outside the bounds of CNT[18–20]. Some of the experimental findings and results from molecular dynamic simulations are at odds with the classical interpretation of nucleation and growth in supersaturated solutions, as they suggest that nucleation is not as simple as the single-step mechanism explained by CNT which assumes both the density and structure change simultaneously.

Electrolytic solutions[21,22], proteins[23,24], colloidal suspensions[25–28], and undercooled metallic systems[29–32] exhibit multi-pathway crystallization where the formation of the nucleus occurs in a more complex manner. Several intermediate phases such as dense liquid precursors, amorphous particles, stable ion clusters, as well as structures with short-range order have been discovered in numerous systems. Such pathways which deviate from the classical description of nucleation and growth, rather support the theory of Ostwald's rule of stages which posits that the first phase that nucleated from a supersaturated solution is not the most stable phase[33]. These intermediate phases also play an important role in the crystallization process and in certain cases can even decide the final crystalline polymorph[28,34]. Such findings have attracted a huge amount of interest among researchers to develop a comprehensive theory to explain these non-classical phenomena. With rapid advancements in materials characterization techniques as well as numerical modeling methods, this field of science has gained a lot of momentum in the past few decades. Computer simulations make a powerful research tool to study structural evolution of the intermediate stages during crystallization when used in combination with experimental characterization techniques such as Raman spectroscopy, X-ray scattering, and electron diffraction. Containerless processing techniques[35] have also shown to provide robust ways to induce high supersaturation in electrolytic solutions, thereby enabling non-contact in situ structural investigation in metastable solutions.

In this paper, we discuss some of the most recent findings on non-classical nucleation mechanisms observed in protein solutions, colloidal suspensions and electrolytic solutions. A detailed discussion of crystallization of biominerals via stable nanometer-sized clusters is also presented. Finally, the need for microgravity environments to study crystallization mechanisms is explained, along with the advantages that containerless processing techniques could provide in this field of research.

2. Classical Nucleation Theory

Based on the thermodynamic concepts derived by Gibbs[36], CNT was initially developed to explain the formation of a liquid phase from supersaturated vapor[37]. CNT was later extended to describe the nucleation of crystals from supersaturated solutions. According to CNT, nucleation proceeds via addition of simple monomeric units such as atoms, ions, or molecules due to their stochastic motion in a supersaturated solution. The clusters thus formed in such a manner begin to grow once the system has surpassed the energy threshold for the formation of a stable nucleus.

The two factors that govern the nucleation event are volume Gibbs free energy (ΔG_v) and interfacial free energy (ΔG_s). ΔG_v is the difference between the Gibbs free energy of the liquid and the crystal phases, while ΔG_s is the energy required to form a new crystal-liquid interface. Hence, the total free energy required for the formation of a nucleus is the sum of ΔG_v and ΔG_s. As the solid phase is more stable than the liquid phase in the supersaturated state, ΔG_v is negative and hence reduces the total free energy, thereby promoting the growth of the nucleus. However, ΔG_s, the penalty for forming a crystal-liquid interface – increases the total free energy of the system and favors dissolution of unstable nuclei. The total free energy for the formation of a spherical nucleus of radius r is given by Eq. (1).

$$\Delta G = \Delta G_v + \Delta G_s = -\frac{4\pi r^3}{3v} k_B T \ln(S) + 4\pi r^2 \sigma, \quad (1)$$

where v is the molecular volume, k_B is the Boltzmann constant, T is the absolute temperature, S is the supersaturation ratio, and σ is the interfacial tension between the crystal surface and the solution. Supersaturation S here is the ratio of the concentration of the solution at the time of nucleation to the solubility of the nucleating crystal phase. For small values of r, the positive ΔG_s term is more dominant as it is proportional to r^2 and the total free energy ΔG of the system increases. As r increases, the contribution of ΔG_v, which is proportional to r^3, increases at a faster rate. ΔG reaches a maximum value (ΔG_c) at a certain value of r and decreases as shown in **Fig. 1**. The radius at the maximum ΔG is termed as the critical radius, r_c. At this radius, the system is in metastable equilibrium and will tend to reduce its free energy by either decreasing or increasing its size. Any infinitesimal change in the system can either lead to dissolution or growth of the nucleus. When $r > r_c$, the ΔG_v term begins to dominate, thereby causing a continuous decline in ΔG and results in the spontaneous growth of a stable nucleus. The dependence of ΔG on r is shown in **Fig. 1**. The expressions of r_c and ΔG_c are given by Eq. (2).

