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Ion-association complexes unite classical and non-classical theories for the biomimetic nucleation of calcium phosphate

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Despite its importance in many industrial, geological and biological processes, the mechanism of crystallization from supersaturated solutions remains a matter of debate. Recent discoveries show that in many solution systems nanometre-sized structural units are already present before nucleation. Still little is known about the structure and role of these so-called pre-nucleation clusters. Here we present a combination of in situ investigations, which show that for the crystallization of calcium phosphate these nanometre-sized units are in fact calcium triphosphate complexes. Under conditions in which apatite forms from an amorphous calcium phosphate precursor, these complexes aggregate and take up an extra calcium ion to form amorphous calcium phosphate, which is a fractal of Ca2(HPO4)32− clusters. The calcium triphosphate complex also forms the basis of the crystal structure of octacalcium phosphate and apatite. Finally, we demonstrate how the existence of these complexes lowers the energy barrier to nucleation and unites classical and non-classical nucleation theories.
According to Classical Nucleation Theory\(^1\)-\(^2\), the first step in the formation of crystalline mineral phases involves the stochastic and dynamic association of ions in solution, overcoming a free-energy barrier to form nuclei that, above a critical size, can grow out to a mature crystal. In addition, the rate equations that derive from this theory show that the first material to precipitate is not necessarily the thermodynamically most stable state, but the kinetically most accessible one, which may later transform into the thermodynamically more stable form. The appearance of amorphous precursors, which are observed for many minerals\(^3\), fits this concept of multistage crystallization\(^4\) that was first put forth by Ostwald and later revised to incorporate the evolution in the understanding of both thermodynamic and kinetic factors\(^5\). One example of such a mineral is calcium phosphate, the main component of bone and tooth and consequently the most studied biomineral, with major applications in dentistry, orthopaedics and reconstructive surgery\(^6\).

Biological calcium phosphate is rather ill-defined, best described as poorly crystalline, highly substituted apatite (AP)\(^7\) and has recently been demonstrated to form via an amorphous precursor phase\(^8\)-\(^10\). Already in the 1960s, biomimetic mineralization experiments were directed at understanding the formation mechanism of this mineral, and resulted in the precipitation of calcium phosphate with X-ray diffraction characteristics identical to those of the biological material\(^11\). These early investigations indicated the presence of a metastable amorphous precursor phase, which was postulated to consist Ca\(_6\)(PO\(_4\))\(_6\) clusters\(^12\). Although initially these observations were met with skepticism\(^13\), recently reports confirmed that the precipitation of calcium phosphate indeed involves the formation of nanometre-sized building blocks\(^14\)-\(^18\). In fact for many different organic\(^19\),\(^20\) and inorganic crystals\(^3\),\(^18\),\(^21\) nucleation models involving pre-nucleation clusters have been proposed. However, owing to their small dimensions, it has so far not been possible to unravel the structural details of the clusters in their native hydrated state nor of the mechanism by which they aggregate. Moreover, the impact of such clusters on the energy barriers that ultimately dictate passage through the amorphous phase has not yet been fully explored\(^22\).

Here, we study the biomimetic precipitation of calcium phosphate, in a buffered solution and under constant ionic strength, where a wide range of in situ analysis techniques provide morphological, structural and chemical information. These experiments reveal that the formerly observed calcium phosphate pre-nucleation clusters in fact are calcium tripolyphosphate ion-association complexes, which can aggregate into branched three-dimensional (3D) polymeric structures. From these polymeric solution structures, nucleation of amorphous calcium phosphate (ACP) occurs through the simultaneous binding of calcium to form \(\sim 1.2\) nm post-nucleation clusters, and their aggregation and precipitation as spherical particles. Continued calcium uptake converts ACP into octacalcium phosphate (OCP) and subsequently into AP, which both contain the calcium tripolyphosphate complex as their basic structural unit. Measuring the rate of calcium phosphate nucleation as a function of supersaturation, we show that under the conditions used here, ACP formation cannot be directly reconciled with classical nucleation theory. However, theoretical considerations show that the thermodynamic barrier for nucleation is dramatically lowered when the existence of pre-nucleation complexes and the particle size dependence of the interfacial free energy are taken into account. With this, the observed non-classical route to ACP formation can be explained using classical theory.

**Results**

**Morphological development during calcium phosphate formation.** Biomimetic calcium phosphate was prepared by the instantaneous addition of a phosphate solution to a gently stirred solution of calcium ions. During the reaction, samples were collected and vitrified on electron microscope grids for high-resolution cryogenic transmission electron microscopy (cryo-TEM) analysis\(^24\). Cryo-TEM images (Fig. 1a – i) showed that the growth of calcium phosphate proceeds via multiple distinct morphologies. Within the first minutes after the addition, strands of nanometre-sized units—not seen in the buffers themselves (Supplementary Fig. S6D)—appeared in the solution (Fig. 1a), and grew out to form a branched polymeric network (Fig. 1b) that subsequently developed nodules (Fig. 1c) with dimensions of 150–200 nm. In agreement with previous reports\(^14\),\(^23\)-\(^25\), these nodules condensed further, transforming into spheres that coagulated and subsequently precipitated after 15–20 min (Fig. 1d). This sequence of events was supported by dynamic light scattering data (Supplementary Fig. S1). High-resolution cryo-TEM revealed that also the spheres consisted of nanometre-sized building blocks. After \(\sim 1\) h, the coagulated spheres transformed, producing a thin ribbon-like morphology (Fig. 1e,f). At this time, a small number of polymeric chains were still present inside the solution, and occasionally the direct assembly of ribbons from these polymers was observed (Fig. 1g). In time, the ribbons evolved into their final shape that consists of elongated plates (Fig. 1h,i), similar to the morphology as described for biomimetic AP\(^26\).

