Prenucleation Clusters and Non-classical Nucleation

by Denis Gebauer* and Helmut Cölfen*

University of Konstanz, Department of Chemistry, Physical Chemistry,
Universitätsstrasse 10, Box 714, D-78457 Konstanz (Germany)
Denis.Gebauer@uni-konstanz.de (+49-(0)7531-88-2169)
Helmut.Coelfen@uni-konstanz.de (+49-(0)7531-88-4063)
Fax: +49-(0)7531-88-3139

Keywords

amorphous precursors, biomineralization, biomimetic mineralization,
crystallization, nucleation, prenucleation clusters

Abstract

In this contribution, we review the recent literature on an alternative crystallization pathway involving stable clusters prior to nucleation and show that the prenucleation cluster pathway is a truly non-classical concept of nucleation. Prenucleation clusters are solutes with "molecular" character in aqueous solution. It becomes evident that the stable clusters may have been concealed by the so-called ion pair concept and activity effects. We show that non-classical nucleation *via* stable prenucleation clusters is a highly valuable concept for a novel understanding of phenomena observed in bio- and biomimetic mineralization, which however may hardly be rationalized by means of classical nucleation theory. The importance to combine experimental and theoretical studies is emphasized.

Introduction

Crystallization is a ubiquitous phenomenon. Many of the solid compounds that we handle everyday are crystalline, and in fact we do lean ourselves on crystals. Our skeletal support is based on crystalline calcium phosphate, carbonated hydroxyapatite, which is the major constituent of bone besides collagen, proteoglycans, and few non-collageneous proteins [1]. A huge amount of industrial products and applications are based on crystallization, ranging from fillers and pigments to construction materials and products of the food industry. Crystallization is the basis of the structure determination of biological macromolecules [2], which is an important discipline of molecular biology.

It appears impossible to reflect the abundance of crystallization phenomena by means of these few examples, but they underline the importance of crystallization for society. Osteoporosis is directly connected to crystallization, while possible treatments of countless other diseases may be based on the structure determination of biological molecular machineries [3,4], and with it, are indirectly linked to crystallization. Not only protein crystallization but also novel bottom-up approaches to advanced materials that may be used in (nano)technology depend on the understanding of the mechanisms that underlie crystallization. In this context, a look at nature has proven to be a highly promising approach. Biominerals, *i.e.* composites of minerals and organics that are formed by organisms (bone, teeth, shells, etc.) [5], show unmatched materials properties, tailor-made in evolution with respect to their particular function [6]. A well-known example is the great toughness of nacre (mother-of-pearl) if compared to geological crystals [7], which is based on a sophisticated microstructure of the biogenic composite. Learning from nature, and mimicking the underlying principles for the design of novel materials or applications

is a successful and well-employed strategy [8–15]. However, numerous studies, especially in the field of bio- and biomimetic mineralization, challenge the classical view of nucleation [16,17] and growth that is presented in crystallization textbooks [18].

The classical view considers the different stages of crystallization to proceed *via* attachment of the basic monomers, which (depending on the crystal) can be atoms, ions or molecules. This notion is incompatible with the particle-mediated processes of so-called non-classical crystallization pathways with nanoparticles as monomers that can lead to the formation of mesoscopically structured crystals, abbreviated mesocrystals [15,19–22]. In some instances, these mesocrystals that also occur in biominerals [20,23–25] may be difficult to discriminate analytically from classically grown single crystals, since the nanoscopic building blocks can be mutually oriented in perfect crystallographic register, and partially fuse crystallographically. Moreover, amorphous precursor [26–28] and liquid precursor [28,29] pathways were identified in biological as well as synthetic systems. These pathways offer the possibility to form crystalline bodies with complex, off-equilibrium morphologies [26,28].

It is evident that the most fundamental step in crystallization is nucleation - the formation of the first nuclei in a system that has become supersaturated. Control of crystallization, in principle, requires nucleation control. However, classical theories cannot explain a number of phenomena associated to nucleation. We will summarize the classical concept to point out why the stable prenucleation cluster pathway is truly non-classical. The term "non-classical nucleation" is used here in order to emphasize that the prenucleation clusters are different to the species envisaged in the classical concept outlined below. We note that there are alternative uses of the term, especially in atomistic theories of nucleation, which dispense the concept of cluster formation

and consider statistical fluctuations of molecule number densities [16]; we do not intend to imply this particular notion by "non-classical nucleation".

In the present paper, we review the field of prenucleation clusters and give an outlook on future work, but also include early and more recent indications for these clusters from the literature. Naturally, there is a certain focus on calcium carbonate, because it is the physicochemically best analyzed system with respect to prenucleation clusters so far. Importantly, our considerations suggest why the prenucleation clusters have been concealed for a long time, even though the crystallization of calcium carbonate has been an extensively studied field for more than 100 years. We also review theoretical modelling and simulation results, because, obviously, these approaches are crucial for an understanding of the complex physical-chemical basis underlying stable cluster formation, non-classical nucleation and crystallization. The assumptions of classical nucleation theories appear too simplified, and eventually, formulation of a revised nucleation theory that includes stable prenucleation clusters without simulation and modelling approaches is hard to imagine. The aim of this review is to give a comprehensive perspective, which shows that the stable prenucleation cluster concept is highly valuable. It promises a more detailed understanding of nucleation in general, formation of polymer-induced liquid precursors, mesocrystals, amorphous intermediates with distinct structures, and of many other observations that may hardly be rationalized by means of classical nucleation theory.

Classical Nucleation Theory (CNT)

CNT is a concept derived in the 1930s by Becker and Döring [30]. This work was based on a first quantitative treatment of nucleation by Volmer and Weber [31], which was in turn based on ideas of Gibbs [32,33], and that was partially improved by

Farkas [34]. CNT was originally derived for the formation of nuclei from supersaturated water vapour and was conceptually transferred to the nucleation of crystals from solution.

CNT assumes that the bulk energy of a nascent nucleus drives nucleation, where its structure is that of the macroscopic bulk material. However, the generation of a phase interface, and with it, of interfacial tension, impedes nucleus growth. Here, the interfacial tension of a macroscopic body with its bulk structure is assumed. This is the very debatable “capillary assumption” [17]. Because the total bulk and surface energies of the nascent particle scale with the cube and the square of the radius of the nuclei, respectively, the bulk energy begins to balance the energetic costs due to the generation of a phase interface at the so-called critical size (Figure 1). Smaller nuclei are thermodynamically unstable and dissolve again. The change in free enthalpy for the formation of (pre-) critical nuclei is positive, hence, their formation is thermodynamically improbable, and can only occur through stochastic fluctuations on microscopic lengthscales. The classical (pre-) critical nuclei can thus be perceived in analogy to the notion of activated complexes in chemical kinetics [35]. In consequence, classical (pre-) critical nuclei are rare species and the underlying cluster size distribution is characterized by an exponentially decaying function with an average of size of monomers or dimers of the underlying molecular units [16,17].

Likely owing to severe simplifications, CNT often fails in quantitative predictions of nucleation phenomena [36], and there have been several reconsiderations of CNT, e.g. [37]. Under purely qualitative considerations, CNT is a very successful concept for the comprehension of nucleation phenomena in general, though.

The Stable Prenucleation Cluster Concept

To begin with, we summarize the basic and important notions established in the first report of stable prenucleation clusters [38]. The basic experiment was based on the addition of dilute calcium chloride solution into dilute carbonate buffer at a constant rate of 10 μL per minute, while the calcium potential was recorded, and the pre-set pH-value of the carbonate buffer was kept constant by means of titration with dilute NaOH solution. The pH-range investigated by means of this experimental setting is limited to a range between pH ~ 9 and pH ~ 10 , because the fraction of carbonate ions in the buffer equilibrium becomes minor below pH 9, and concurrent precipitation of calcium hydroxides can occur at the high pH limit [38]. The amount of calcium detected throughout the course of this experiment is shown in Figure 2, *a priori* assuming that the solutions are ideal (that is, the activity coefficient is assumed to be 1). That this assumption is justified will be rationalized below. At all pH values, the amount of free calcium increases linearly until a critical point is reached, and nucleation occurs (indicated by arrows in Figure 2). After nucleation and a short period of equilibration, the amount of calcium is essentially independent of further added calcium; this is, because a phase interface has been established, and the dissolution equilibrium of the formed particles determines the ion product in solution. After nucleation, the amount of free calcium decreases with pH since the fraction of carbonate in the buffer increases with pH, too. Thus, the resulting solubility product is constant in different pH-intervals (cf. below). Before nucleation, that is, in the solution single-phase system, a significant amount of calcium is bound (Figure 2). It is very important to note that this is already true for the undersaturated solution where no thermodynamic driving force for nucleation exists. This already indicates that the prenucleation clusters, in which the ions are bound, are non-classical species.

As per post-nucleation, the pre-nucleation calcium binding increases with pH, indicating that it is based on an interaction with carbonate. Evidence of thermodynamic equilibrium between calcium and carbonate (in pre- and post-nucleation stages) is thus inferred from the effect of pH on the calcium binding according to the principle of Le Chatelier [38]. Thermodynamic equilibrium brings about reversibility, which may be easily tested: As long as nucleation is avoided, the pre-nucleation calcium binding can be reversibly influenced by changing the pH, by diluting or adding calcium [39]. Importantly, as-observed thermodynamic equilibrium implies that the activation barrier of the formation of this calcium carbonate species is insignificant compared to thermal energy. The binding of carbonate ions during the pre-nucleation stage (as well as after nucleation) can be determined by means of pH titration. In this context, it is important to note that titration techniques in general are not affected by activity effects as the activity coefficients of titrants and analytes cancel out in the solution where titrant and analyte are combined (the general equation of titration is $v_t \cdot V_t \cdot c_t \cdot \gamma_t = v_a \cdot V_a \cdot c_a \cdot \gamma_a$, where indexes 't' and 'a' refer to titrant and analyte, respectively, with stoichiometric factor, v , volume, V , concentration, c , and activity coefficient, γ . Only average activity coefficients are accessible in solution, which are valid for the cations and anions, i.e. $\gamma_t = \gamma_a = \gamma$).