$$r_c = \frac{2v\sigma}{k_B T \ln(S)}, \quad \Delta G_c = \frac{16\pi v^2 \sigma^3}{3(k_B T \ln(S))^2} \quad (2)$$

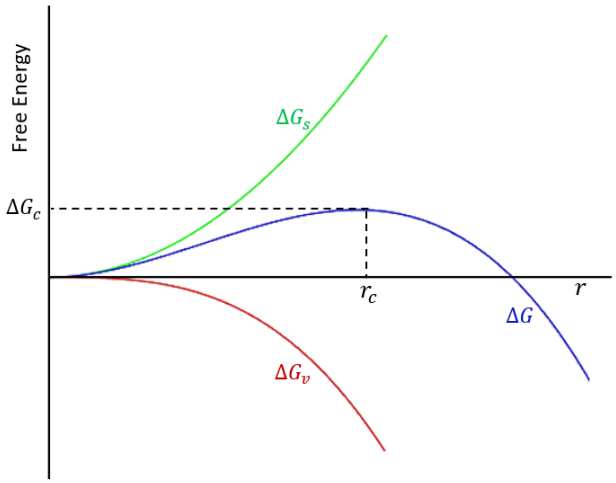

Figure 1. Graphical representation of the dependence of free energy (ΔG_c) of the system on the radius of the nucleus (r).

The nucleation rate or the number of nuclei per unit volume per unit time can be determined by the Arrhenius rate equation given by Eq. (3)[38].

$$J = A \exp\left(-\frac{\Delta G_c}{k_B T}\right) = A \exp\left(-\frac{16\pi v^2 \sigma^3}{3{k_B}^3 T^3 (\ln(S))^2}\right), \quad (3)$$

where A is the pre-exponential factor and it depends on the frequency of collision between molecules/ions [39,40].

Based on these equations, CNT can be applied to predict the nucleation rates of various material systems. However, the predicted and experimental values of nucleation rates have been reported to differ by several orders of magnitude[41,42]. Therefore, it is essential that the underlying assumptions on which CNT was built are closely scrutinized[18]. One of the major assumptions is the "capillarity approximation" that treats the nanoscopic clusters that form the nucleus to be structurally the same as the bulk crystal[43], i.e. the nuclei that are formed in the supersaturated solution and the final crystal have similar properties. The validity of this assumption has been challenged, particularly in cases where crystallization proceeds via multiple intermediate phases where the nucleating phase eventually transforms into the most stable polymorph[21,44]. The solubility of the nucleating phase could differ largely from that of the final polymorph, which leads to errors in determining the value of ΔG_c.

3. Complex Nucleation

In the recent years, there have been many findings[21,26,45,46] on crystallization pathways that do not agree with the traditional explanations based on CNT which considered nucleation to occur via single-step addition of monomeric units such as atoms, ions, or molecules. More complex pathways have been discovered where nucleation involves intermediate metastable[46] or possibly even stable phases[21]. Although there is some disagreement, these observations can be broadly classified as non-classical nucleation pathways in that the change in two order parameters, density and structure, occurs not simultaneously but sequentially through various higher-order intermediate phases ranging from amorphous precursors to nanocrystals (**Fig. 2**)[46]. In this section, such non-classical behaviors observed in protein solutions, colloidal suspensions and electrolytic solutions will be briefly discussed.