**The evolution of structure.** During the reaction both the calcium concentration ([Ca\(^{2+}\)]\(^\text{aq}\)) and the pH were monitored, revealing that the morphological transitions observed with cryo-TEM corresponded to distinct, concomitant drops in the free [Ca\(^{2+}\)]\(^\text{aq}\) and pH (Fig. 2a). This implies that each of the four subsequent morphologies (polymeric aggregates, spheres, ribbons and elongated plates) represents a separate phase with its own characteristic calcium solubility. As no changes in the free [Ca\(^{2+}\)]\(^\text{aq}\) or pH occur during the first minutes of the reaction (see also Supplementary Fig. S2), we must consider the first drop in [Ca\(^{2+}\)]\(^\text{aq}\) or pH as the nucleation point, that is, the point where the first new phase appears from solution. Hence, we consider the polymeric network of nanometre-sized units found before the first phase transition as pre-nucleation species, and the spherical aggregates of nanometre-sized building blocks after this transition as post-nucleation species. The nodules consequently present a transition state at which both the chemical transformation and the aggregation are only partially completed.

In agreement with previous reports\(^14\),\(^15\) low-dose selected area electron diffraction (LDSAED, Fig. 3a) demonstrated that both in the pre-nucleation and the post-nucleation stage no (long-range) structural order was present, characterizing the precipitate of coagulated spherical aggregates as ACP. Analysis of the third phase (ribbons) revealed the appearance of a broad band at a lattice spacing (\(d\)) of 0.26–0.33 nm that became more pronounced in the fourth phase (plates).

Wide-angle synchrotron X-ray scattering (WAXS) measurements (Fig. 4a) on samples of this stage gave proof of a structure very similar to OCP. Only the (100) peak, which corresponds to planes with a large \(d\)-spacing of 1.85 nm along the thinnest dimension of the OCP plate-like morphology\(^27\), was missing. This can be explained by the small thickness of the OCP-like plates in our samples (\(\approx 1.4\) nm, see small-angle synchrotron X-ray scattering (SAXS) data Fig. 4b), which is less than the \(d\)-spacing between the (100) planes in OCP crystals. LDSAED from plates aged for 1 month revealed a peak at \(d = 0.28\) nm.
corresponding to the (211)/(112) planes of AP (Fig. 3a), implicating that, as in previous reports, the OCP-like structure acted as a precursor for AP. These structural developments were supported by in situ Fourier transform infrared spectroscopy (FTIR, Fig. 3b), which further indicated significant chemical transformations between the different morphological stages.

**Chemical development through calcium binding.** By monitoring the pH and the free \([Ca^{2+}]\), the release of protons and the changes in the concentration of bound calcium could be determined (Fig. 2b). From these data and taking into account the applied physicochemical conditions and the relevant chemical equilibria (see also Supplementary Methods), we calculated the concentration of the different calcium-bound phosphate species (\(H_2PO_4^-\), \(HPO_4^{2-}\) and \(PO_4^{3-}\)) in the subsequent stages of the reaction (Fig. 2c). Combining the information on the calcium and phosphate concentrations at different time points, and taking into account that also calcium phosphate ion pairs are formed, the calcium/phosphate (Ca/P) ratios of the different phases were calculated (Fig. 2d). In addition, formal charges were derived (see also Supplementary Table S1). For the pre-nucleation species \((Ca/P = 0.3–0.4)\), this yielded an ion-association complex with the formula \([Ca(HPO_4)_{x_1}(H_2PO_4)_{x_2}]^{4-}\) of which 57% was in its protonated state \([Ca(HPO_4)_{x_1}(H_2PO_4)_{x_2}]^{3-}\).

To determine the exact speciation of the pre-nucleation complex chemistry, a titration and a dilution experiment were performed. Upon titrating small \(Ca^{2+}\)-containing aliquots to a high phosphate concentration \([P]\), a constant ratio of free to bound \(Ca^{2+}\) was measured during the first 20 min (Supplementary Fig. S4). As initially \([P]\) is approximately constant, the concentration of all pre-nucleation species (both complexes and ion pairs) according to the overall equilibrium in the pre-nucleation stage (Fig. 5a) is independent of \([P]\), that is: \(K_{eq} \propto [\text{pre-nucleation species}]/[Ca^{2+}]^{x} = [Ca(HPO_4)_x(H_2PO_4)_y]^{2x-2y-2}/[Ca^{2+}]^x\) with \(x\) being the number of \(Ca^{2+}\) ions inside a pre-nucleation species and \(K_{eq}\) the equilibrium constant of the pre-nucleation species. In the pre-nucleation stage, all bound calcium must be present either as complexes or ion pairs. A constant ratio of free to bound \(Ca^{2+}\) therefore implies \(x = 1\), and hence that all pre-nucleation species contain only one calcium ion (see Supplementary Methods: titration experiment).

By subsequently monitoring \([Ca^{2+}]\) upon dilution of the pre-nucleation stage—with all equilibrium constants of all pre-nucleation species remaining unchanged—the composition of the pre-nucleation species could be calculated (Supplementary Methods: dilution experiment). This revealed a dynamic equilibrium between pre-nucleation complexes with ions and ion pairs (mainly \(CaHPO_4\)) in solution according to the reaction scheme presented in Fig. 5b.

For these pre-nucleation complexes, ab initio calculations indicated a triangular arrangement of phosphates around the calcium ion. This results in a disc-like shape with a diameter of 1.1 nm and a height of 0.5 nm (Fig. 5c,d, Supplementary Fig. S7).