The evaluation shows that within minor deviations, equal amounts of calcium and carbonate are bound before and after nucleation (Figure 3). For the phase precipitated after nucleation, this is expected since bicarbonate does not play a role in solid calcium carbonates under the given conditions, and there may be only minor deviations from stoichiometry owing to the requirement of electroneutrality of phases. This means that carbonate titration confirms the calcium potential measurements independently. In turn, this shows that the *a priori* assumption of ideal solution states

does hold, that is, the reduction of calcium concentration is not due to a decrease in ion activity, but due to 1:1 binding of calcium and carbonate. This notion, as well as the fact that binding of bicarbonate does not play a role in the prenucleation stage at pH 9 - 10 (which was tacitly assumed above), has been corroborated by means of mass-spectrometric evidence [40].

An important consequence of the above is that the prenucleation clusters have to be considered to be solutes. This originates from the fact that as soon as a phase interface is present, the ionic concentrations are determined by the dissolution equilibrium across this phase interface. In equilibrium (that is, solution/solid equilibrium but not necessarily equilibrium according to Gibbs' phase rule), the "concentration of a phase interface" is of no importance. Hence, after nucleation, a constant solubility product is established: solubility products are independent of the amount of precipitate (there is a correlation with the size of the precipitated particles through Gibbs-Thomson effects that becomes important at the nanoscale). There is the possibility that the prenucleation species are off-equilibrium species though, for instance particles that are kinetically trapped and cannot grow or dissolve. However, this possibility can be excluded owing to the thermodynamic reversibility of the observed ion binding (cf. above). Hence, the prenucleation state conforms to the thermodynamics of a single-phase system, that is, the prenucleation species do not have a phase interface.

The ion binding as determined by potential measurements and pH-titration does not allow for the size determination of the prenucleation species; the as-observed ion binding could well be due to the formation of the classical ion pair (cf. below). The size determination, and with it, the notion of prenucleation *clusters*, is based on analytical ultracentrifugation (AUC) of solutions drawn at different times in the

prenucleation stage. Reproducibly, a second species besides ions is detected, which is significantly larger than ion pairs (Figure 4). Assuming spherically shaped clusters with the density of amorphous calcium carbonate (ACC), the determined sedimentation coefficients can be transformed into average cluster diameters, which are approximately 2 nm for the basic cluster species, corresponding to roughly 70 ions combined in a single cluster on average (a thorough discussion is given in ref. [38, supporting online material, pages 7-12]). Since the density of ACC can be regarded to be an upper limit of the density of prenucleation clusters, the as-determined diameter of 2 nm poses a lower limit in terms of size (cf. below).

Moreover, the time-dependent sedimentation profiles of the solutions detected at different times in the prenucleation stage conform to the sedimentation of multicomponent systems, wherein distinctly larger clusters are detected with good statistical difference closer to the point of nucleation (Table 1). This is evidence that nucleation proceeds *via* cluster-cluster aggregation rather than *via* growth of single clusters, since this would be indicated by a continuously increasing cluster size of the smallest species, which is not observed. As discussed below, this cluster-aggregation based mechanism has been corroborated for heterogeneous nucleation by means of cryogenic transmission electron microscopy. AUC can be employed to determine the density of sedimenting species by changing the density of the solvent [41], and yet unpublished data utilizing H₂O/D₂O mixtures in AUC analyses of cluster solutions indicate that the density of the clusters is even lower than that of ACC. This implies that the clusters contain a large amount of water, and the data suggest that the basic cluster size is in the range of 3 nm rather than 2 nm in diameter. It is important to note that AUC evidences the "molecular" character of the prenucleation clusters [42]. We note that the timescale of AUC experiments is in the range of hours, and since the

prenucleation cluster dynamics are expected to be orders of magnitude faster, it is impossible to infer reliable cluster size distributions from AUC experimentation (as opposed to macromolecules with covalent bonds). Thus, we emphasize that under these conditions, the cluster size determined by AUC is an average value, and that the underlying cluster size distribution could be very broad. However, cryo-TEM analyses indicate a rather narrow size distribution of pre-nucleation clusters (cf. below).

The average size determined by AUC is significantly larger than the average size of the ion association species considered in CNT, showing that the pre-nucleation clusters are non-classical species. The *classical* cluster size distribution profile decays exponentially, and the average size of classical pre-critical nuclei is thus generally given by the monomer or dimer of the molecular units. The pre-nucleation clusters are larger than classical pre-critical nuclei, because they are considered to be thermodynamically stable, as opposed to the metastable critical nuclei (cf. below). If the clusters were metastable, they could not be detected by means of AUC, because in that case the average size would be that of ions or ion pairs, which cannot be discriminated by means of AUC.

The pre-nucleation cluster equilibrium can be evaluated assuming a multiple-binding equilibrium [43] that does not require knowledge of the cluster concentration and stoichiometry that are inaccessible through potential measurements and titration. Evaluation of the ion binding by means of this speciation model shows that the carbonate ions in the buffer of the pre-nucleation stage in fact have more than one binding site for calcium ions, underlining that the data do not conform to the ion pair concept. Consistent with the observation that, depending on pH, a majority of calcium ions is bound in the pre-nucleation clusters (Figure 2), the physicochemical

characterization shows that the clusters are thermodynamically stable with respect to the state of free ions as well as ion pairs (equilibrium constant $K \gg 1$, *i.e.* $\Delta_R G < 0$). The stability of the clusters, though, depends on the pH level (Figure 5). Hence, the state of clusters represents a global minimum in free enthalpy in the solution phase, but not in the solution/solid two-phase system. The solution state can become metastable with respect to formation of the solid phase, here, through addition of more and more calcium ions, and eventually nucleation occurs. It is interesting to note that calcium carbonate is nucleated only if the solubility of ACC is exceeded, and indeed, ACC is formed initially (see below). However, it is possible that crystalline modifications are nucleated directly from prenucleation clusters if not the solubility of ACC but that of crystalline modifications is exceeded [44]. This pathway may be kinetically inhibited, and require substantial waiting periods in the case of homogeneous nucleation, or, for instance, the presence of suitable phase interfaces to accelerate the process in the heterogeneous case. Hence, our observations do not necessarily mean that ACC is *always* nucleated initially as an intermediate phase.

At lower pH, the clusters are thermodynamically more stable than the clusters that are formed at higher pH-values (Figure 5). In the following section, we summarize the indirect evidence that these different cluster stabilities may in fact correspond to different cluster structures.

Evidence of Distinct Structures in Prenucleation Clusters

We emphasize that up to now, no direct structural characterization of prenucleation clusters has been achieved. However, strong indirect evidence is mounting that distinct structures are present in prenucleation clusters. Evaluation of the ion products of the nucleated phases reveals that more stable ACC (ACCI, more stable is equivalent to less soluble) is nucleated from more stable clusters at lower pH

values, while a less stable ACC (ACCII) is nucleated from less stable clusters at higher pH values (Figure 6) [38]. We note that the solubilities of these ACCs are one order of magnitude lower than previously determined [45]. If ACCI and ACCII are quenched from metastable solutions of calcium carbonate of different pH by a sudden change of solvent, nanoparticles of ACC are obtained [46] that show nanostructural features in the range of 2 nm in certain focus planes in transmission electron microscopy (TEM) (Figure 7). These nanostructural features may be based on prenucleation clusters, preserved owing to the manner of precipitation through quenching.

Structural characterization of the ACCs by means of solid-state NMR, extended X-ray absorption fine structure (EXAFS) analyses (Figure 8) and IR spectroscopy evidence short-range structural analogies in calcite/ACCI and vaterite/ACCII [46] as was speculated previously [38]. Consequently, the notion of proto-calcite and proto-vaterite ACCs (abbreviated pc-ACC and pv-ACC, respectively) has been introduced [46]. The presence of distinct short-range structures in ACCs is well-known from biogenic [26,47,48], but also synthetic specimens [28,49], and the results suggest that these proto-crystalline structures are intrinsic to amorphous precursors. That is, the proto-structures in ACC are likely based on distinct structures in prenucleation clusters, which are carried over to ACC from the clusters upon quenching. The presence of distinct structures in prenucleation clusters is also indirectly evidenced by distinct differences in the time of nucleation found at different pH-values in the presence of peptides that specifically bind to different structures of calcium carbonate [50]; peptides that bind vaterite inhibit nucleation more efficiently at pH values that correspond to vaterite-like structures present in prenucleation

clusters. At pH-values that suggest calcite-like structures in prenucleation clusters, nucleation is inhibited less efficiently by these peptides.

The challenge in this context is to conceive of a structural form that relates to vaterite or calcite with regard to the short-range order but does not exhibit the properties of bulk structures (cf. below). Similar scenarios of structural "pre-formation", on the level of molecular precursors, have been suggested, for example, for the formation of metastable phases of zinc oxide [51], or of indium sulfide [52] and gallium sulfide [53] films formed *via* vapor deposition.