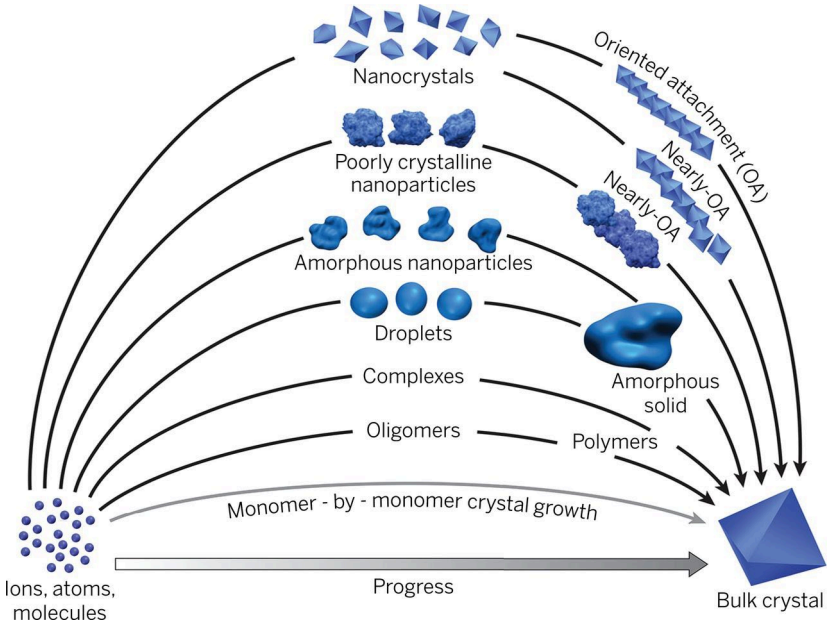

Figure 2. Non-classical nucleation pathways include higher-order intermediate phases ranging from ion complexes to nanocrystals, which is in contrast to nucleation via addition of monomers according to CNT. From Ref. 46. Reprinted with permission from AAAS.

3.1. Protein solutions

Two-step nucleation pathways were initially proposed for crystallization in protein solutions. One of the earliest studies that showed the presence of an alternate pathway for nucleation was by means of a computational study by ten Wolde and Frenkel[47] using Monte Carlo simulations. They showed that close to the critical point of liquid-liquid phase separation, protein crystallization occurred in a two-step process. The first step was the formation of disordered dense liquid clusters caused by density fluctuations, which was followed by structural ordering within these clusters that gave rise to a crystalline phase. A similar phenomenon was depicted by several theoretical studies too[48,49]. The results from experimental studies have also helped to corroborate the two-step mechanism in protein solutions. Galkin and Vekilov showed that the presence of this dense liquid phase affected the kinetics of protein crystallization[45]. In their experiments, lysozyme solutions of concentrations 50 mg/mL and 80 mg/mL were prepared, and the temperature of the solutions was gradually lowered. At around 12°C (for 50 mg/mL solution) and 15°C (80 mg/mL solution), liquid-liquid separation occurred, and the solutions turned cloudy. Structure fluctuations were observed within this dense liquid phase that led to formation of crystalline nuclei. It was also revealed that the nucleation rate depended on the temperature, and was maximum at the critical point. Similar experiments were performed with 20 – 80 mg/mL lysozyme solutions containing 4% w/v NaCl in the presence of 0.05 M sodium acetate buffer[50]. Supersaturation was achieved by decreasing the temperature. The rate of crystal nucleation was observed to be 8-10 orders of magnitude slower than nucleation of dense liquid droplets, thereby concluding that the formation of crystalline nuclei is the rate determining step[50]. Another

experimental study showed that protein nucleation occurred via mesoscopic clusters that are stable with respect to the parent liquid and metastable with respect to the emerging crystal phase[51]. Experimental methods such as Brownian microscopy, dynamic and static light scattering, and laser confocal microscopy were used to characterize the protein clusters. When crystallization occurred in solutions containing these dense, liquid-like clusters, protein crystals showed 3D multilayer growth, while such a mechanism was not observed in solutions in which clusters were removed. These clusters also significantly affected the nucleation rate of protein crystals. When the mesoscopic clusters were filtered from solutions of glucose isomerase (70 mg/mL protein, 100 mM HEPES, 200 mM magnesium chloride and 5.5% PEG 1000) and lysozyme (60 mg/mL protein, 50 mM sodium acetate, 25 mg/mL sodium chloride), a 100- and 10-fold reduction in nucleation rate was observed respectively[51]. A more detailed review of non-classical nucleation mechanisms in crystallization of proteins can be found elsewhere[20].