**Figure 1 | Morphological transformations of CaP during time as observed by Cryo-TEM.** (a) Polymeric strands from nanometre-sized units (<2 min), (b) branched polymeric assemblies (2–20 min), (c) nodules (10–20 min), (d) aggregated spheres (15–60 min), (e) aggregated spheres + ribbons (60–80 min), (f) ribbons (80–110 min), (g) direct assembly of ribbons from polymeric aggregates (60–110 min) (lines denote alignment of complexes), (h) elongated plates (>110 min), (i) plates (1 month), scale bar, 50 nm (insets are lower magnification images: scale bar, 100 nm). Similar morphological transformations were found in solutions without Tris-buffer (see Supplementary Fig. S13).
Detailed analysis of the high-resolution cryo-TEM images gave $1.3 \pm 0.3$ nm for the diameter and $0.9 \pm 0.3$ nm for the height of the complexes (Supplementary Fig. S8A–D). These TEM values agree well with the calculated values if we take into account that electron beam. The high charge calculated for the pre-nucleation complexes seems incompatible with their rapid aggregation into polymeric assemblies. However, previous work showed the aggregation of highly charged solutes to be driven by the gain in entropy associated with the release of hydration water\(^{22}\) and also the formation of linear supramolecular polymers of charged monomeric units has been described\(^{33}\).

For the ribbons (Ca/P = 1.0) a composition of \([\text{Ca}_6(\text{HPO}_4)_4(\text{PO}_4)_2]^{2-}\) was found, which developed into \(\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4\) for the elongated plates (Ca/P = 1.33). The latter ratio was confirmed by induction-coupled plasma-optical emission spectroscopy (ICP-OES) measurements (marked in Fig. 2b,c) and is consistent with their identification as OCP by FTIR spectroscopy. As WAXS revealed an OCP-like structure for the ribbons, their formula \([\text{Ca}_6(\text{HPO}_4)_4(\text{PO}_4)_2]^{2-}\) (Ca/P = 1.0) points to a Ca-deficient OCP. This implies that the OCP structure precedes the development of the OCP chemistry (Ca/P = 1.33), which is only fully completed in the stage of the plates. Moreover, Fig. 2a shows that the drop of the pH occurs after the drop in the free calcium concentration. This suggests that the binding of the calcium ions to the negatively charged Ca-deficient OCP is a first step that takes place, before their incorporation in the OCP lattice and the concomitant release of protons.

Mechanism of aggregation. The high charge calculated for the pre-nucleation complexes seems incompatible with their rapid aggregation into polymeric assemblies. However, previous work showed the aggregation of highly charged solutes to be driven by the gain in entropy associated with the release of hydration water\(^{22}\) and also the formation of linear supramolecular polymers of charged monomeric units has been described\(^{33}\).

Also the observed small negative zeta potential of $\sim -3.5$ mV (Fig. 6) seems to contradict the proposed high charge of the polymeric structures. Still, this value is in complete accordance with values calculated for a 3D assembly of complexes in which hydrodynamic shielding prevents the detection of charge in the interior of the partially penetrable aggregate with hydrodynamic skin depth $\varepsilon$ (Fig. 6, see also Supplementary Methods,
Upon the formation of the spherical nodules and aggregated spheres (Fig. 6, Structures 2 and 3), the zeta potential diminishes further as they represent (an aggregate of) opaque structures. The aggregation process was also investigated using dual-axis cryo-electron tomography in combination with 3D box counting (Fig. 5e, Supplementary Fig. S9). This revealed the 3D structure as well as the fractal dimension ($D_f = 2.2 \pm 0.1$) of the polymeric assemblies, indicating a reaction-limited aggregation (RLCA) process. Based on the structure of the ion-association complexes, it is likely that their aggregation involves hydrogen bonding (see Fig. 5c). This will be most efficient in the conformation that allows double, bidentate hydrogen bond formation between two complexes, as suggested for protonated phosphate groups in solution. This explains the reaction-limited nature of the aggregation process.

Binding of calcium and associated loss of structural water during the transformation into post-nucleation species leads to a further cross-linking of the complexes, and a concomitant densification of their packing (Figs 1c,5e). Indeed, analysis of the aggregates of post-nucleation clusters in ACP revealed a fractal dimension of $2.7 \pm 0.1$ (Supplementary Fig. S9), corresponding to a denser, more ordered packing than for the polymeric assemblies in the pre-nucleation stage. This reorganization of the cluster packing also demonstrates the dynamic nature of the interactions in the 3D polymeric structures, in line with the proposed hydrogen bond formation between the pre-nucleation complexes.

From the ACP spheres an OCP-like structure evolves, for which the observed thickness ($\sim 1.4 \text{ nm}$) is in accordance with the structural characteristics of OCP—that is, a zig–zag arrangement of rows of calcium triphosphate complexes fused through binding of additional calcium ions (Fig. 5e).

**Figure 3** | **Electron diffraction and FTIR analysis.** (a) Radially averaged electron diffraction data at different stages compared with the tris-buffered saline and crystalline nano-AP, showing the uprising of the OCP signal (arrow) (inset: corresponding raw electron diffraction data and morphology in Cryo-TEM, scale bar, 200 nm). (b) In situ FTIR of the calcium phosphate, spectra recorded at different time points in the P–O stretch region. Dotted lines + arrow indicate the shift of the upcoming $\text{PO}_4^{3-}$-stretch vibration from 1,021 to 1,030 cm$^{-1}$. In the first two stages, the signals observed correspond to vibrations of $\text{HPO}_4^{2-}$ and $\text{H}_2\text{PO}_4^{-}$ (pre-nucleation) and $\text{HPO}_4^{2-}$ (post-nucleation), respectively. The $\text{PO}_4^{3-}$-stretch vibration at 1,021 cm$^{-1}$ characteristic for OCP first appears in the stage of the ribbon-like structures and further develops in the stage of the plates, which upon aging shifts to 1,030 cm$^{-1}$ confirming the conversion into AP.