Relevance of Proto-Structures in Polymorph Selection

The crystallization of the proto-crystalline ACC is not "pre-determined", that is, *e.g.*, proto-calcite ACC does not necessarily transform into calcite [46]. This is due to kinetic control in the calcium carbonate system; the differences in stability among the different polymorphs are actually small and comparable to thermal energy [54], while the distribution of structural environments in the proto-structured ACCs is broad, and also low amounts of vaterite-like sites are found in the proto-calcite ACC (NMR, Figure 8). A randomly formed nanoparticle of vaterite that is slightly larger than a nanoparticle of calcite can be actually thermodynamically more stable than the particular calcite nanocrystal (Gibbs-Thomson effect). Kinetic control of crystallization, hence, is well-known to the experimentalist; changes of extrinsic parameters, as simple as the choice of glassware, may distinctly influence the outcome of experiments. Even though the transformation of ACC [55] *in vivo* can be unambiguous in terms of polymorphism [56], there are also reports that biogenic ACC transforms in an ambiguous manner, *e.g.* apparent in the formation of mainly aragonite and traces of vaterite from ACC in the shell of the freshwater snail *Biomphalaria glabrata* [47].

These observations suggest that proto-crystalline structures in biogenic and synthetic ACCs are not the sole basis of polymorph selection, but that the combination of pre-structuring with additives is important, as these specimen always contain a certain amount of organic additives (note that pc-ACC and pv-ACC are additive-free [46]). Distinct proto-crystalline structures in amorphous intermediates [46], but probably also distinct structures in prenucleation clusters (as suggested by the indirect evidence outlined above), render specific interactions with additives or surfaces possible [50,57], in this way facilitating polymorph selection. An example is given by the influence of poly(acrylic acid), which is a polyanion commonly used as scale inhibitor as discussed in ref [57], on crystallization of calcium carbonate. Even at very low concentrations of polymer that lead to complexation of minor (in fact, at this polymer concentration undetectable) amounts of free calcium ions, nucleation is effectively inhibited (Figure 9). This is conform to the notion that nucleation of calcium carbonate can proceed *via* aggregation of prenucleation clusters, and not *via* clustering of free ions as assumed in CNT, as this effect can only be explained by colloidal stabilization of the prenucleation species. We note that the prenucleation clusters are considered to be thermodynamically stable, however, the *solution* with the clusters can become metastable when it has become supersaturated. By colloidal stabilization of clusters, here, we thus mean stabilization of the cluster containing solution against cluster aggregation and with it, phase separation yielding nanoparticles of ACC. Furthermore, the precipitated particles are also stabilized against growth, probably due to surface-adsorbed polymer, because the nucleated particles do not grow. The particles are kinetically stabilized (cf. above) and the solubility product is not maintained (Figure 9). The amount of calcium in solution increases again upon addition of further calcium. Eventually, again, a critical state is

reached, nucleation occurs for a second time, and this process is repeated (Figure 9). After each successive nucleation event, the effect becomes weaker and weaker, indicating that the polymer additive is consumed during the process. Interestingly, the ion product drops to the value corresponding to the pv-ACC after the first two nucleation events, but it reaches the value of the solubility of pc-ACC after the third nucleation (Figure 9). Since phase-pure calcite is obtained, as opposed to the reference experiment at the same pH and after the same time of ripening, this means that even though both proto-structured ACCs appear to be nucleated in parallel, the less stable pv-ACC is stabilized against transformation. This is likely to be due to specific interactions with poly(acrylic acid), and the proto-vaterite precursor dissolves for the benefit of more stable phases, here pc-ACC and/or calcite. Precursors with proto-calcite structuring may crystallize though, and in this manner we suggest that polymorph selection is facilitated in this simple synthetic system. There exist different possibilities of similar specific additive interactions with the proto-crystalline structures that may become efficient before, during and after nucleation and lead to polymorph selection; interestingly it appears that in all cases investigated so far, the particular polymorph is stabilized that shows the weakest interaction with the respective additive [50,57,58]. Importantly, the different modes of additive action can be categorized and quantified, which we regard as an important step for a novel understanding of the processes that underlie additive-controlled crystallization [57]. Similar mechanisms may be efficient in biomineralization processes that lead to the formation of phase-pure polymorphs from proto-structured amorphous intermediates. We emphasize that the results strongly suggest that proto-crystalline features in ACC are not induced by additives, that is they are intrinsic to ACC, and are most likely based on the underlying equilibria of prenucleation clusters, from which the ACCs are

formed [46]. It appears that the additives merely stabilize certain structures and in this way control the course of mineralization, that is by a combination of thermodynamic and kinetic means [54,59]. In this way, unambiguous transformation of proto-structured ACC *in vivo* is rather based on the presence of bio(macro)molecules that specifically interact with these structures than on the proto-structuring alone. It is important to note that some synthetic ACCs do not display distinct proto-crystalline structuring [60,61]. This is readily explained by the prenucleation cluster equilibrium; the unstructured synthetic ACCs were prepared from high levels of supersaturation, thus precipitated immediately from solution, while the prenucleation clusters did not equilibrate. However, equilibrated prenucleation clusters appear to be the basis for the proto-structuring.

Cryogenic Transmission Electron Microscopy Studies

Cryogenic transmission electron microscopy (cryo-TEM) has proven to be a highly valuable and powerful tool when it comes to the analyses of non-classical nucleation and crystallization phenomena. In fact, it is possible to obtain *in situ* images of the prenucleation clusters, as was first shown for calcium carbonate by Sommerdijk *et al.* [62]. Imaging of freshly prepared calcium bicarbonate solutions from the Kitano method [63], which is exactly saturated with respect to calcite, reveals the presence of prenucleation clusters (Figure 10 a, b) that are approximately 0.6 nm in diameter on average (Figure 10 c), whereas the cluster size distribution, though based on limited statistics, is rather narrow, in between approximately 0.5 and 1.1 nm. The clusters appear to not grow upon generation of supersaturation (they are of comparable sizes in all images which were taken at different solution states prior and post nucleation), as opposed to the classical picture (cf. above) and are still present after nucleation [62], corroborating the notion that they form at any given

concentration of calcium and carbonate ions according to the law of mass action [38]. AUC shows that nucleation of the nanoparticles of ACC in solution (which afterwards attach to an organic template and crystallize) proceeds *via* aggregation of prenucleation clusters [62] in analogy to the discussion above. We note the discrepancy between the prenucleation cluster sizes determined from AUC (~ 3 nm in diameter on average, see above) and from cryo-TEM (~ 0.6 nm in diameter on average). The size determined by means of AUC is statistically far more reliable than the size determined by means of cryo-TEM, as the size of *every* cluster in the investigated ensemble is averaged. However, the size determined in AUC relies on knowledge of cluster density, which can be estimated based on bulk material densities. Since these can be considered to be higher than the cluster density, the as-determined size is in fact a lower limit, because species with higher densities sediment faster. When the sedimentation coefficient remains unaltered, and the densities are varied, thus, a lower density corresponds to a larger size (cf. above). On the other hand, cryo-TEM of specimen that are composed of elements with low molar mass is essentially based on phase contrast that arises from coherent interference of scattered and transmitted beams [64]. Thus, cryo-TEM cannot display hydration layers, which are included in AUC, and may not be able to display cluster structures below a certain threshold in density that is too close to that of the background (the prenucleation clusters should *not* be considered to be particles, cf. below). *Cum grano salis*, a distinct part of prenucleation clusters could be invisible in cryo-TEM.

Cryo-TEM investigations of calcium phosphate nucleation from simulated body fluid, which is supersaturated with respect to amorphous calcium phosphate, have now corroborated the existence of prenucleation clusters of calcium phosphate [65], which has been suggested in an earlier study [38]. Interestingly, the

prenucleation clusters only gather in simulated body fluid to form more dense structures if an extrinsic interface is present, and densifying on the interface, amorphous intermediates of calcium phosphate are heterogeneously nucleated, which eventually crystallize (Figure 11). The fascinating cryo-TEM images and tomographs show that the crystallization pathway established for the homogeneous nucleation of calcium carbonate in solution does hold for the heterogeneous nucleation of calcium phosphate from simulated body fluid. The aggregation-based mechanism appears to be highly relevant for possible treatments of pathological crystallization as in osteoporosis. For example, blood (serum) proteins from the fetuin family bind prenucleation clusters and in this way inhibit pathological mineralization of soft tissues [66–68].

The size of stable prenucleation clusters of calcium phosphate is determined to be approximately 1 nm by means of cryo-TEM [65] and the prenucleation clusters were discussed in terms of Posner clusters [69]. Posner clusters represent structural units ($\text{Ca}_9(\text{PO}_4)_6$) determined for the *bulk* structure of amorphous calcium phosphate through analyses of the pair distribution function obtained from X-ray diffraction. Posner speculated that these clusters may be abundant in solution and precursors to amorphous calcium phosphate. Thus, nanostructural features of amorphous calcium phosphate are based on the aggregation of prenucleation clusters according to the results presented in Figure 11. We emphasize that Sommerdijk *et al.* state that the prenucleation clusters may be different from Posner clusters in terms of composition [65] and therefore, in our opinion, different in terms of structure. This is almost certainly the case, as structural identity of solution and bulk structures appears to be unlikely; inferring structural identity based on an agreement in size is not possible (as to the size of prenucleation clusters in cryo-TEM, cf. above).

In another seminal study, Sommerdijk *et al.* showed that the synergy of collagen functions with inhibitors of hydroxyapatite nucleation actively controls mineralization, which is important for the fundamental understanding of the formation of bone [70]. It was shown that nucleation inhibition by poly(aspartic acid) is based on the formation of rather loosely packed assemblies of calcium phosphate prenucleation clusters, which are stabilized with respect to the situation in absence of the polymer (Figure 12). The inhibition of nucleation appears to be sufficient for the internal mineralization of collagen fibers as in bone, indicating that the ability to mediate mineralization might be intrinsic to type I collagen fibrils. We note that at the pH level of the study (pH 7.4) [70], poly(aspartic acid) is largely uncharged [71]. Thus, it can be expected that the degree of stabilization against nucleation by poly(aspartic acid) is much more pronounced at levels above pH ~8.