3.2. Colloidal suspensions

Colloidal systems offer a greater advantage to study the multi-step pathways. Their larger size and ease of characterization enable examining particle aggregation and phase transitions with single-particle resolution, in addition to tunable interactions among the particles[27,52,53]. Zhang and Liu observed two-step crystallization in a colloidal suspension of 0.03% polystyrene particles dispersed in deionized water[26]. The surface potential of the particles was set to -72 mV by adding 10^{-4} M Na_2SO_4 and the sample was sealed between two conducting glass plates. In the presence of long-range attraction induced by an alternating electric field, the colloidal particles were transported to the surface of the plates where they crystallized. The first step was the formation of amorphous dense liquid droplets. Within this dense liquid phase, sub-crystalline nuclei were observed, which then transformed to stable crystalline nuclei. However, the mechanism of formation of the sub-crystalline nuclei in the amorphous dense liquid phase was different from the formation of stable crystalline nuclei. Thermal fluctuations within the amorphous phase led to formation of several sub-crystalline nuclei simultaneously, and coalescence of multiple sub-crystalline nuclei within a single dense liquid droplet resulted in a large stable crystalline nucleus[26]. Based on the findings from an earlier work by Noro et al.[54], the long-range forces played an important role in the two-step crystallization pathway[26]. A similar two-step mechanism was reported during freezing crystallization in a colloidal system consisting of 0.7 μm polystyrene spheres with a polydispersity of 3.5%[27]. Depletion attraction between the colloidal spheres was induced by the addition of 0.04 M hexaethylene glycol monodocecyl ether ($C_{12}E_6$). When the samples were heated to a temperature between 26 and 29℃, the depletion attraction increased due to the presence of the non-ionic surfactant $C_{12}E_6$ and resulted in freezing of the colloidal spheres[27]. The process was observed under a microscope, and it was revealed that at low concentration (area fraction of 17%), a single-step pathway consistent with CNT was preferred. However, in a sample with an area fraction was 30%, amorphous clusters containing up to 30 particles were formed before rapid crystallization. A $3.5k_BT$ reduction in free-energy barrier was observed relative to the classical pathway followed at low concentrations.

In addition to the huge body of evidence supporting the appearance of a dense amorphous precursor phase, there have been several simulation and experimental studies that have reported structures with local order that acted as precursors to the final crystal phase[28,55,56]. Tan and co-workers observed body centered cubic (bcc), hexagonal closed packing (hcp) and face centered cubic (fcc) symmetries in the precursors in their experiments with poly(methyl methacrylate) colloids. The system was composed of 17% nitrobenzoxadiazole-dyed poly(methyl methacrylate) colloids of size 2.2 μm suspended in a mixture of non-polar and weakly polar solvents, with a polydispersity of less than 2.5%. Colloidal crystals were shear-melted and recrystallized at a supercooling of $\Delta T = T_m - T = 0.2T_m$ to $0.4T_m$ below the melting temperature, T_m[28]. The local order of the precursor phases corresponded to that of the emerging nuclei, with the hcp-like component dominating the other two in the prenucleation stage[28]. Strong pathways from hcp-like precursors to either hcp, bcc or fcc solid was also observed. This dominance of hcp-like precursors was attributed to the structural similarity between the disordered liquid phase and the hcp-like precursor. It was also noted that this local order in the liquid precursor phase was short-ranged, which extended to not more than the first shell[28]. In an interesting work by Fang et al., a crystal intermediate was observed during crystallization in a 2D binary mixture of colloids comprising two kinds of polystyrene-poly (ethylene oxide) copolymers – particle A was grafted with a DNA sequence *S*, and particle B with two DNA sequences *S* and *S**[56]. The interactions between particles were altered by varying α, which is the fraction of *S* in particle B, from 0 to 0.5. Using bright-field microscopy, both one-step and two-step crystallization pathways were observed, depending on the value of α. At α values of 0 and

0.5, one-step crystallization to crystals with square and hexagonal symmetry was observed. On the other hand, for $\alpha = 0.3$, a crystal with square symmetry nucleated first and grew switching between square and hexagonal symmetries, before final transformation to a crystal with complete hexagonal symmetry. From these findings, it is evident that concentration[27] and inter-particle interactions[26,56] play a key role in deciding the crystallization pathway in colloidal suspensions.