**Thermodynamics of a growth mechanism based on complexes.** This study, as well as other recent research, has demonstrated that the formation of AP can occur through the conversion of an...
amorphous precursor phase\textsuperscript{36} that forms via a cluster-growth mechanism\textsuperscript{14,15}. We now must assume that it also involves the aggregation of pre-nucleation complexes. To investigate the consequences of this nucleation-complex-based mechanism on the energy barriers associated with AP formation, we used \textit{in situ} AFM, which allows one to directly monitor mineralization processes on surfaces with sub-nanometre detail\textsuperscript{37}.

To avoid competitive nucleation in the bulk solution, the substrates used for these AFM experiments were coated with collagen. Owing to its widespread role as an organic matrix for calcium phosphate nucleation in biological dentine and bone, we reasoned collagen would provide a low interfacial energy and thus high nucleation rates at low supersaturation. Using these collagen substrates, we found it possible to measure the nucleation rates of calcium phosphate within minutes to hours, depending on the supersaturation, \( \sigma \) (see Methods for details on solubility and calculating supersaturations), with no concomitant nucleation in the solution. At values of \( \sigma_{\text{AP}} \geq 3.08 \) (\( \sigma_{\text{ACP}} \leq -0.23 \)), the smallest features detected were composed of AP (Fig. 7a – c). Note that the solution is undersaturated with respect to ACP; thus any ACP that forms is owing to inherently unstable and transient fluctuations, which we would not see by AFM. However, for values of \( \sigma_{\text{AP}} \geq 3.36 \) (\( \sigma_{\text{ACP}} \geq 0.04 \)), the first phase to form was ACP, which transformed first to OCP and then to AP (Fig. 7d – f), consistent with the cryo-TEM results (see also Supplementary Fig. S10). In both cases, the first particles to form were 1–2 nm in size and grew with time. Moreover, the morphologies observed by AFM and subsequent \textit{ex situ} TEM (Fig. 7d – f, insets) at \( \sigma_{\text{AP}} \geq 3.36 \) were similar to those seen by cryo-TEM; nm-size particles forming spheroidal aggregates that transform first into spherulites of OCP and, finally, into plate-like crystals of AP.

To analyse these data, as a starting point we assume that nucleation occurs through ion-by-ion growth in accordance with classical nucleation theory. Then the interfacial energy \( \alpha \) of the nucleating phase can be determined from the number density of nucleation events versus time using the classical expressions (Fig. 7g)\textsuperscript{1,2}.

Applying this analysis to heterogeneous nucleation of hemispherical particles, we obtain values of 40 and 90 mJ m\textsuperscript{-2} for \( \alpha_{\text{ACP}} \) and \( \alpha_{\text{AP}} \), respectively (see Supplementary Methods for details). Based on solubilities, the values of \( \alpha_{\text{ACP}} \) and \( \alpha_{\text{AP}} \) in bulk solution are expected to be about 150 and 180 mJ m\textsuperscript{-2}, respectively\textsuperscript{38}. Consequently, the experimental values of \( \alpha \) appear reasonable for nucleation on a substrate that promotes nucleation. However, the magnitudes of the thermodynamic barriers to nucleation implied by these values demonstrate a dramatic failure of the classical expressions for ion-by-ion nucleation. In these expressions, \( \Delta G_{r} \), (see caption of Fig. 7) scales with \( \alpha^{3}/\sigma^{2} \). Taking the values of \( \sigma \) for AP and ACP at the conditions where the first phase to appear changes from the former to the latter (\( \sigma_{\text{AP}} = 3.36 \), \( \sigma_{\text{ACP}} = 0.04 \)), we find that \( \Delta G_{r} \), for ACP formation should be about 600 times that for AP formation. In addition, as shown by Fig. 8a, which is a plot of \( \Delta G \) versus \( R \) using the value of \( \alpha_{\text{ACP}} \) determined from the data in Fig. 7g, in this scenario the value of the barrier to ACP nucleation is more than three orders of magnitude greater than kT. Moreover, the critical radius \( R_{c} \) given by the particle radius at which the free-energy change in Fig. 8a reaches a maximum, should be \( \sim 10 \) nm, which is much larger than the 1–2 nm sizes of the first features observed experimentally. Similarly, for the homogeneous nucleation of ACP under the conditions used in the cryo-TEM experiments (\( \sigma_{\text{AP}} = 3.8 \) and \( \sigma_{\text{ACP}} = 0.69 \)) and using the interfacial energies in bulk solution, we calculate that \( \Delta G_{r} \) is expected to be yet three times larger than for nucleation on collagen, and 18 times that of AP in the same solution. To put these barriers in perspective, even if the pre-factor A was equivalent to the attempt frequency for atomic vibrations at room temperature (6 \( \times 10^{12} \) Hz), the barrier would have to be of order 30 kT or less to get significant rates of nucleation. Consequently, ACP should never form under these conditions.

This analysis does not take into account any size dependence in the effective interfacial energy. In reality, below some size limit \( R_{\infty} \) in the nm range this energy must diminish, reaching zero at the length scale of ion pairs. This size dependence can have significant consequences for both the stability of phases\textsuperscript{3} and the barriers to nucleation\textsuperscript{29}. However, the effect only becomes a factor of importance when \( R_{c} \) is comparable to \( R_{\infty} \), whereas the 7 nm critical radius for ACP formation predicted by classical theory is well above that at which size dependence should be a factor. Even if we set the interfacial energy to zero at the dimension of an ion pair (\( \sim 4 \) Å) and assume it reaches its bulk value only at a particle diameter greater than 5 nm, which is about two times the typical dimension considered appropriate\textsuperscript{39}, the barrier remains insurmountable. As shown in Fig. 8b, which is a...
plot of $\Delta G$ versus $R$ using the same parameters as in Fig. 8a with the addition of a size dependent $z$, for a reasonable range of $R_N$, little or no change in $\Delta G_c$ or $R_c$ is predicted in the range of supersaturations relevant to the AFM experiments. For example, when $\sigma_{ACP} = 0.15$ for $R_N$ ranging from 1–3 nm, the barrier is still over 500 kT. For homogeneous nucleation at the supersaturation used in the TEM experiments, owing to the much larger value of $a_N$ (100 versus 40 mJ m$^{-2}$), the barrier is much larger still.