Modelling and Simulation

Computer simulation is a very important approach to the understanding of nucleation and of the very early stages of crystal growth, since it is difficult to access these steps experimentally. Moreover, it can be used to corroborate or extend experimental findings, and in this way shed light on the underlying (atomistic) mechanisms. For example, experimentalists commonly scratch surfaces of beakers to promote nucleation. The answer as to why this works may be given by simulation rationalizing why nucleation is orders of magnitude faster in a groove than on a flat surface [72]. Further, kinetic Monte Carlo simulations were used to reproduce experimentally observed crystal growth of urea, showing that it might eventually be used for the computer-aided design of crystals [73]. On the other hand, molecular dynamics simulations could explain the morphology of barite crystals that were grown at different supersaturation levels, indicating that dissolution of specific

surfaces was assisted by adsorbed anions [74]. A comprehensive review of simulation and modelling approaches to (non-classical) nucleation is beyond the scope of this paper, we refer to ref. [75], *e.g.*, and focus on the calcium carbonate system, in particular on non-classical aspects of its nucleation and crystallization.

Looking at the growing body of experimental evidence of initial nucleation of ACC in biological systems, Quigley and Rodger concluded that a missing step in our understanding was the structure and relative energetics of amorphous and crystalline nanoparticles of calcium carbonate [76]. In their seminal computational study, the free energy landscape of an ACC nanoparticle (2 nm, 75 formula units) was explored, showing that a distinct minimum in free energy was given by an amorphous state, while a less stable state corresponding to a vaterite-like crystalline structure was also accessible. In a broader subsequent study, the stability of ACC nanoparticles was attributed to effects of nanoconfinement, and a stable state was found for calcite-like nanoparticles as small as 2 nm [77]. The first molecular simulations of the crystallization of nanoparticles of ACC were presented by Freeman *et al.*, who showed that the chicken eggshell protein ovocleidin-17 facilitated the structural transition to calcite [78]. Based on this computational study, a catalytic cycle was proposed as a mechanism for the first stage of eggshell formation (Figure 13). Simulations of the crystallization of large nanoparticles of ACC to calcite on self-assembled monolayers indicated that the monolayer was able to adjust to the structure of the growing crystals, while ionization of the monolayer was essential to induce crystallization [79].

These simulations of nanoparticles of ACC provide insight into the very early stages of crystal growth, however, they do not directly address nucleation itself. The process of nucleation of ACC, though coming from a unrealistically high level of

supersaturation (from an experimentalist's point of view), was first considered by Tribello *et al.*, who investigated the aggregation of ACC in solution from its constituent ions [80]. They found that nanoparticles of misaligned domains of vaterite and aragonite formed, whereas addition of further ions to these "amorphous" species was virtually barrier free. Growth of calcite nanoparticles, though, was associated with a large barrier, and it was concluded that at high levels of supersaturation, ACC was formed owing to its fast kinetics of growth. With limited statistics, Hassan showed that nucleation and crystal growth by attachment and coalescence of clusters is in principle possible [81]. These results for sodium chloride indicate that small clusters, which would otherwise dissolve, might be stabilized by hydration processes [81], though there may be issues in terms of timescales and the respective model used.

Raiteri and Gale pointed out that the different simulations of ACC outlined above had employed force fields that failed in terms of an accurate description of the aqueous calcium carbonate system [82], both in terms of obtaining the correct relative stabilities of crystalline phases and the free energy of solvation of the ions, and developed a force field that renders reliable simulation possible [83]. In their study, most of the experimental findings on prenucleation clusters [38] could be corroborated for small nanoparticles of ACC. Importantly, it could be shown that very small nanoparticles of ACC can be thermodynamically stable, and the basic results of the physicochemical model of speciation based on a multiple-binding equilibrium [38] could be verified [82]. Moreover, the simulations showed that the incorporation of water into nanoparticles of ACC was driven by thermodynamic stability to an extent that was dependent on the particle size. Similar to the findings of Tribello *et al.* [80], Raiteri and Gale found that the growth process of different nanoclusters of ACC was

effectively barrier-free and limited by diffusion [82]. Consequently, it was argued that formation of nanoclusters of ACC was the actual nucleation step (Figure 14).

We note that this notion contradicts the experimental findings. Figure 2 shows that a nucleation event is clearly detectable, requiring a certain level of supersaturation, which proves the presence of a barrier to nucleation, separating solution and solution/solid states. The experimental and theoretical findings can be reconciled at the high pH-limit (which is the actual situation in the simulations), where the nucleation event may not be detectable anymore, extrapolating the decreasing drop in free calcium upon nucleation at higher pH-values (Figure 2). However, this experimental observation indicates that the barrier to nucleation is overcome owing to concentration effects (increase in carbonate), but not that nucleation is barrier-free at high pH values. Moreover, the essentially constant average size of prenucleation clusters in cryo-TEM (cf. above), which is independent of the level of supersaturation, contradicts a barrier-free growth of this species. These observations can be summarized in the "prenucleation cluster conundrum": Why are prenucleation clusters thermodynamically stable, but do not grow?

A clue to this conundrum appears to be that the *structure* of prenucleation clusters *does not* relate to macroscopic bulk structures; this notion is discussed in detail in a forthcoming publication [84]. Consequently the barrier associated to nucleation may be based on the requirement of structural rearrangements in prenucleation clusters towards bulk structures. A rather low barrier may separate solution and bulk state in an aggregation-based pathway, while internal structural rearrangement may be associated with a larger barrier. This can explain why ACC is often, but not always, nucleated as an intermediate phase in the homogeneous case through aggregation (cf. above).

Prenucleation Cluster Concept in Comparison to Other Phenomena

The above implies that prenucleation clusters are solutes, do not have a phase interface, and should thus not be considered to be "classical particles", which moreover probably do not resemble macroscopic bulk structures. It appears that both fundamental assumptions of CNT (*i.e.* there is an interfacial tension associated to small ionic aggregates in solution, and the structures of these aggregates relate to macroscopic bulk structures) are wrong for the non-classical nucleation pathway *via* prenucleation clusters. A related idea has been introduced by Askhabov with the quataron concept, where nano-sized pre-critical clusters are regarded to differ from the known states of a substances that arise under non-equilibrium conditions [85,86], as opposed to prenucleation clusters that are considered to be equilibrium species, though. When it comes to non-classical views on nucleation, there is a large body of literature that suggests or supposes the presence of species other than monomers (that is, atoms, ions or molecules) that play a role during nucleation and growth of crystals. In this section, we reflect merely two examples in more depth, which were chosen because they are useful for contemplating the introduced concept of stable prenucleation clusters. Considering the thermodynamics of common biominerals, Navrotsky pointed out that the small differences among the free enthalpies of nanoscopic phases rendered controlled thermodynamic and mechanistic pathways possible that were different to conventional nucleation and growth envisaged in CNT [54]. Concluding, Navrotsky argued that the assembly of nanoparticles or clusters might be a clue to pathways to biomineralization, where both thermodynamic and kinetic factors were important. In our opinion, non-classical nucleation *via* prenucleation clusters and amorphous intermediates as discussed above completely complies with this notion. On the other hand, reviewing organic nanoparticles in the

aqueous phase, Horn and Rieger pointed out that there was a considerable need for research in the area of nucleation theory [87], and, based on experimental observations, suggested the existence of a precursor in solution that exists prior to the formation of critical nuclei (Figure 15). In this context, the authors referred to advances in protein crystallization, which are today known as the two-step mechanism in protein nucleation. In the following section, we will discuss this concept and compare it to the notion of stable prenucleation clusters. In a second section, we discuss the connection between prenucleation clusters and ion pairing. We are then able to suggest in a third section, why stable prenucleation clusters may not have been suggested earlier.

The Two-Step Mechanism in Protein Crystallization

The two-step mechanism of nucleation [88] was experimentally found for the nucleation of proteins from solution under certain conditions [89], while the basic mechanism was first suggested by means of simulation by ten Wolde and Frenkel [90]. They showed that close to a metastable fluid-fluid critical point, the pathway of formation of a crystal nucleus can drastically change (Figure 16). Similar mechanisms were accessed by means of other theoretical studies and simulation [91,92]. Vekilov has recently argued that the two-step mechanism of nucleation applies to other systems besides proteins, *e.g.*, to nucleation of organic crystals [93], of colloidal crystals [94], and of biomineral crystals that form *via* prenucleation clusters [36]. In the two-step nucleation mechanism of proteins from solution, two barriers must be overcome (Figure 17). In a first step, a dense liquid is nucleated, and in a second step, crystals are nucleated within this dense liquid. The dense liquid is formed as an intermediate phase that is metastable with respect to the protein crystals, but can be either stable or metastable with respect to the proteins in solution (Figure 17),

depending on the level of supersaturation, it may even be unstable. In protein crystallization, it appears that the dense liquid is in fact a metastable phase, with respect to the solution as well as the final crystals [36].