3.3. Electrolytic solutions

Electrolytic solutions that consist of cations and anions which are much smaller than colloidal particles or polymers have also exhibited non-classical nucleation pathways. Molecular dynamics simulations on crystallization of sodium chloride were conducted to study the crystallization behavior in supersaturated solutions[57]. The simulations were carried out at 27℃ and 1 atm, with 56,000 water molecules and 4000 ion pairs. The concentration of the solution was 3.97 m, which is close to the equilibrium concentration for models using SPC/E water. Prior to nucleation, a disordered region of salt concentration higher than the surrounding solution was noticed at a time of 3 ns. At 7 ns, ordering of the ions was observed within the dense region. The nascent nucleus developed a less ordered arrangement and consisted of a few water molecules, instead of being structurally similar to anhydrous crystalline sodium chloride as predicted by CNT. This result can be explained by the Ostwald's rule of stages as the structure and composition of the nucleus was similar to those of the supersaturated (metastable) solution rather than the final (stable) crystal[33]. The water molecules were gradually expelled from the nucleus as it progressed towards the stable anhydrous phase. Similar to the outcome from simulations, experimental results on crystallization of calcium sulfate showed the evidence for the occurrence of mesoscopic clusters at a concentration of 0.09 m (S ~6)[58]. Supersaturation was achieved by evaporation of water in a climatic chamber at a temperature of 22±1℃ and varying relative humidity. These clusters were spherical, from which it was expected that they were liquid in nature and not crystalline. Although these results were reproducible, the short lifetime of ~1-2 seconds of these clusters hindered further investigation of their structure and role crystallization.

The size of the intermediate phases being much smaller, calls for characterization techniques such as X-ray scattering in order to reveal structural information in the atomic scale. Electrostatic levitation with in-situ Raman spectroscopy and synchrotron X-ray diffraction techniques was used by Lee et al. to investigate the crystallization mechanisms in highly supersaturated potassium dihydrogen phosphate solutions[22]. A ~2-3 mm sized droplet of undersaturated aqueous solution of potassium dihydrogen phosphate was levitated in an electric field generated between two electrodes at ambient temperature of 25±0.2℃ and relative humidity of 42±2%. Evaporation of water from the levitating droplet resulted in an increase in the solution concentration. The Raman peak of PO_2 at 1077 cm^{-1} which was initially symmetric for an undersaturated solution, broadened and became asymmetric with increasing concentration which could possibly be due to polymerization of the ions. In addition to the $P(OH)_2$ at 879 cm^{-1} which corresponds to monomers, the emergence of a new solution phase with local structural order was inferred from a new peak at 896 cm^{-1} that appeared at a concentration of 5.66 m (S ~3.08). Additional evidence was obtained from X-ray scattering data obtained at increasing concentrations. For scattering vector $q < 2$ $Å^{-1}$, with increasing supersaturation an increase in the intensity of the scattering signal could be observed, which is due to the formation of medium-range networks. This complemented the results from the Raman peak at 1077 cm^{-1} becoming asymmetric due to local ordering. In addition to strong evidence for the formation of ordered clusters in the solution, the dependence of the final crystalline polymorph on the level of supersaturation at the time of nucleation could clearly be observed. A solution nucleating below 5.5 m (S ~3), crystallized into a stable crystal with tetragonal structure, while a solution nucleating close to 5.88 m (S ~3.2) crystallized into a metastable crystal. This interesting result, similar to that shown by Fang et al. for colloidal systems[56], underpins the role of nucleation pathways in polymorph selection. Levitation experiments on supersaturated sodium chloride solutions with in-situ X-ray scattering and Raman spectroscopy were conducted by Hwang et al[59]. They observed the presence of locally ordered solute-rich regions of size ~1-2 nm. Evolution of hydration structure of the ions as a function of supersaturation was studied using Raman spectroscopy. Results showed that at higher supersaturation, breakage of hydration structure led to increased ion-ion interactions, thereby inducing structural ordering in the solution prior to nucleation.

