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# Confinement Increases the Lifetimes of Hydroxyapatite Precursors

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**KEYWORDS**

Amorphous calcium phosphate, octacalcium phosphate, biomineralization, bio-mimetic, bio-inspired, crystallization

**ABSTRACT**

The mineral component of bone is a carbonated, non-stoichiometric hydroxyapatite (calcium phosphate) that forms in nanometer confinement within collagen fibrils, the principal organic constituent of bone. We here employ a model system to study the effect of confinement on hydroxyapatite precipitation from solution under physiological conditions. In common with earlier studies of calcium carbonate and calcium sulfate precipitation, we find that confinement significantly prolongs the lifetime of metastable phases, here amorphous calcium phosphate (ACP) and octacalcium phosphate (OCP). The effect occurs at surprisingly large separations of up to 1 μm, and at 0.2 μm the lifetime of ACP is extended by at least an order of magnitude. The soluble additive poly(aspartic acid), which in bulk stabilizes ACP, appears to act synergistically with confinement to give a greatly enhanced stability of ACP. The reason for the extended lifetime appears to be different from that found with $CaCO_3$ and $CaSO_4$, and underscores both the variety of mechanisms whereby

confinement affects the growth and transformation of solid phases, and the necessity to study a wide range of crystalline systems to build a full understanding of confinement effects. We suggest that in the case of ACP and OCP the extended lifetime of these metastable phases is chiefly due to a slower transport of ions between a dissolving metastable phase, and the more stable, growing phase. These results highlight the potential importance of confinement on biomineralisation processes.

## INTRODUCTION

Although typically studied in bulk solution, crystallization is a phenomenon that is strongly dependent on the environment in which it occurs. Surfaces, including particulate impurities, can affect nucleation and growth processes through their chemistry and topography,[1, 2] and an improved understanding of their action will ultimately afford us greater control over crystallization. Environment is particularly important for the many crystallization processes – such as frost heave, the templated growth of nanomaterials and biomineralization – which occur within confined volumes. It is well-recognized that confinement can affect many features of crystal growth, such as the size and morphology, polymorph, orientation and single-crystal vs. polycrystalline structure of crystals, although most work has focused on the freezing transitions of liquids,[3] or the crystallization of organic/ molecular crystals.[4, 56] A perfect example of a process which occurs in confinement is biomineralization.[7, 8] Despite this, with the exception of control over morphology,[9-12] the effects of confinement on the formation of biominerals, or indeed inorganic substances has received little attention, possibly partly due to the experimental challenges associated with carrying out systematic studies of the precipitation of poorly soluble compounds in small volumes. Recent work has provided strong evidence, however, that confinement can also have significant effects on the precipitation of compounds such as calcium carbonate[13] and calcium sulfate, sometimes at surprisingly large length scales.[14-19]

In this article, we investigate the effects of confinement on the precipitation of calcium phosphate (CaP), the principal inorganic component of bones and teeth. Confinement plays a key role in the formation of bone, which begins with the growth of ultrathin platelets of nonstoichiometric carbonated hydroxyapatite (HAP) within gaps in the collagen fibrils.[20] These 2-6 nm thick platelets are the smallest known biogenic crystal[21, 22] and are organized so

that their c-axes are preferentially oriented with respect to the long axes of the collagen fibrils.[23, 24] Hierarchical organization of these components over multiple length scales then results in a remarkable composite material with mechanical properties optimised for its function.[25, 26, 27]

How organisms achieve such remarkable control over mineral formation has been the subject of intense interest, and it is widely considered that both the collagen matrix and non-collagenous proteins act in consort to achieve effective mineralization. The non-collagenous proteins are essential to normal bone formation,[28-30] and in vitro experiments have shown that acidic non-collagenous proteins can inhibit or promote nucleation and growth depending on the experimental conditions, in addition to modifying crystal polymorphs, sizes and shapes.[31-37] The collagen matrix, in turn, plays a key role in determining the size and shape of the HAP precipitates, and their orientation with respect to the collagen has largely been considered to derive from a structural relationship between these phases.[22, 38]