It is important to note that the notion of two-step nucleation, as summarized above, cannot be transferred to the nucleation of crystals forming *via* prenucleation clusters: As thoroughly discussed above, analyses of the solution thermodynamics show that the prenucleation clusters *are not nucleated* and *do not represent a second phase*, but that they coexist with ions in a single-phase system. Consistently, the barrier separating the state of prenucleation clusters and the state of free ions is negligible compared to thermal energy. Therefore, prenucleation clusters are thermodynamically stable *solutes* as is also evident from their pH dependent formation equilibrium (Fig. 2) [38], while the dense protein liquid in the two-step mechanism is considered to be a *metastable phase that has been nucleated* [36]. On the other hand, the nucleation of amorphous nanoparticles of biominerals from the prenucleation clusters mainly appears to proceed *via* aggregation of the clusters, while in the two-step mechanism, protein crystals are considered to form *via* structural transitions (that is, a second nucleation event) within the dense protein liquid. The free energy landscape presented in Figure 17, thus does not characterize calcium carbonate crystallization *via* prenucleation clusters, as was argued elsewhere [36]. The free energy profile characterizing the formation of calcium carbonate from prenucleation clusters as outlined above, in contrast to the notion of CNT is shown in Figure 18. It is important to note that according to this free energy landscape, solid calcium carbonate is nucleated in a single step, while the prenucleation clusters are stable with respect to the free ions in the solution state that constitutes a single-phase system [38].

An analogy between the nucleation of biomineral crystals and the two-step mechanism in protein crystallization can be seen in the fact that an intermediate (metastable) phase is nucleated first in both cases, that is, amorphous biomineral nanoparticles (but not prenucleation clusters which are solutes) and dense protein liquid. However, the nature and speciation of the amorphous intermediate nanoparticles in biomineral formation and of the dense liquid in protein crystallization appear to be fundamentally different. In our opinion, the different length scales involved (~20-30 nm in the case of amorphous mineral intermediates, as opposed to hundreds of nm in the case of droplets of dense liquid proteins), and the different energetics as well as (an-)isotropy of ion-ion and of macromolecule-macromolecule interactions underline that the phenomena must be physically different. For example, amorphous-to-crystalline transitions may be determined by macromolecular folding kinetics in two-step nucleation [95], whereas in case of the amorphous-to-crystalline transitions in minerals, no covalent bonds are involved and ionic coordinations have to be rearranged only locally.

Ion Pairing

It has been known at least since the early 1930s that in solution, salts of calcium with bivalent cations behave as if they were incompletely dissociated, and the possibility that complexes of carbonate with calcium (or magnesium) form was suggested [96, further references therein]. This effect is known as ion pairing, and in the course of scientific history, it has been rejected and re-suggested repeatedly. An overview of the different treatments of ion pair formation of calcium carbonate is given by Gal *et al.* [97], noting that several unfortunate events could have contributed to a failure of the ion pair concept, which in their opinion is basically a large body of contradictory results on the underlying equilibrium constant of ion pair formation.

Even though it was suggested that, taking ion pairs into account, the kinetics of scale formation can be explained in a better way [97], the meaning of the existence of this species for nucleation itself was not addressed explicitly. The chemistry of calcium and carbonate ions does not constrain the formation of ionic aggregates beyond dimers, which was however excluded in the ion pair concept. From a historical point of view, the shortfalls of the ion pair concept may thus be due to the fact that the concepts of ion pairs and of CNT were developed in parallel in the early 1930s, probably unaware of each other (as to activity, cf. next section). Later considerations of the ion pair concept include the idea of distinct precursors to the different polymorphs of calcium carbonate, suggesting the existence of a series of various hydrated forms of ion pairs [97]. However, it remains unclear how different structures can be encoded in these different hydrated forms if no species bigger than dimers are formed, and how these are carried over to the solid phase upon nucleation - especially in case of anhydrous polymorphs. Structural "pre-formation" requires the formation of species that are significantly larger than dimers.

Stable prenucleation clusters concealed by ion pairing & activity

Is there room for calcium carbonate prenucleation clusters, looking at a system that has been analyzed for more than 100 years? How could they be missed? One answer is that the prenucleation clusters could have been concealed by the ion pair concept. Interestingly, the average size of the basic prenucleation clusters determined by AUC and cryo-TEM is largely independent of the level of supersaturation and does not conform to the notion of an exponentially decaying cluster size distribution that is envisaged within CNT. In fact, CNT predicts an excess of prenucleation species to be either monomers or dimers, and that the average of the cluster size distribution depends on the level of supersaturation, both contrary to the experimental findings.

On the other hand, the fact that the distinct decrease of calcium activity in carbonate solutions may be due to ion pair formation has been rejected repeatedly [96,97]. Activity coefficients are used to correct for deviations from ideal behaviour in real systems, that is, in the case of ionic solutions, mainly for Coulomb interactions between cations and anions, which would not matter in the case of perfect solutions. Basically, the problem of activity in electrolyte solutions is as old as the concept of their dissociation in solution, introduced by Arrhenius [98,99]. A quantitative theory for the determination of the activity in electrolyte solutions based on Coulomb interactions was developed by Debye and Hückel [100]. Following the introduction of the Debye-Hückel theory, several authors noted that it was based on an oversimplified physical model that might apply only to sufficiently dilute solutions [101, further references therein]. Hence, several reconsiderations of electrolyte theory were presented, for instance, based on virial developments for the osmotic pressure [102]. In an early paper, Redlich pointed out that the shortfall of the Debye-Hückel theory might be due to effects of hydration or of the formation of complex ions, but that such secondary phenomena should only be important in certain cases, however, not in general [103]. Further, importantly, Redlich argued that there must be a theoretically significant and experimentally accessible criterion that permits to draw a sharp line between interacting ions (i.e. activity effects) and undissociated ions that remain as "molecules" in solution (i.e. the notion of ion pairs, or prenucleation clusters). The experimental evidence of the molecular character of prenucleation clusters is given by AUC, which was historically also used to prove the existence of macromolecules [42]. As the decrease in calcium concentration is due to 1:1 binding of calcium and carbonate, formation of complex clusters with "molecular" character is observed, but not a decrease in ionic activity. Both scenarios, *i.e.* binding and decreased activity, are

in principal based on Coulomb interactions, but are in effect physically and chemically different. Moreover, consistent with the considerations above, this indicates that prenucleation clusters of calcium carbonate have been misleadingly regarded as ion pairs so far.

In summary, our considerations strongly suggest that calcium carbonate (and likely, also the other biominerals that form prenucleation clusters) has to be considered to be a "weak electrolyte" that is not completely dissociated. Within experimental accuracy, the dilute solutions of calcium carbonate behave like ideal solutions if the formation of rather large ionic aggregates on the basis of equilibrium thermodynamics is taken into account. We note that special care needs to be taken when activity coefficients are calculated according to the Debye-Hückel theory, its quantitative reconsiderations or to empirical approaches like the Davies equation [104] for ions that form prenucleation clusters. As discussed above, the activity coefficients obtained in this manner are not accurate and in part, they actually correct for prenucleation cluster formation, that is, the formation of prenucleation clusters is erroneously treated as a decrease in ion activity. However, with increasing ionic strength (in presence of, *e.g.*, additional (poly)electrolytes, buffers, etc.) activity effects will play a significant role, and have to be carefully elaborated.

Conclusions and Outlook

Calcium carbonate and phosphate can be nucleated *via* an aggregation-based pathway that cannot be reconciled with the notion of CNT (Figure 19) [105]. When ions meet in solution based on stochastic collisions, they form stable prenucleation clusters, the structures of which probably do not relate to the macroscopic bulk. Isothermal titration calorimetry investigations of prenucleation cluster formation revealed an endothermic process meaning that the driving force for prenucleation

cluster formation must be entropic [106]. The entropy increase upon prenucleation cluster formation may be caused by release of ion hydration water similar to the formation of micelles, but we expect further insight from simulation and modelling approaches. Experimentally, the prenucleation clusters and the non-classical pathway of nucleation need to be characterized in more detail, in terms of cluster structure, composition, thermodynamics, formation dynamics, kinetics of nucleation taking stable clusters into account, etc. In our opinion, combination of theoretical and experimental means will provide crucial insight into the processes that underlie non-classical nucleation. Possibly, the prenucleation cluster concept is of broader importance and could apply to a number of other minerals besides calcium carbonate and phosphate. The generality of the concept should be tested, which can then lead to a novel view on the mechanism of crystal nucleation in general. Again, we note that with respect to non-classical nucleation and crystallization, currently, the notion is established that crystallization of calcium carbonate always proceeds *via* the nucleation of an amorphous intermediate, but it is possible that crystalline calcium carbonate is directly nucleated from stable prenucleation clusters under certain conditions [44].

Relevance to Applications

Calcium carbonate is not only the most abundant biomineral, but also a very important industrially used material. Understanding of non-classical nucleation mechanisms can render the development of novel pigments, fillers or advanced functional materials possible that are tailor-made with respect to the particular application. For example, nano-structured construction materials based on calcium carbonate, e.g. concrete, may facilitate revolutionary possibilities in architecture. In turn, unwanted crystallization of calcium carbonate in industrial heating and cooling

circuits, but also in laundry machines or dishwashers, causes enormous costs, and development of novel anti-scalant strategies appear to be possible. Moreover, it is evident that much more carbonate is bound in stable chemical species taking prenucleation clusters into account, which can provide a better understanding of geological CO₂ cycles. For example, ocean acidification may be much more critical than previously assumed. As to calcium phosphate, the significance of the prenucleation cluster concept is clearly given by its relevance to osteoporosis, which is a serious problem in the aging western societies. A novel understanding of bone formation can render the development of new treatments possible. Thinking ahead, we can obtain a better understanding of biomineralization pathways in general, and of their control by specialized bio(macro)molecules, which can possibly be adapted for the design of synthetic crystallization additives. Since it appears to be possible that the presented concept of non-classical nucleation is of broader significance, the principles found for polymorph selection may also apply to a variety of other compounds.