Some of the observations of complex nucleation mechanisms in protein solutions, colloidal suspensions and electrolytes were discussed in this section. Several intermediate phases preceding the formation of a nucleus that are shown in **Fig. 2** have been reported for these systems. In the next section, some of the findings

on the formation of stable ion clusters during the crystallization of biominerals will be discussed in detail. A summary of the intermediate phases observed in each of the three systems is presented in **Table 1**.

Table 1. Summary of intermediate phases observed during the crystallization of proteins, colloids, and electrolytes

Material system	Reported intermediate phases
Proteins	Dense liquid phases[23,24,45,47,50] Mesoscopic clusters[51]
Colloids	Amorphous liquid droplets[26] Amorphous clusters[27,52] Precursors with short-range order[28] Metastable crystal intermediate[56]
Electrolytes	Dense liquid regions[57,59] Mesoscopic clusters[58] Pre-nucleation clusters[21,60,61] Ionic polymers[65]

4. Pre-nucleation Clusters

A non-classical pathway, yet quite different from those described in the previous section has been observed in the crystallization of biominerals such as calcium carbonate and calcium phosphate[21,60,61]. A cluster of ions precedes the formation of amorphous phases in both these systems, and the aggregation of these clusters results in the nucleation of one of the polymorphs, as opposed to CNT where nucleation occurs as a consequence of aggregation of monomeric such as ions.

Calcium carbonate is known to exist in three different crystalline forms namely, calcite, vaterite and aragonite, with calcite being the most stable polymorph[62]. A metastable Amorphous Calcium Carbonate (ACC) phase has also been reported to form during the precipitation of crystalline calcium carbonate[63]. Recent experiments on calcium carbonate crystallization by Gebauer et al. have evidenced the presence of stable clusters of ions which aggregate to form ACC, which then transforms to one of the crystalline polymorphs[21]. In their experiments, a dilute solution of calcium chloride was added to a dilute carbonate buffer, and the concentration of free Ca^{2+} in the mixture was measured and compared with the dosed Ca^{2+}. It was observed that the measured free Ca^{2+} in the solution was smaller than the dosed amount, indicating that a considerable fraction of the Ca^{2+} bind with the CO_3^{2-}. In the pre-nucleation stage, about 35% of the dosed Ca^{2+} were bound at a pH of 9, and this number increased to 70% at a pH of 10. Analytical Ultracentrifugation (AUC) experiments revealed that these cluster of ions were ~2 nm in diameters and each cluster comprised of 70 Ca^{2+} and CO_3^{2-} ions. These clusters were eventually named as Pre-nucleation Clusters (PNCs). However, certain characteristics of these PNCs differentiate them from other precursor species such as amorphous liquid precursors and ion pairs discussed in the previous section[44,64].

PNCs are considered as stable solutes that do not have an interface with the surrounding solution, i.e., they are not a separate phase but co-exist along with the ions in the solution[21,44]. Thermodynamic analysis of the equilibrium constants of formation of PNCs revealed that they are stable solute species, which possess minimum Gibbs energy. This characteristic of PNCs particularly differentiates them from the dense liquid metastable precursor phase that was observed in the crystallization of proteins[45]. In Gebauer's experiments, larger PNCs of size ~5-6 nm were detected in the early post-nucleation stage[21]. In addition to this, smaller clusters that were seen in the pre-nucleation stage could not be detected. It was inferred from this result that nucleation occurs as a result of aggregation of PNCs and not via growth. If nucleation were to proceed via growth of clusters, the sedimentation profiles of AUC experiments would indicate small clusters that grew in size over time[44].