The experiments described here provide a systematic study of the effects of confinement on the precipitation of CaP, both in the absence of additives, and in the presence of poly(aspartic acid), which is often used as a model for the acidic non-collagenous proteins.[37, 39, 40] The experiments are carried out with a crossed-cylinder apparatus[14, 16] in which two glass half-cylinders are in contact with their axes at a right angle. This configuration, based on that of the surface force apparatus,[41] is equivalent to a sphere-on-a-flat (Figure 1a) and provides an annular wedge which enables study of a continuous range of confinement levels (defined by the separation of the surfaces, SS) in one experiment. The surface separation SS is calculated from the cylinder radius R and the distance to the contact between the cylinders D. These experiments build on recent results in which we used the rod-shaped pores of track etch

membranes to show that confinement alone can direct the orientation of HAP,[42, 43] and demonstrate that both octacalcium phosphate (OCP) and amorphous calcium phosphate (ACP) can be effectively stabilized in confinement. Consideration of the mechanism by which confinement increases the lifetime of these phases suggests that is distinct from that found with $CaCO_3$ and $CaSO_4$, and emphasizes the need to study multiple systems to fully understand the effects of confinement on crystallization processes, and therefore to employ confinement as a route to controlling crystallization.

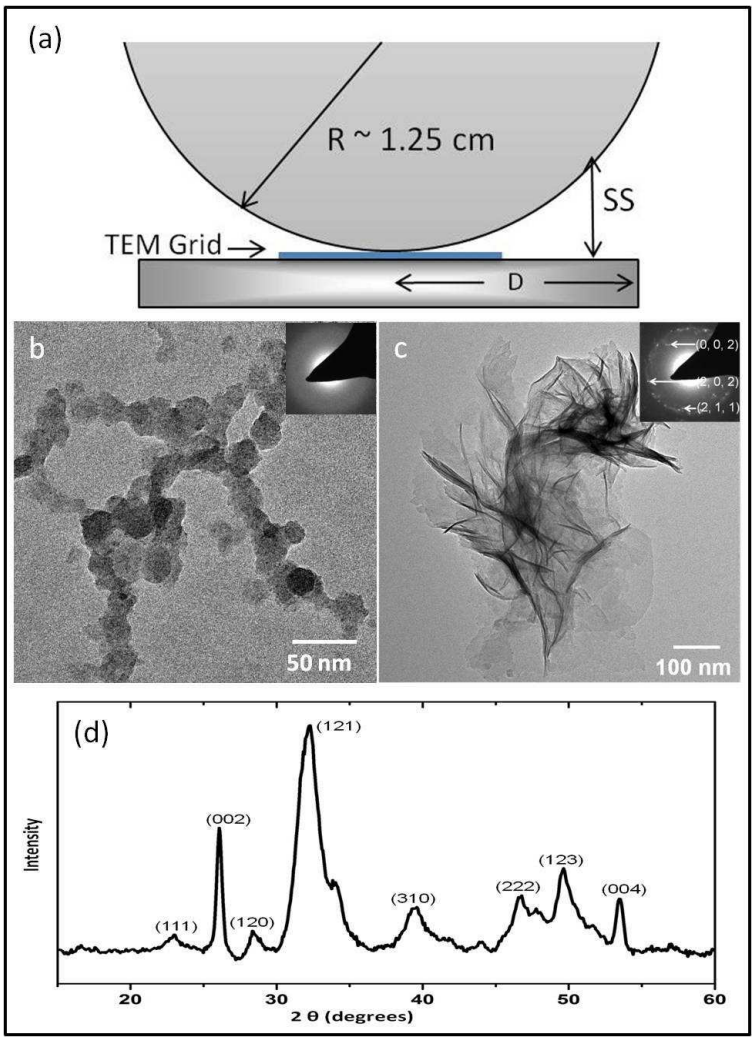


**Figure 1.** (a) Schematic drawing of crossed-cylinder apparatus, and (b,c) TEM images and electron diffraction patterns of CaP particles precipitated from a buffered solution of calcium phosphate at 37 °C. (b) After 1 h, the particles were amorphous. (c) After 5 h all precipitates were HAP, as confirmed by XRD (d).

## EXPERIMENTAL SECTION

**Precipitation of Calcium Phosphate.** Following Olszta et al.[39], 8.77g NaCl, 6.61g Tris-HCl and 0.96g Tris-base (Sigma-Aldrich) were dissolved in 1L 18 MΩ $cm^{-1}$ Millipore water to prepare a Tris-buffered saline solution of pH 7.68 at 25 °C. A solution 9 mM in $CaCl_2$ and 4.2 mM in $K_2HPO_4$ was prepared by dissolving $CaCl_2 \cdot 2H_2O$ and $K_2HPO_4 \cdot 3H_2O$ (both Sigma-Aldrich) in the buffer solution. NaOH was used to adjust the pH of both solutions to 7.4 at 37 °C. Polyaspartic acid sodium salt solution (Poly-(α,β)-DL-aspartic acid sodium salt MW 2 - 11 kDa) was added to the $K_2HPO_4$ solution at 10 µg $ml^{-1}$.