Acknowledgement

We acknowledge numerous discussions on the prenucleation cluster concept with many colleagues on various occasions. We would like to express special thanks to M. Antonietti, L. Bergström, J.J. De Yoreo, P. Fratzl, J.D. Gale, P.U.P.A. Gilbert, W.J.E.M. Habraken, J.H. Harding, N. Hedin, M. Kellermeier, A. Navrotsky, D. Quigley, J. Rieger, N.A.J.M. Sommerdijk, and K. Tauer.

References

- [1] P. Fratzl, H.S. Gupta, E.P. Paschalis, P. Roschger, J. Mater. Chem. 14 (2004) 2115-2123.
- [2] M. Benvenuti, S. Mangani, Nat. Protoc. 2 (2007) 1633-1651.
- [3] J. Sussman, P. Spadon, NATO Science for Peace and Security Series A: Chemistry and Biology: From Molecules to Medicines: Structure of Biological

Macromolecules and Its Relevance in Combating New Diseases and Bioterrorism, Springer in cooperation with NATO Public Diplomacy Division, Dordrecht, 2009.

- [4] F. Schlünzen, R. Zarivach, J. Harms, A. Bashan, A. Tocilj, R. Albrecht, A. Yonath, F. Franceschi, *Nature* 413 (2001) 814-821.
- [5] H. Lowenstam, S. Weiner, *On Biomineralization*, Oxford University Press, New York, 1989.
- [6] B.L. Smith, T.E. Schaffer, M. Viani, J.B. Thompson, N.A. Frederick, J. Kindt, A. Belcher, G.D. Stucky, D.E. Morse, P.K. Hansma, *Nature* 399 (1999) 761-763.
- [7] J.D. Currey, *Proc. R. Soc. London, Ser. B* 196 (1977) 443-463.
- [8] N.A.J.M. Sommerdijk, H. Cölfen, *MRS Bull.* 35 (2010) 116-121.
- [9] P. Fratzl, S. Weiner, *Adv. Mater.* 22 (2010) 4547-4550.
- [10] P. Fratzl, *J. R. Soc. Interface* 4 (2007) 637-642.
- [11] H.J. Gao, B.H. Ji, I.L. Jäger, E. Arzt, P. Fratzl, *Proc. Natl. Acad. Sci. U.S.A.* 100 (2003) 5597-5600.
- [12] S. Mann, D.D. Archibald, J.M. Didymus, T. Douglas, B.R. Heywood, F.C. Meldrum, N.J. Reeves, *Science* 261 (1993) 1286-1292.
- [13] S. Mann, *Biomimetic Materials Chemistry*, VCH, New York, 1996.
- [14] A.-W. Xu, Y. Ma, H. Cölfen, *J. Mater. Chem.* 17 (2007) 415.
- [15] F.C. Meldrum, H. Cölfen, *Chem. Rev.* 108 (2008) 4332-4432.
- [16] D. Kashchiev, *Nucleation : Basic Theory with Applications*, Butterworth-Heinemann, Oxford, 2000.
- [17] A.E. Nielsen, *Kinetics of Precepitation*, Pergamon Press, New York, 1964.
- [18] J. Mullin, *Crystallization*, 4th ed., Butterworth - Heinemann, Oxford, 2001.
- [19] H. Cölfen, M. Antonietti, *Angew. Chem. Int. Ed.* 44 (2005) 5576-5591.
- [20] H. Cölfen, M. Antonietti, *Mesocrystals and Nonclassical Crystallization*, John Wiley & Sons, Ltd., Chichester, 2008.
- [21] H. Cölfen, S. Mann, *Angew. Chem. Int. Ed.* 42 (2003) 2350-2365.
- [22] M. Niederberger, H. Cölfen, *Phys. Chem. Chem. Phys.* 8 (2006) 3271.
- [23] M. Rousseau, E. Lopez, P. Stempfélé, M. Brendlé, L. Franke, A. Guette, R. Naslain, X. Bourrat, *Biomaterials* 26 (2005) 6254-6262.
- [24] Y. Oaki, A. Kotachi, T. Miura, H. Imai, *Adv. Funct. Mater.* 16 (2006) 1633-1639.
- [25] I. Sethmann, R. Hinrichs, G. Wörheide, A. Putnis, *J. Inorg. Biochem.* 100 (2006) 88-96.
- [26] L. Addadi, S. Raz, S. Weiner, *Adv. Mater.* 15 (2003) 959-970.
- [27] J. Aizenberg, D.A. Muller, J.L. Grazul, D.R. Hamann, *Science* 299 (2003) 1205-1208.
- [28] L.B. Gower, *Chem. Rev.* 108 (2008) 4551-4627.
- [29] L.B. Gower, D.J. Odom, *J. Cryst. Growth* 210 (2000) 719-734.

- [30] R. Becker, W. Döring, *Ann. Phys.* 24 (1935) 719-752.
- [31] M. Volmer, A. Weber, *Z. Phys. Chem.* 119 (1925) 277-301.
- [32] J.W. Gibbs, *Trans. Connect. Acad. Sci.* 3 (1876) 108-248.
- [33] J.W. Gibbs, *Trans. Connect. Acad. Sci.* 3 (1877) 343-524.
- [34] L. Farkas, *Z. Phys. Chem.* 125 (1926) 236-242.
- [35] H. Eyring, *Chem. Rev.* 17 (1935) 65-77.
- [36] P.G. Vekilov, *Nanoscale* 2 (2010) 2346-2357.
- [37] J. Lothe, G.M. Pound, *J. Chem. Phys.* 36 (1962) 2080-2085.
- [38] D. Gebauer, A. Völkel, H. Cölfen, *Science* 322 (2008) 1819-1822.
- [39] D. Gebauer, H. Cölfen, *Unpublished Data* (2010).
- [40] S.E. Wolf, L. Müller, R. Barrea, C.J. Kampf, J. Leiterer, U. Panne, T. Hoffmann, F. Emmerling, W. Tremel, *Nanoscale* 3 (2011) 1158-1165.
- [41] H.G. Müller, F. Herrmann, in: J. Behlke (Ed.), *Analytical Ultracentrifugation - Progress in Colloid and Polymer Science*, Steinkopff, Darmstadt, 1995, pp. 114-119.
- [42] T. Svedberg, R. Fåhræus, *J. Am. Chem. Soc.* 48 (1926) 430-438.
- [43] G. Scatchard, *Ann. N.Y. Acad. Sci.* 51 (1949) 660-672.
- [44] J.J. DeYoreo, *Personal Communication* (2011).
- [45] L. Brečević, A.E. Nielsen, *J. Cryst. Growth* 98 (1989) 504-510.
- [46] D. Gebauer, P.N. Gunawidjaja, J.Y.P. Ko, Z. Bacsik, B. Aziz, L.J. Liu, Y.F. Hu, L. Bergström, C.W. Tai, T.K. Sham, M. Edén, N. Hedin, *Angew. Chem. Int. Ed.* 49 (2010) 8889-8891.
- [47] B. Hasse, H. Ehrenberg, J.C. Marxen, W. Becker, M. Epple, *Chem. Eur. J.* 6 (2000) 3679-3685.
- [48] Y. Politi, Y. Levi-Kalisman, S. Raz, F. Wilt, L. Addadi, S. Weiner, I. Sagi, *Adv. Funct. Mater.* 16 (2006) 1289-1298.
- [49] R.S.K. Lam, J.M. Charnock, A. Lennie, F.C. Meldrum, *CrystEngComm* 9 (2007) 1226-1236.
- [50] D. Gebauer, A. Verch, H.G. Börner, H. Cölfen, *Cryst. Growth Des.* 9 (2009) 2398-2403.
- [51] C. Lizandara Pueyo, S. Siroky, S. Landsmann, M.W.E. van den Berg, M.R. Wagner, J.S. Reparaz, A. Hoffmann, S. Polarz, *Chem. Mater.* 22 (2010) 4263-4270.
- [52] A.N. MacInnes, W.M. Cleaver, A.R. Barron, M.B. Power, A.F. Hepp, *Adv. Mater. Opt. Electron.* 1 (1992) 229-233.
- [53] A.N. MacInnes, M.B. Power, A.R. Barron, *Chem. Mater.* 5 (1993) 1344-1351.
- [54] A. Navrotsky, *Proc. Nat. Acad. Sci. U.S.A.* 101 (2004) 12096-12101.
- [55] A.V. Radha, T.Z. Forbes, C.E. Killian, P.U.P.A. Gilbert, A. Navrotsky, *Proc. Nat. Acad. Sci. U.S.A.* 107 (2010) 16438-16443.