The failure of the classical picture can be understood when the presence of the pre-nucleation complexes and the complex pathway to the crystalline state observed by cryo-TEM are taken into account. The origin of the barrier in the classical nucleation equation is the introduction of an excess free energy—over that of the bulk solid—that is associated with creation of new surface. However, the pre-nucleation complexes themselves—though they are solution species—have a certain excess free energy over that of the free ions that is associated with their surface. (In this regard, they differ from the pre-nucleation clusters postulated in the calcium carbonate system, which are said to be lower in free energy than the free ions.) Evidence for this surface energy can be found in the fractality of the polymeric assemblies, whose packing of complexes in the reaction limited (RLCA) range differs substantially from the open diffusion limited (DLCA) geometry described for structures at the zero surface tension limit.$^{40}$ When the complexes combine to form a larger particle, the elimination of this excess free energy $\Delta G_{Ex}$ must be taken into account and this can have a dramatic effect on the size of the free-energy barrier, as well as the critical radius. To evaluate the effect, we can express the change in free energy as a function of particle radius $R$ using:

$$\Delta G = -\frac{4\pi R^3}{3\sigma} kT \sigma + 4\pi R^2 \alpha(R) - N\Delta G_{Ex}$$

where $N$ is the number of complexes that combine to form the particle and $f$ is a geometric factor that depends on nucleus shape,
equalling 1 and 1/2 for spherical and hemispherical nuclei, respectively. This explicitly shows that there is a reduction in $\Delta G$ owing to any excess free energy of the complexes over that of the free ions. For the case of ACP nucleation on collagen, the number of disk-shaped complexes of radius $r$ (with height = radius) that must combine to form an ACP nucleus is given by $N = (4\pi/3)(R/r)^3$. Attributing $\Delta G_{\text{Ex}}$ exclusively to the complex surface free-energy $\pi/2a$ implies that $\Delta G_{\text{Ex}} = 4\pi R^2\zeta_{\text{comp}}$. This leads to a nucleation barrier of:

$$\Delta G_c = \frac{16\pi \sigma^2 \zeta^2}{3(kT)^2} \left(1 + \frac{4\pi \sigma}{rRT\eta}\right)^{-2}, \quad \eta = \frac{\xi_{\text{comp}}}{a}$$