Molecular dynamics simulations showed that the PNCs comprise chains of ionic polymers and they were held together as a consequence of ion-ion interactions[65]. These chains of ionic polymers should not be confused with similar nomenclature used for organic polymers where covalent bonds are prevalent. Results also showed continuous breakage and restructuring of these chains in the PNCs, which reveal the dynamic nature

of PNCs. Several configurations such as branched structures, rings and chains could be observed among the ions in the PNCs.

Another interesting revelation is the presence of short-range order in the PNCs that corresponded to the final crystalline polymorph. The aggregation of PNCs was succeeded by nucleation of ACC, which then transformed into either calcite or vaterite[21]. The ion products of the nucleated ACC phases as well as results from polarized light microscopy showed that two types of ACC phases nucleated. Stability of the PNCs had an influence on the stability of the following ACC phase and on the final crystal phase. PNCs that were more stable yielded a more stable ACC phase, which transformed to calcite, while less stable PNCs resulted in nucleation of less a stable ACC phase that transformed to vaterite. Transmission Electron Microscopy (TEM) images of the quenched ACC phases revealed nanostructural features in the length scale of 2 nm[34]. Further evidence for short-range order in the ACC phases was obtained from Extended X-ray Absorption Fine Structure (EXAFS), infrared spectroscopy and nuclear magnetic resonance spectroscopy[34]. The more stable ACC phase showed short-range structural features that were closely related to calcite, while the less stable ACC phase exhibited features that could be linked to the structure of vaterite. It could thus be inferred that these structural features in ACC were transferred from the corresponding PNCs.

More supporting evidence for PNC-based pathways was obtained from other experimental work on calcium carbonate and calcium phosphate. Pouget et al. investigated the template-controlled crystallization of calcium carbonate on stearic acid monolayer[60]. Combining cryogenic Transmission Electron Microscopy (cryo-TEM) and low-dose Selected Area Electron Diffraction (SAED), they showed the existence of PNCs in the nucleation process. PNCs in the size range of 0.6-1.1 nm were observed in the cryo-TEM images, and over time, they aggregated to form larger clusters (~30 nm). SAED patterns suggested that these clusters were indeed ACC, and were present throughout the solution and not just on the organic layer, indicative of homogeneous nucleation of ACC. These ACC clusters that nucleated on the organic layer grew in size and nanocrystalline domains were observed inside the amorphous particles. The structure of these domains corresponded to vaterite. A similar pathway was observed during the crystallization of calcium phosphate on arachidic acid monolayer[61]. Aggregation of PNCs followed by nucleation of Amorphous Calcium Phosphate (ACP) and further transformation to a crystalline form was observed[61]. However, in this case, the different stages involved in the aggregation of PNCs before the formation of ACP were distinguishable. PNCs which were present as loose networks in the solution, formed densified spherical domains once they were in contact with the monolayer. In a later stage, these spherical domains transformed into ACP particles.

5. Need for Microgravity Experiments

The effect of gravitational force on crystallization of proteins, colloids and electrolytes is well known[66–68]. In ground-based experiments, concentration gradients in protein solutions result in convective transport of molecules, which interferes with the crystallization behavior[66]. It induces fast transport of protein molecules to the surface, thereby not allowing enough time for the molecules to orient themselves for structured crystal growth. However, in the microgravity conditions, the transport of molecules is dominated by diffusion which is slower than convective transport, thereby resulting in crystals with good crystallinity[69]. Another factor that hinders systematic investigation of crystallization on earth, especially in solutions of macromolecules is sedimentation of crystals to the bottom of the container[66]. Due to the difference in the densities of the crystals and the solution, the nucleated crystals tend to sediment on the bottom of the container, and the growth of these crystals is influenced by the surface of the container. This will affect the crystallization behavior and alter the morphology of the growing crystal[70]. Such effects can be avoided in experiments conducted in microgravity conditions.