- [56] Y. Politi, R.A. Metzler, M. Abrecht, B. Gilbert, F.H. Wilt, I. Sagi, L. Addadi, S. Weiner, P.U.P.A. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* 105 (2008) 17362-17366.
- [57] D. Gebauer, H. Cölfen, A. Verch, M. Antonietti, *Adv. Mater.* 21 (2009) 435-439.
- [58] A. Verch, D. Gebauer, M. Antonietti, H. Cölfen, *Phys. Chem. Chem. Phys.* 13 (2011) 16811-16820.
- [59] B. Aziz, D. Gebauer, N. Hedin, *CrystEngComm* 13 (2011) 4641-4645.
- [60] F.M. Michel, J. MacDonald, J. Feng, B.L. Phillips, L. Ehm, C. Tarabrella, J.B. Parise, R.J. Reeder, *Chem. Mater.* 20 (2008) 4720-4728.
- [61] H. Nebel, M. Neumann, C. Mayer, M. Eppele, *Inorg. Chem.* 47 (2008) 7874-7879.
- [62] E.M. Pouget, P.H.H. Bomans, J.A.C.M. Goos, P.M. Frederik, G. de With, N.A.J.M. Sommerdijk, *Science* 323 (2009) 1455-1458.
- [63] Y. Kitano, *Bull. Chem. Soc. Jpn.* 35 (1962) 1973-1980.
- [64] H. Friedrich, P.M. Frederik, G. de With, N.A.J.M. Sommerdijk, *Angew. Chem. Int. Ed.* 49 (2010) 7850-7858.
- [65] A. Dey, P.H.H. Bomans, F.A. Müller, J. Will, P.M. Frederik, G. de With, N.A.J.M. Sommerdijk, *Nat. Mater.* 9 (2010) 1010-1014.
- [66] A. Heiss, W. Jahnen-Dechent, H. Endo, D. Schwahn, *Biointerphases* 2 (2007) 16-20.
- [67] A. Heiss, V. Pipich, W. Jahnen-Dechent, D. Schwahn, *Biophys. J.* 99 (2010) 3986-3995.
- [68] W. Jahnen-Dechent, A. Heiss, C. Schäfer, M. Ketteler, *Circ. Res.* 108 (2011) 1494-1509.
- [69] A.S. Posner, F. Betts, *Acc. Chem. Res.* 8 (1975) 273-281.
- [70] F. Nudelman, K. Pieterse, A. George, P.H.H. Bomans, H. Friedrich, L.J. Brylka, P.A.J. Hilbers, G. de With, N.A.J.M. Sommerdijk, *Nat. Mater.* 9 (2010) 1004-1009.
- [71] E. Kokufuta, *Biosystems* 9 (1977) 211-214.
- [72] A.J. Page, R.P. Sear, *J. Am. Chem. Soc.* 131 (2009) 17550-17551.
- [73] S. Piana, M. Reyhani, J.D. Gale, *Nature* 438 (2005) 70-73.
- [74] S. Piana, F. Jones, J.D. Gale, *J. Am. Chem. Soc.* 128 (2006) 13568-13574.
- [75] J. Anwar, D. Zahn, *Angew. Chem. Int. Ed.* 50 (2011) 1996-2013.
- [76] D. Quigley, P.M. Rodger, *J. Chem. Phys.* 128 (2008) 221101.
- [77] D. Quigley, C.L. Freeman, J.H. Harding, P.M. Rodger, *J. Chem. Phys.* 134 (2011) 044703.
- [78] C.L. Freeman, J.H. Harding, D. Quigley, P.M. Rodger, *Angew. Chem. Int. Ed.* 49 (2010) 5135-5137.
- [79] D. Quigley, P.M. Rodger, C.L. Freeman, J.H. Harding, D.M. Duffy, *J. Chem. Phys.* 131 (2009) 094703.
- [80] G.A. Tribello, F. Bruneval, C. Liew, M. Parrinello, *J. Phys. Chem. B* 113 (2009) 11680-11687.

- [81] S.A. Hassan, J. Chem. Phys. 134 (2011) 114508.
- [82] P. Raiteri, J.D. Gale, J. Am. Chem. Soc. 132 (2010) 17623-17634.
- [83] P. Raiteri, J.D. Gale, D. Quigley, P.M. Rodger, J. Phys. Chem. C 114 (2010) 5997-6010.
- [84] R. Demichelis, P. Raiteri, J.D. Gale, D. Quigley, D. Gebauer, Nat. Commun. 2 (2011) 590.
- [85] A.M. Askhabov, Geochim. Cosmochim. Acta 73 (2009) A59-A59.
- [86] A.M. Askhabov, M.A. Ryazanov, Dokl. Akad. Nauk 362 (1998) 630-633.
- [87] D. Horn, J. Rieger, Angew. Chem. Int. Ed. 40 (2001) 4330-4361.
- [88] D. Erdemir, A.Y. Lee, A.S. Myerson, Acc. Chem. Res. 42 (2009) 621-629.
- [89] P.G. Vekilov, Cryst. Growth Des. 4 (2004) 671-685.
- [90] P.R. ten Wolde, D. Frenkel, Science 277 (1997) 1975-1978.
- [91] N. Duff, B. Peters, J. Chem. Phys. 131 (2009) 184101.
- [92] R.P. Sear, J. Chem. Phys. 131 (2009) 074702.
- [93] B. Garetz, J. Matic, A. Myerson, Phys. Rev. Lett. 89 (2002) 175501.
- [94] M.E. Leunissen, C.G. Christova, A.-P. Hynninen, C.P. Royall, A.I. Campbell, A. Imhof, M. Dijkstra, R. van Roij, A. van Blaaderen, Nature 437 (2005) 235-240.
- [95] S. Chung, S.-H. Shin, C.R. Bertozzi, J.J. De Yoreo, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 16536-16541.
- [96] I. Greenwald, J. Biol. Chem. 141 (1941) 789-796.
- [97] J.-Y. Gal, J.-C. Bollinger, H. Tolosa, N. Gache, Talanta 43 (1996) 1497-1509.
- [98] S. Arrhenius, Z. Phys. Chem. 1 (1887) 631.
- [99] S. Arrhenius, J. Am. Chem. Soc. 34 (1912) 353-364.
- [100] P. Debye, E. Hückel, Phys. Z. 24 (1923) 185-206.
- [101] H.S. Frank, P.T. Thompson, J. Chem. Phys. 31 (1959) 1086.
- [102] J.E. Mayer, J. Chem. Phys. 18 (1950) 1426.
- [103] O. Redlich, Chem. Rev. 39 (1946) 333-356.
- [104] C.W. Davies, Ion Association, Butterworth, Washington, D.C., 1962.
- [105] F.C. Meldrum, R.P. Sear, Science 322 (2008) 1802-1803.
- [106] A. Verch, Pränukleationscluster Und Ihre Wechselwirkungen Mit Additiven, Universität Potsdam (Ph.D. thesis), 2010.

Figures

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Figure 9.

Figure 10.

Figure 12.

Figure 13.

Figure 14.

Figure 15.

Figure 16.

Figure 17.

Figure 18.

Figure 19.

Figure Captions

Figure 1. Free energy *versus* radius of nuclei in classical nucleation theory. The bulk energy (green trace) and the surface energy (red trace) scale with the cube and the square of the radius, respectively. Hence, summing up (blue trace), the energetically unfavourable surface generation has started to get balanced by the bulk energy at the critical size. Nuclei that are bigger than the critical size grow without limit, smaller ones dissolve again.

Figure 2. Free calcium in 25 mL 10 mM carbonate buffer in a range between pH 9.00 and pH 10.0 during addition of 10 mM calcium chloride solution at a constant rate of 10 μ L per minute. Arrows indicate the time of nucleation of calcium carbonate.

Figure reproduced utilizing data presented in ref. [38].

Figure 3. Averaged amount of bound calcium as calculated from the difference between the detected amount of calcium and the added amount of calcium (cf. Figure 2) from a sample of three single measurements at each pH level (error bars depict ± 1 standard deviation) at the five pH-levels investigated as indicated (black traces). As averaging is not possible during nucleation, nucleation periods are left out. The carbonate titrations (red traces) require offset corrections due to initial pH-electrode tune-in. Slope corrections are most likely due to in-diffusion of CO₂, the rate of which increases with increasing pH, while the resulting changes in carbonate buffer concentrations would be negligible [38, supporting online material page 7]. The corrections (green traces) conform to the electroneutrality requirement of the nucleated phase and strongly suggest that calcium and carbonate binding are equal during the pre-nucleation stage as well. Figure reproduced utilizing data presented in ref. [38].

Figure 4. Diffusion corrected distribution of sedimentation coefficients after 90 minutes reaction time at pH=9.00 shown for a single experiment. Besides the free ions, clusters can be detected as indicated. Figure reproduced utilizing data presented in ref. [38].

Figure 5. Gibbs standard energy of reaction for the formation of a calcium-carbonate unit *within* prenucleation clusters. At low pH-values, more stable clusters are formed than at higher pH-values. Figure reproduced utilizing data presented in ref. [38].

Figure 6. Time development of the free ion product during the prenucleation stage and nucleation of calcium carbonate. Shown are averaged values of three measurements. Because averaging is not appropriate during nucleation, the particular developments are indicated by dashed lines. Two different phases of amorphous calcium carbonate with solubility products of $\sim 3.1 \times 10^{-8} \text{ M}^2$ (ACCI/pc-ACC, more stable) and $\sim 3.8 \times 10^{-8} \text{ M}^2$ (ACCII/pv-ACC, less stable) are found, corresponding qualitatively to the changing stability of the prenucleation clusters at respective pH levels (Figure 5). The solubilities of vaterite, aragonite, and calcite are also given [45]. From Ref. [38].

Reprinted with permission from AAAS.

Figure 7. Transmission electron microscopy images of ACCI (pc-ACC) and ACCII (pv-ACC) at various magnifications. Insets in (b) and (e) are selective area electron diffraction (SAED) patterns obtained from an area slightly larger than the particular image sections, SAED scale bars: 5 nm^{-1} . SAED patterns are shown as negatives to make weak features clear. Arrows in (f) indicate nanostructural features. Reprinted with permission of John Wiley and Sons from Ref. [46].