Comparison of equation 2 with the classical expression shows that $\Delta G_c$ is now given by the product of the free-energy barrier for nucleation directly from molecular species and a correction term that is always $\leq 1$ and whose value scales with the ratio of the complex surface energy to that of the newly nucleated phase. Moreover, because the surface energy terms dominate at low $\sigma$, the impact on $\Delta G_c$ is enormous, even when the surface energy of the complexes is significantly less than that of the nucleating particles. A best fit of equation 2 to the data in Fig. 7 for a value of $\zeta_{\text{comp}}$ in the range of 100–150 mJ m$^{-2}$ gives a value of about 1/15 for $\eta$. For ratios of the complex-to-bulk surface energy ($\eta$) of this size or larger, Fig. 8c shows that both $\Delta G_c$ and $R_c$ are dramatically reduced. In fact, for sufficiently large values, the barrier can be completely eliminated so that nucleation proceeds downhill in free energy. For example, when $\sigma_{\text{ACP}} = 0.15$, the barrier disappears for all values of $\zeta_{\text{comp}}$ greater than 10% of $\zeta_{\text{m}}$, or conversely, when $\zeta_{\text{comp}}$ is greater than 10% of $\zeta_{\text{m}}$, it is eliminated for all $\sigma_{\text{ACP}} \geq 0.15$. Figure 8c also shows that, for the slightly greater value of $\sigma_{\text{ACP}} = 0.4$, there is no barrier for $\zeta_{\text{comp}}$ greater than only 6.7% of $\zeta_{\text{m}}$. Thus the aggregation of the pre-nucleation complexes followed by binding of Ca$^{2+}$ may avoid the insurmountable thermodynamic barrier faced during ion-by-ion nucleation of ACP predicted in Fig. 8a,b. As a result, the pathway to the final crystalline state should be controlled by kinetic barriers associated with the chemical reactions and structural rearrangements revealed by cryo-TEM. As this analysis describes the heterogenous nucleation of ACP on a complex size and therefore their interactions deviate from the DLVO theory derived for larger colloidal particles. Hence, the ion-association complexes are radii, $\xi$ = hydrodynamic skin dept, and $D_{12}$ are fractal dimensions. Zeta potentials were measured in transmission at a 50 V effective voltage (average over 100 runs). The Von Smoluchowski equation was applied, considering the high ionic strength used ($I = 0.2$ M) and size of the polymeric aggregates (>100 nm). Importantly, for the described complexes the distances between the charged moieties inside the complex are not small with respect to the complex surface energy to that of the newly nucleated phase. Moreover, because the surface energy terms dominate at low $\sigma$, the impact on $\Delta G_c$ is enormous, even when the surface energy of the complexes is significantly less than that of the nucleating particles. A best fit of equation 2 to the data in Fig. 7 for a value of $\zeta_{\text{comp}}$ in the range of 100–150 mJ m$^{-2}$ gives a value of about 1/15 for $\eta$. For ratios of the complex-to-bulk surface energy ($\eta$) of this size or larger, Fig. 8c shows that both $\Delta G_c$ and $R_c$ are dramatically reduced. In fact, for sufficiently large values, the barrier can be completely eliminated so that nucleation proceeds downhill in free energy. For example, when $\sigma_{\text{ACP}} = 0.15$, the barrier disappears for all values of $\zeta_{\text{comp}}$ greater than 10% of $\zeta_{\text{m}}$, or conversely, when $\zeta_{\text{comp}}$ is greater than 10% of $\zeta_{\text{m}}$, it is eliminated for all $\sigma_{\text{ACP}} \geq 0.15$. Figure 8c also shows that, for the slightly greater value of $\sigma_{\text{ACP}} = 0.4$, there is no barrier for $\zeta_{\text{comp}}$ greater than only 6.7% of $\zeta_{\text{m}}$. Thus the aggregation of the pre-nucleation complexes followed by binding of Ca$^{2+}$ may avoid the insurmountable thermodynamic barrier faced during ion-by-ion nucleation of ACP predicted in Fig. 8a,b. As a result, the pathway to the final crystalline state should be controlled by kinetic barriers associated with the chemical reactions and structural rearrangements revealed by cryo-TEM. As this analysis describes the heterogenous nucleation of ACP on a complex size and therefore their interactions deviate from the DLVO theory derived for larger colloidal particles. Hence, the ion-association complexes...
Figure 7 | Results of in situ AFM investigation of ACP and AP nucleation kinetics. (a–c) Nucleation of HAP on collagen at (a) $t = 270$, (b) 1,020 and (c) 5,280 s, respectively, where $\sigma_{AP} = 3.08$, $\sigma_{OCP} = 1.51$ and $\sigma_{ACP} = -0.23$. (d) Nucleation of ACP on collagen followed by transformation to (e) OCP and then (f) AP at (d) $t = 552$, (e) 3,660 and (f) 6,000 s, respectively, where $\sigma_{AP} = 3.36$, $\sigma_{OCP} = 1.76$ and $\sigma_{ACP} = 0.04$. Inserts are TEM images of mineral phase (a) AP, (b) ACP (e) OCP and (f) AP. Scale bars in AFM images are 100 nm. (g) Dependence of the steady-state nucleation rate $J_0$ on time $t$ at six different supersaturations. The supersaturations corresponding to the numeric labels are, 1: $\sigma_{AP} = 3.08$, $\sigma_{OCP} = 1.51$, $\sigma_{ACP} = -0.23$; 2: $\sigma_{AP} = 3.24$, $\sigma_{OCP} = 1.65$, $\sigma_{ACP} = -0.08$; 3: $\sigma_{AP} = 3.31$, $\sigma_{OCP} = 1.71$, $\sigma_{ACP} = -0.02$; 4: $\sigma_{AP} = 3.45$, $\sigma_{OCP} = 1.83$, $\sigma_{ACP} = 0.128$; 5: $\sigma_{AP} = 3.46$, $\sigma_{OCP} = 1.84$, $\sigma_{ACP} = 0.129$ and 6: $\sigma_{AP} = 3.47$, $\sigma_{OCP} = 1.85$, $\sigma_{ACP} = 0.1295$. Analysis of the data using classical nucleation theory, which predicts that $J_0 = A \cdot \exp(-\Delta G_f/kT) = A \cdot \exp(-8\pi\sigma^2J/3kT)$, where $A$ is the kinetic constant related to diffusional, steric and any other kinetic barriers, $\Delta G_f$ is the free-energy barrier to nucleation, $\sigma$ is the interfacial energy, $k$ is Boltzmann’s constant and $T$ is the absolute temperature, gives $\sigma_{ACP} = 40 \text{ mJ m}^{-2}$ and $\sigma_{AP} = 90 \text{ mJ m}^{-2}$ (see Supplementary Information Analysis of nucleation data: fitting of classical nucleation rate equation for details.) We note that nucleation on a substrate is completely equivalent to nucleation in the bulk solution in terms of this equation, which does not distinguish between heterogeneous and homogeneous nucleation except through the differences in $\sigma$ and the numerical factor $8/3$.

Figure 8 | Effect of supersaturation, size dependent surface energy, and cluster excess free energy on classical nucleation barrier. (a) Dependence of free-energy change $\Delta G_f$ on radius of nucleus $R$ based on the classical expression for heterogeneous ion-by-ion nucleation $\Delta G_f = -(2\pi R^2/3\sigma) \cdot kT + 2\pi R^2 \sigma$ of hemispherical ACP for $\sigma_{ACP} = 40 \text{ mJ m}^{-2}$ (derived from Fig. 7), values of $\sigma$ as specified next to the curves, and other symbols as defined in Fig. 7. (See Supplementary Information Analysis of nucleation data: fitting of classical nucleation rate equation for details.) (b) Impact of size-dependent interfacial free energy on the barrier for heterogeneous ion-by-ion nucleation of hemispherical ACP, as based on equation 1. Many forms for this dependence have been proposed; however, the exact dependence chosen has only a minor effect on the predicted change in nucleation rates. Here, we assume an exponential dependence, $\sigma_{ACP}(R) = \sigma_{\infty} [1 - \exp(-R/R_0/R_\infty)]$, where $\sigma_{\infty}$ is the surface energy for the bulk phase, $R_0$ is on the order of the molecular radius of the nucleating species and $R_\infty$ is the characteristic particle radius at which the interfacial energy approaches the bulk value. Typical literature values for $R_0$ are $\sim 2 \AA$, which we use here.39 Curves are shown for $R_\infty = 1 \text{ nm}$ (blue lines), $2 \text{ nm}$ (red lines) and $3 \text{ nm}$ (green lines), assuming $\sigma_{ACP} = 0.15$ (solid lines) or 0.25 (dashed lines) and $\sigma_{\infty} = 40 \text{ mJ m}^{-2}$. All other parameters are as in Fig. 8a. Comparison of the solid curves of (b) with the red curve in (a) shows that the size dependence of surface energy shifts the free-energy barrier to slightly lower values and decreases $R_\infty$ by a minor amount. (c) Dependence of free energy on nucleus size and ratio of complex to ACP surface energy ($\sigma_{comp}/\sigma_{ACP}$) according to equations 1 and 2 during heterogeneous nucleation of hemispherical ACP through aggregation of 1.1 nm disk-shaped complexes for $\sigma_{ACP}(R) = \sigma_{\infty} [1 - \exp(-R/R_0/R_\infty)]$ with $\sigma_{\infty} = 100 \text{ mJ m}^{-2}$, $R_0 = 2 \AA$ and $R_\infty = 1 \text{ nm}$, where values of $\sigma_{comp}/\sigma_{ACP}$ and the corresponding $\Delta G_{f\infty}$ are specified in the legend. Note that here $\sigma_{\infty}$ refers to the interfacial free energy of bulk ACP in solution, which we have conservatively taken as $100 \text{ mJ m}^{-2}$. Heavy solid curves: $\sigma_{ACP} = 0.15$, light solid curves: $\sigma_{ACP} = 0.4$.