**Crossed-Cylinder Experiment**. A crossed-cylinder apparatus[14, 16] was used to study precipitation in confinement. 50 mL buffered $K_2HPO_4$ solution with or without additive was mixed with 50 mL buffered $CaCl_2$ solution. The crossed-cylinder apparatus, with or without a TEM grid placed between the glass cylinders at their contact point (Figure 1a), was immersed in the mixed solution in a beaker and was kept in an oven at 37°C for between 3 h and 6 days. The experiment was terminated by removing the crossed-cylinder apparatus from solution, flushing with ethanol and drying with nitrogen with the surfaces were still in contact. The TEM grid was rinsed with ethanol and dried with filter paper after careful separation of the crossed cylinders.

**Control Experiments**. Control experiments were performed by carrying out the equivalent reactions in bulk solution. Buffered 9 mM $CaCl_2$ and 4.2 mM $K_2HPO_4$ solutions were combined in a crystallization dish, glass slides were placed on the bottom, and crystallization was allowed to proceed from solution. For the additive experiments, PAsp was dissolved in the $K_2HPO_4$ solution prior to mixing with the $CaCl_2$ solution. The precipitates were isolated by filtration through a 0.2 µm filter membrane, washing with ethanol and air-dried before

characterisation. Copper TEM grids were dipped in the solution, withdrawn, washed in ethanol and dried on filter paper.

**Characterisation Methods.** For Scanning Electron Microscopy (SEM) and electron diffraction, glass cylinders with deposited calcium phosphate particles were mounted on SEM stubs using conducting carbon tape, and were then sputter-coated with 5 nm Pt. The samples were examined using a Phillips XL–30 ESEM or a Leo 5000-SEM operating at 3 kV. For Transmission Electron Microscopy (TEM) studies, the area on the Cu grids around the contact point of the half-cylinders was found by identifying a region with scratches, devoid of precipitates. Imaging was carried out with a Phillips Tecnai FEG-TEM operating at 200 kV. PXRD was carried out using a Bruker D8 Advanced diffractometer equipped with an X-ray source emitting Cu $K\alpha_1$ radiation. Samples were placed on a silicon wafer, and XRD data were collected in an angular range between $15^o$ and $60^o$ in intervals of $0.02^o$, with a scan rate of $1^o$ $min^{-1}$.

## RESULTS

The influence of confinement on CaP precipitation was demonstrated by comparison with control experiments in which CaP was precipitated in bulk solution. These showed that amorphous calcium phosphate (ACP) was the main product after 1 h, where the ACP particles were around 20 nm in diameter. Their amorphous character was confirmed by the absence of crystalline reflections in electron diffraction patterns (Figure 1b). After 2 h some 30 nm ACP particles could still be observed (not shown), but after 5 h all the CaP had transformed into thin lamellae of hydroxyapatite (HAP), as confirmed by electron diffraction

(Figure 1c, inset), and powder X-Ray Diffraction (PXRD) (Figure 1d), where the (121) reflection at 31.78° is diagnostic.[44]