Figure 8. Spectra of calcite (dashed blue lines), vaterite (dashed red lines), pc-ACC (blue lines), and pv-ACC (red lines). a) ^{13}C solid-state NMR spectra recorded through

single pulses at a magnetic field of 9.4 T and a MAS rate of 8.0 kHz. b) Fourier Transform of calcium *K*-edge EXAFS plotted in the *R* space (lines as in (a)). The expected interactions of the first three coordination shells are indicated. The black arrow marks the position of peaks, which may relate to the coordination of structural water. The lack of distinct features above $R = 3\text{\AA}$ shows that there is no intermediate-range order beyond the first coordination sphere. The vertical lines are a guide for the eye. The spectral features in (a) and (b) evidence short-range structural analogies calcite/pc-ACC(ACCI) and vaterite/pv-ACC(ACCII) in the carbonate and calcium environments, respectively. Reprinted with permission of John Wiley and Sons from Ref. [46].

Figure 9. Time-developments of the free calcium amount in presence of poly(acrylic acid) (PAA) as compared to the reference at pH 9.75 (top), and of the free ion product in presence of 10 mg/L poly(acrylic acid) after the first nucleation event (bottom). After the first and second nucleation event, the ion product drops to a value corresponding to the solubility product of ACCII (pv-ACC, proto-vaterite ACC), while it drops to the solubility product of ACCI (pc-ACC, proto-calcite ACC) after the third nucleation event, and pure-phase calcite is obtained. Reproduced utilizing data presented in ref. [57].

Figure 10. (a) High-resolution cryo-TEM image of a fresh 9 mM $\text{Ca}(\text{HCO}_3)_2$ solution after image processing. Prenucleation clusters are observed, while an arbitrary number of clusters is highlighted by black circles. Scale bar, 20 nm. (b) Nonfiltered images representing the zone delimited by the red square in (a), all prenucleation clusters present are highlighted by black circles. Sizes below the detection limit of 0.45 nm (3 times the pixel size) are considered noise. Scale bar, 5 nm. (c) Distribution of particle diameters *d* of the prenucleation clusters determined from the cryo-TEM images. (d)

Radial integration of the diffraction patterns of vitrified aqueous solutions of (1) ACC (green trace), (2) 9 mM $\text{Ca}(\text{HCO}_3)_2$ (red trace), (3) water (blue trace), and (4) 10 mM CaCl_2 (black trace). Vertical (black) lines are drawn to indicate the shifts of the diffraction rings of the different samples with respect to that of a vitrified film of pure water. From Ref. [62]. Reprinted with permission from AAAS.

Figure 11. (a–e) Two-dimensional projection images, (f–j) computer-aided three-dimensional visualizations of tomograms of different stages of the surface-induced nucleation of amorphous calcium phosphate from simulated body fluid. (a, f) Stage 1, control experiment in the absence of a monolayer. (b, g) Stage 2, (c, h) stage 3, (d, i) stage 4, the inset selected area diffraction (SAED) pattern in (d) shows that the spherical particles attached to the monolayer are amorphous. (e, j) stage 5, the inset SAED in (e) may be indexed as carbonated hydroxyapatite with a $[110]$ zone axis. The preferred nucleating face is (110) , indicated by the yellow arrow in the inset of (j). For clarity (markers of the surface obscuring the image), the same area for two- and three-dimensional images is only used in (e, j). Markers and gold beads are indicated by red and blue arrows, respectively. Scale bars, 50 nm. Reprinted by permission from Macmillan Publishers Ltd: NATURE MATERIALS [65], copyright 2010.

Figure 12. (a) Cryo-TEM image of aggregates of calcium phosphate formed after 10 min of reaction without poly(aspartic acid). Scale bar: 100 nm. (b) Higher magnification of the area marked in (a), scale bar: 50 nm. (c) Cryo-TEM image of aggregates of calcium phosphate formed after 10 min of reaction in the presence of poly(aspartic acid). Inset: Higher magnification of the marked area. Scale bar: 50 nm. (d) Cryo-TEM image of calcium phosphate aggregates formed after 6 h of reaction in the presence of poly(aspartic acid). Scale bar: 50 nm. The polymer stabilizes rather

loose aggregates of prenucleation clusters approximately 1 nm in size, and densification of the precipitates may occur slower in presence of the polymeric inhibitor of nucleation. Reprinted by permission from Macmillan Publishers Ltd: NATURE MATERIALS [70], copyright 2010.

Figure 13. The Ovocleidin-17 (O-17) catalytic cycle. OC-17 binds to a small amorphous calcium carbonate (ACC) nanoparticle, induces the nanoparticle to crystallize as calcite; the calcite begins to grow, causing desorption of the OC-17 and making it available to bind to another ACC nanoparticle. This enables rapid formation of many calcite crystals, as required for the polycrystalline mammillary layer in eggshells. Reprinted with permission of John Wiley and Sons from Ref. [78].

Figure 14. Schematic representation of the free energy profile for the nonclassical formation of calcium carbonate from ion pairs in solution. Note that nucleation is suggested to occur barrier-free, in contrast to experimental observations (cf. Figure 18). Here the two lines for ACC represent the boundaries of a distribution of possible curves depending on the water content. The final transformation of ACC to a crystalline polymorph can occur at variable reaction coordinate depending on the environmental conditions. Reprinted with permission from Ref. [82]. Copyright 2010 American Chemical Society.

Figure 15. Scheme illustrating different stages of the process of particle formation, emphasizing open questions. The existence of a precursor to critical nuclei is suggested. Reprinted with permission of John Wiley and Sons from Ref. [87].

Figure 16. Contour plots of the free-energy landscape along the path from the metastable fluid to the critical crystal nucleus for a system of spherical particles with short-range attraction. The curves of constant free energy are drawn as a function of

the number of connected particles, N_p , and the number of solid-like particles belonging to a given crystal nucleus, N_{crys} , and are separated by $5 k_B T$ (Boltzmann constant k_B , temperature T). (A) The free-energy landscape well below the critical temperature T_c ($T/T_c = 0.89$). The lowest free-energy path to the critical nucleus is indicated by a dashed curve. This curve corresponds to the formation and growth of a highly crystalline cluster. (B) As (A), but for $T = T_c$. In this case, the free-energy valley (dashed curve) first runs parallel to the N_p axis (formation of a liquid-like droplet), and then moves toward a structure with a higher crystallinity (crystallite embedded in a liquid-like droplet). The free-energy barrier for this route is much lower than the one in (A). From Ref. [90]. Reprinted with permission from AAAS.

Figure 17. Free energy profiles during two-step nucleation. A dense liquid forms from solution through density fluctuations, overcoming the barrier defined by the critical free enthalpy ΔG_1^* , that is the first nucleation step. The dense liquid may be either metastable (upper) or stable (lower) with respect to the solution state. Overcoming ΔG_2^* , that is the second nucleation step, crystals form within the dense liquid. The dense liquid is metastable with respect to the crystals in both cases. Ref. [36] - Reproduced by permission of The Royal Society of Chemistry.

Figure 18. Free energy profile of nucleation of calcium carbonate. In the classical point of view, metastable clusters are formed, resulting in a barrier to nucleation characterized by the critical free enthalpy ΔG^* . In fact, however, stable clusters form with a negligible barrier compared to thermal energy; the clusters are solutes and separated from the solid state by a barrier that is significant compared to thermal energy, i.e. the actual nucleation step is the formation of solid CaCO_3 from the clusters. In the solution state, the state of prenucleation clusters represents a minimum in free enthalpy, and the clusters are thermodynamically stable. The solution state,

though, may become metastable with respect to solid calcium carbonate, and nucleation then appears to proceed via aggregation of the prenucleation clusters. From Ref. [38]. Reprinted with permission from AAAS.

Figure 19. According to classical nucleation theory, calcium carbonate nucleation proceeds by addition of ions to a single cluster (top). Calcium carbonate forms *via* a different mechanism, in which nucleation of ACC occurs by aggregation of stable, amorphous, precritical clusters (bottom). The nucleated ACC phase subsequently crystallizes to generate the final stable crystal product. From Ref. [105]. Reprinted with permission from AAAS.

Tables

Table 1 Clusters in analytical ultracentrifugation, AUC (pH = 9.00). Ions and three cluster species can be observed. $t_{\text{obs.}}$ gives the time, at which the species can be observed (cf. Figure 2; 90 minutes is close to nucleation, 120 minutes is the early post-nucleation stage), brackets give the ratio of number of observations to the total number of AUC experiments performed at the particular time. N gives the absolute number of experiments averaged and index ‘av.’ indicates averaged values $\pm 1\text{-}\sigma$ -standard deviation. s sedimentation coefficient, $d(\text{ACC})$ hydrodynamic diameter calculated from the sedimentation coefficient and utilizing the density of ACC, $d(\text{ikaite})$ hydrodynamic diameter calculated with the density of ikaite. The semicolons separate the two determined parameters for cluster III. The values marked in red are not considered, since they can be attributed to evaluation artefacts. Data taken from ref. [38].

	Ions	cluster I	cluster II	cluster III
$t_{\text{obs.}} \text{ (min.)}$	0(6/6), 15(3/3), 75(4/4), 90(5/5), 120(3/3)	0(2 /6), 75(3/4), 90(5/5)	75(1/4), 90(2/5)	120(2/3)
N	21	8	3	2
$s_{\text{av.}} \text{ (S)}$	0.11 ± 0.05	1.4 ± 0.8	5 ± 1	7; 10
$D_{\text{av.}} \text{ (10}^{-7} \text{ cm}^2 \text{ s}^{-1})$	118 ± 7	59 ± 12	29 ± 8	11; 39
$d_{\text{av.}}(\text{ACC})$	-	2.1 ± 0.6	3.9 ± 0.4	5; 6
$d_{\text{av.}}(\text{ikaite})$	-	1.6 ± 0.4	3.1 ± 0.3	4; 4