collagen substrate, an extension for homogenous nucleation, as observed in cryo-TEM, was derived and is given in the Supplementary Note 1.

Nothing in the above analysis requires the nucleating phase to be ACP. Thus, the impact of the pre-nucleation complexes on the free-energy barrier to ACP formation should also be manifest. However, to go directly from these complexes to the ordered crystal requires both more extensive chemical transformations than needed for ACP formation, as well as structural transformations that are not a factor in ACP nucleation. Hence, the kinetic barriers to AP formation are likely to exceed those for ACP formation and, once the ACP phase boundary is crossed,
ACP quickly becomes the dominant phase to emerge from the supersaturated solution.

Discussion

In 1974, Betts and Posner demonstrated that ACP consisted of subunits already containing some of the structural features of AP and proposed these subunits—later termed Posner’s clusters—to be $\text{Ca}_9\text{P}_4\text{O}_{16}$. They assigned peaks in the radial distribution functions from X-ray data to the atom pairs $\text{P}–\text{O}$ (1.5 Å), $\text{O}–\text{O}$ and $\text{Ca}–\text{O}$ (2.3 Å), and $\text{Ca}–\text{Ca}$, $\text{Ca}–\text{P}$ and $\text{P}–\text{P}$ (3–6 Å). These values agree well with the distances derived from the ab initio calculations for the pre-nucleation complexes, which are $\text{P}–\text{O}$ (1.6 ± 0.1 Å), $\text{Ca}–\text{O}$ (2.5 ± 0.1 Å), $\text{Ca}–\text{P}$ (3.0 ± 0.1 Å) and $\text{P}–\text{P}$ (5.1 ± 0.3 Å) (see also Supplementary Table S3). Actually, the Posner’s cluster can be seen as two deprotonated pre-nucleation complexes in which all negative charges are compensated by complexing calcium ions. And although the Posner’s cluster became regarded as a spherical particle with a diameter of 9.5 Å, in the original paper the cluster was proposed to ‘be large enough to contain atom pairs with about 9.5 Å spacing’. Moreover, a small but significant peak at 12.1 Å was attributed to ‘interparticle distances’, but perfectly describes the close packing of the present 1.2 nm post-nucleation clusters in ACP.

The Ca/P ratios of ACP reported in the literature vary between 1.1 and 1.6 (ref. 36), hence distinctly differ from the value of 0.67 calculated from our pH and Ca-ISE measurements. However, as it was demonstrated recently that drying of ACP can strongly affect the structure of the resulting material, the ACP in solution was isolated by filtration, washing and drying (Supplementary Methods, analysis of calcium phosphate precipitate). For this material, energy dispersive X-ray analysis revealed a Ca/P ratio of 1.5 (Supplementary Table S4), demonstrating that our isolated ACP does not deviate from the isolated materials described in the literature. However, the different Ca/P ratios found for the ACP in solution and the isolated ACP underline the fact that concentration and isolation of hydrated ACP leads to phase changes in the material.

The presence of HPO$_4^{2–}$ in ACP—as implied by the post-nucleation cluster chemistry—is different from the structure of $\text{Ca}_9\text{P}_4\text{O}_{16}$ commonly considered for ACP. However, infrared and solid-state NMR studies have demonstrated the presence of HPO$_4^{2–}$ in synthetic ACP while others indicated its presence in the non-apatitic components of bone.

By combining cryo-TEM with several in situ analysis techniques and ab initio calculations, we conclude that the previously reported nanometre entities termed pre-nucleation clusters for calcium phosphate are in fact soluble ion-association complexes $[\text{Ca(HPO}_4)_3]^{4–}$ that form aggregates in solution. Above the solubility limit of ACP, these aggregated pre-nucleation complexes take up calcium ions from solution to form insoluble post-nucleation clusters of $[\text{Ca}_2\text{HPO}_4]^{2–}$ that precipitate as ACP. Our results show, in contradiction to the previously postulated cluster-growth mechanism, that the clusters do not actually represent a fixed structural unit, but that the chemistry of these building blocks progresses stepwise towards the composition of the final product, AP. However, the calcium triphosphate structure of the initial complexes is retained throughout these steps. The fact that the previously identified pre-nucleation clusters are merely ion-association complexes of one calcium and three phosphate ions places them in the realm of inorganic solution chemistry. Nonetheless, the existence of these pre-nucleation complexes, which have an excess free energy, fundamentally alters the nucleation pathway by making amorphous phases accessible at concentrations for which classical nucleation theory would predict the exclusive formation of the more stable crystalline phases. Nevertheless, the extended nucleation theory we present here is well capable of explaining these results. The observed formation of polymeric assemblies bears strong resemblance to the formation of, for example, the liquid-like clusters proposed for lysozyme, of mass fractals as proposed for glycol and the liquid-like ionic polymers suggested for calcium carbonate. This strongly suggests that the polymerization of basic building blocks that we describe here as the first step in crystal formation is not unique for the crystallization of calcium phosphate, but is a more general phenomenon in both organic and inorganic systems. It is likely that also these ‘non-classical’ nucleation mechanisms can be explained by the extended nucleation theory presented here.