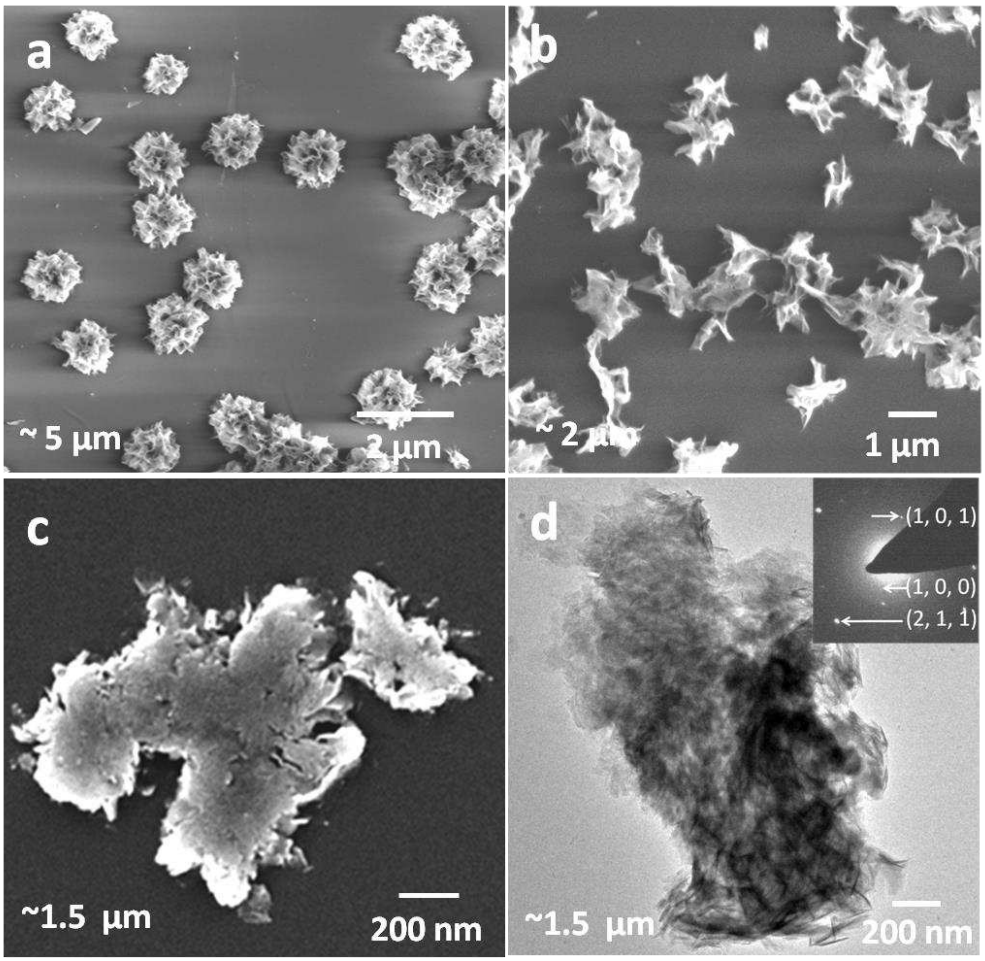


**Figure 2.** (a-c) SEM and (d) TEM images of CaP particles precipitated from buffered solution at 37 °C after 3 days, in the crossed cylinders apparatus at surface separations of (a) 5 µm, where most of the precipitates were HAP as in bulk solution, (b) 2 µm, where a smaller number of HAP particles formed, (c and d) 1.5 µm, when flattened aggregates of HAP were observed, as shown by electron diffraction.

The effects of confinement on CaP precipitation were then investigated under identical solution conditions for incubation times of up to 3 days using the crossed-cylinder apparatus. The entire apparatus was immersed in a supersaturated solution of CaP, 9 mM $CaCl_2$ and 4.2 mM $K_2HPO_4$ solutions at pH 7.4 at 37 °C, with background NaCl to give the ionic strength of

physiological fluids. The precipitates that formed on the glass cylinders or on a Cu TEM grid inserted between the cylinders at surface separations ranging from 100 nm to 5 μm were characterized using SEM and TEM. At surface separations (SS) greater than 5 μm the particles grew as flower-like clusters of HAP crystals ≈1.5 μm in size (Figure 2a), identical to those produced in bulk solution. Closer to the contact point at SS ≈ 2 μm, there were HAP clusters of a similar size, but which were more open in structure, as is indicative of reduced aggregation (Figure 2b). At SS ≈ 1.5 μm, where the surface separation is comparable to the size of the clusters, the CaP precipitates appeared with flattened surfaces (Figure 2c). Electron diffraction confirmed that all these precipitates were HAP, as confirmed from the measured d-spacings of 5.26 Å (101), 8.10 Å (100) and 2.83 Å (211), and were thus identical in polymorph to those formed in bulk solution (Figure 2d).

A significant influence of confinement on both the morphology and polymorph of the CaP precipitates was observed at small surface separations. In the region where SS ≈ 1 μm, most of the precipitates appeared as thin flat, plates, up to several hundred nanometres in size (Figure 3a). Electron diffraction demonstrated that the plates were octacalcium phosphate (OCP) by the observation of peaks distinct from those of HAP. Sequential transformation of ACP to OCP to HAP has previously been observed within the confines of cross-linked gelatine nanoparticles,[45] and also in the complete absence of soluble additives, using reaction solutions containing very high concentrations of calcium and phosphate.[38] With a further decrease in the surface separation to SS ≈ 0.5 μm, nanospheres were observed alongside the platelets (Figure 3b). The plates were again shown to be OCP using electron diffraction, while the nanospheres were amorphous (Figure 3b). Very close to the contact point, in the region where SS ≈ 0.2 μm, only nanoparticles with diameters of 30-50 nm were found (Figure 3c), that were once again shown to be amorphous by electron diffraction. It is emphasized

that these ACP particles were still present after 3 days in the confined system, while significant crystallization of ACP was noted after 2 h in bulk solution, and no amorphous particles could be found after 5 h.