Parabolic flights, sounding rockets and drop tower techniques have been widely used to realize microgravity conditions. Drop towers provide microgravity environments for ~9 seconds[71]. Parabolic flights have been used to study crystallization, but the duration of microgravity is restricted to around 22 seconds[72]. Crystallization experiments on monodispersed polystyrene particles were conducted in microgravity conditions for 20 seconds using parabolic flights, and the crystallization behavior was compared with experiments performed using ground-based methods[73]. Sounding rockets provide microgravity conditions for slightly longer durations of about ~ 5-6 minutes[74]. Ishikawa et al. carried out experiments on colloidal crystallization using sounding rockets under microgravity conditions that lasted 6 minutes[75,76]. They observed the formation of percolation clusters or ordered domains that had an extended network throughout the solution[76]. It was also reported that these clusters were not as compact as the final crystal[76]. Although such

techniques have been widely used to access microgravity environments, these short intervals of microgravity are not sufficient enough for investigation of structural evolution of the supersaturated solutions. The International Space Station (ISS) on the other hand can achieve constant microgravity from months to years[77].

Containerless processing techniques have been employed by materials scientists for more than three decades owing to the plethora of advantages they offer over conventional materials processing methods. With the absence of substrates and container walls, we can get rid of heterogeneous nucleation sites that lower the free energy barrier for nucleation as well as the uncertainties in nucleation behavior induced by the surface of the container. Some of the well-known and widely used containerless processing techniques are Electrostatic Levitation (ESL), Electromagnetic Levitation (EML), acoustic levitation and Aerodynamic Levitation (ADL). Non-contact techniques have been used in the measurement of creep properties of refractory materials, investigation of solidification pathways of alloys, structural studies of undercooled melts and measurement of thermophysical properties[78-80]. More recently, containerless methods have been developed to study the multi-pathway crystallization mechanisms in highly supersaturated electrolytic solutions[59,81]. The results from such containerless processing can farther be extended microgravity experiments by isolating crystallization from the influence of gravity. Currently, the Japanese Aerospace Exploration Agency (JAXA) is conducting a series of containerless experiments using the Electrostatic Levitation Furnace (ELF) aboard the International Space Station[82-85]. JAXA has been studying the thermophysical properties of various kinds of molten metallic alloys and oxides in collaboration with several universities in Japan and in the United States. Meanwhile, the European Space Agency (ESA) and the German Aerospace Center (DLR) are in charge of space experiments using their ISS-EML[86-91]. With numerous international collaborators, non-classical nucleation and thermophysical properties of various metallic alloys have been studied[30,92-94]. Both ELF and ISS-EML have served as effective containerless processing tools for processing metals in microgravity environments. Meanwhile, studies on nucleation and crystallization in microgravity have been conducted using small confinement which could have affected the intrinsic physics[95-97]. By utilizing the technical know-hows of ISS-EML and ELF, levitation methods in microgravity conditions can be extended to soft matter as well, which would help overcome the limitations of conventional experiments used to study crystallization. At this point, developing a containerless processing capability for soft matter at ISS would greatly benefit to better understanding the nucleation and growth of various soft matter of high industrial significance.

6. Summary

Crystallization is a frequently occurring phenomenon in nature. It is also a fundamental step in many industrial applications. Despite its ubiquity and importance, crystallization has not been completely understood and several critical questions still remain unanswered. For more than a century, nucleation and growth have been described based on the CNT, which states that nucleation is a single-step process which occurs via addition of monomers in a supersaturated solution. CNT assumes that the structure of the nucleus is identical to the final crystal. However, the drawbacks of CNT, which are a consequence of many simplifying assumptions were later recognized and challenged by researchers. More recently, researchers have observed that nucleation in proteins, colloidal suspensions and electrolytic solutions did not proceed in a single step, but rather occurred in two or more steps that involved higher order species such as ion complexes, dense liquid amorphous phases, or nanocrystalline particles which were structurally different from the final crystalline phase. Although there has been mounting evidence in favor of multi-step pathways to nucleation, more investigation is indeed required to understand such non-classical behaviors and the role of the intermediate phases in polymorph selection. It has been observed that gravitational force interferes with the transport of molecules in the solution and the sedimentation of particles on the substrate surfaces affects the crystallization behavior of materials. To overcome such limitations, containerless processing techniques in microgravity environments provides a promising alternative to ground-based methods.

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Conflicts of interest

The authors declare no conflict of interest.

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