Methods

Mineralization reaction. The mineralization reaction is performed in a Tris-buffered saline, consisting of 50 mM Trizma base (Sigma-Aldrich, USA) and 150 mM NaCl (Merck, Germany) in ultrapure water (Millipore, USA) set at pH 7.40 using HCl (25%, Merck). A 10 mM calcium stock and a 10 mM phosphate stock were prepared by dissolving CaCl$_2$ $\cdot$ 2H$_2$O (Sigma) and K$_2$HPO$_4$ (Merck) in Tris-buffered saline, after which the pH was adjusted to 7.40 using 0.1 M NaOH (Merck) or 0.1 M HCl. The reaction was simulated at 18.5°C in a 50 ml beaker or 10 ml sample vial, to which 14.7 or 3.53 ml calcium stock was added. The calcium stock was stirred gently at 200 r.p.m. before adding 10.3 or 2.47 ml phosphate stock ($\text{Ca/P} = 1.43$). The reaction was followed under stirring for typically 1–4 h. According to Termine et al., conditions were chosen to achieve initial phase separation of ACP within 15–20 min.

Cryogenic Transmission Electron Microscopy. Samples (3 μl) were vitrified at different time points using an automated vitrification robot (FEI Vitrobot™ Mark III, FEI Company). R2/2 Quantifoil Jena grids (Quantifoil Micro Tools GmbH, Germany) were surface plasma treated before the vitrification procedure using a Cressington 208 carboncoater. The cryo-TEM experiments were performed on a FEI Tecnai 20 (type Sphaer, LaBa, 200 kV, 1 x 1 K Gatan CCD camera) TEM on the TU/e CryoTitan (FEI) (FEG, 300 kV, Gatan Energy Filter, 2 K x 2 K Gatan CCD camera, www.cryotem.nl) or on the Vanport Eindhoven Titan Krios (FEI) (FEG, 300 kV, Falcon direct electron detector). A cryo-holder (Gatan Inc., USA) operating at ~−170°C was used with the Tecnai 20.

LDSÃÄÄED was performed on the FEI Tecnai 20 (type Sphaer) TEM (camera length 520 mm, selected area 0.29 μm). Radial averaging of the diffraction pattern using DiffTools® in Digital MicrographTM software (version 3.10.0, Gatan) and the Radial Profile Angle plug-in in Image/TEM software (version 1.42a, NIH, USA). A 1/4 background correction was performed to diminish the contribution of the zero-loss beam. As a standard for crystalline AP, nanocrystalline HAP suspension in ethanol (Berkeley Advanced Biomaterials Inc., USA) was diluted using 0.01 M acetic acid to a concentration of 600 mg ml$^{-1}$. Dual-axis cryo-Electron tomography was performed on the TU/e CryoTitan (FEI). Two orthogonal tilt series were recorded between negative tilt angles of −70° to −65° and positive tilt angles 60°—70° (magnification ×30,000, defocus range 3.5–0 μm, total dose 45–54 electrons Å$^{-2}$). The tilt step was 2° between 0–45° and 1° above 45°. Volumes were reconstructed using Imtek 3D v. 3.0 software (FEI Company).

Monitoring pH and calcium concentration in solution. Calcium concentration and pH were monitored using a calcium-sensitive electrode (Ca-ISE, Metrhom Ltd., Switzerland) and a pH glass electrode (LF Micro glass electrode, Metrhom Ltd.) connected to a Titramed™ titration device (Metrhom Ltd.) and analysed online using Tiamo™ software (Metrhom Ltd.). The Ca-ISE (Ca$^{2+}$) was calibrated by the 10 mM calcium stock used in the trapping experiment (pH = 7.4) at room temperature with an addition rate of 0.1 ml min$^{-1}$ using a Dosino dosing device (Biorad) was used. 2D scattering patterns were collected using a MarxMosaic CCD detector (Mar, Evanston, USA). Radial integration of the 2D scattering patterns with the software Fit2D (A. Hammersley, ESRF, Grenoble, France) gave the

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spherically averaged scattering intensity as a function of the modulus of the scattering vector \( Q \), with \( Q = 4 \pi \sin(\theta)/\lambda \), 20 being the scattering angle and \( \lambda \) the wavelength. The scattering profiles were corrected for dark current, primary intensity and sample transmission. An additional background signal resulting from the glass capillary was subtracted.

**In situ infrared measurements.** Infrared spectra were recorded by taking 1 ml samples from a 25 ml reaction solution using horizontal-attenuated reflection (ATRMaxITM, PIKE Technologies, USA) employing a ZnSe crystal and a FTIR spectrometer (ExcaliburTM, Varian Inc., USA) equipped with a MCT-detector (500 scans, resolution 2 cm\(^{-1}\)). The tris-buffered saline background was subtracted using Varian resolutions 4.0 software.

**In situ AFM solution preparation.** High-purity Na\(_2\)HPO\(_4\) (99.95%), KCl (99.99%), NaCl (99.99%) and HCl (1.043 N) from Sigma-Aldrich, CaCl\(_2\) (99.9%) from Alfa Aesar and ultrapure water (18.2 M\(\Omega\) cm) were used for all experiments. Infrared spectra were recorded from growth solution to ethanol. The resulting samples were then dispersed in water and the undried substrate was used for the nucleation study. In situ AFM: determination of mineral phase

**References.**


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Author contributions


Additional information

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