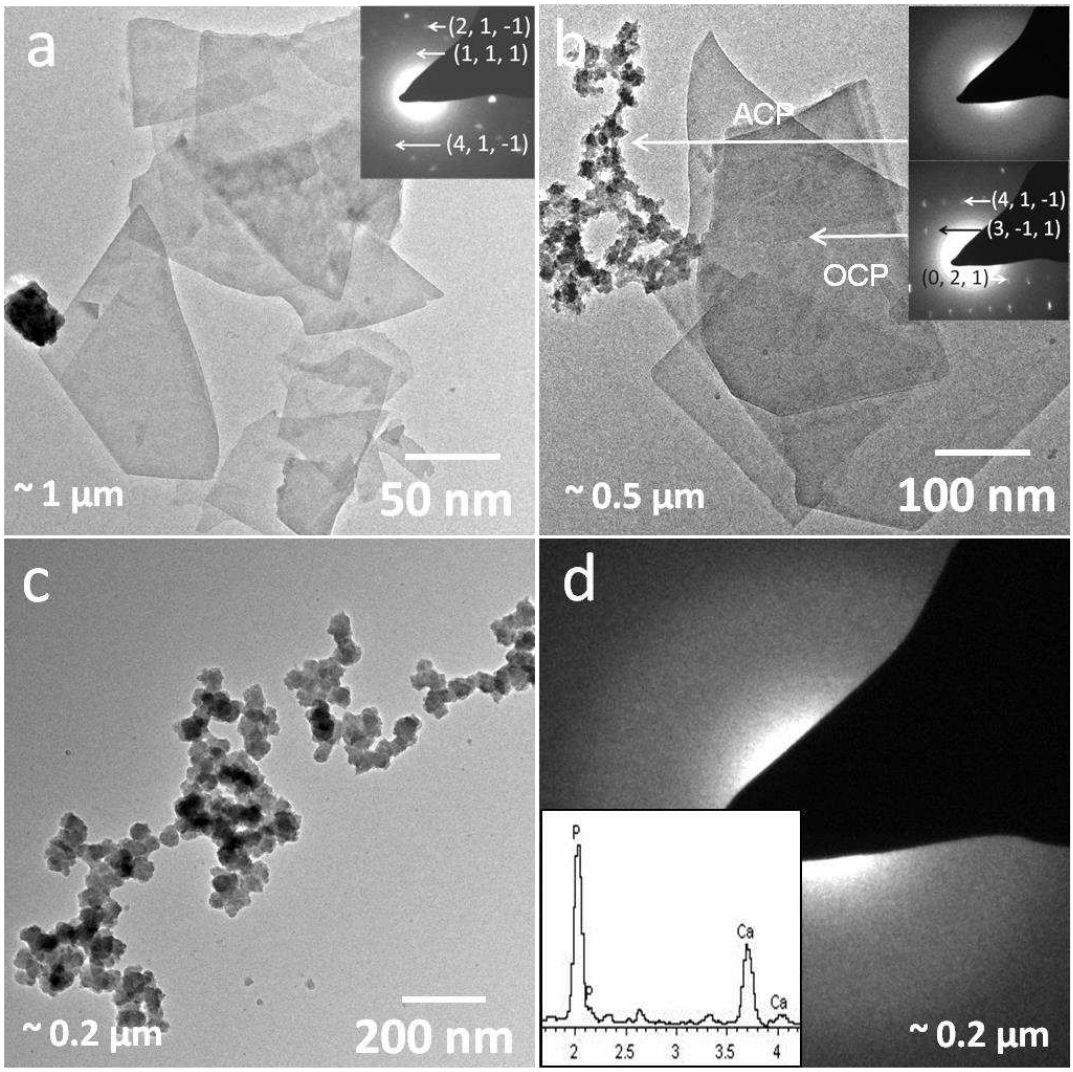


**Figure 3.** TEM images of CaP particles precipitated after 3 days in the crossed cylinders apparatus at surface separations of (a) 1 μm. The particles appeared as thin plates, which were shown to be OCP by electron diffraction. (b) 0.5 μm, the precipitates shown as mixture of both amorphous calcium phosphate (ACP) and octacalcium phosphate (OCP). (c) and (d) 0.2 μm, all the precipitates were ACP nanoparticles with 30-50 nm in diameter.

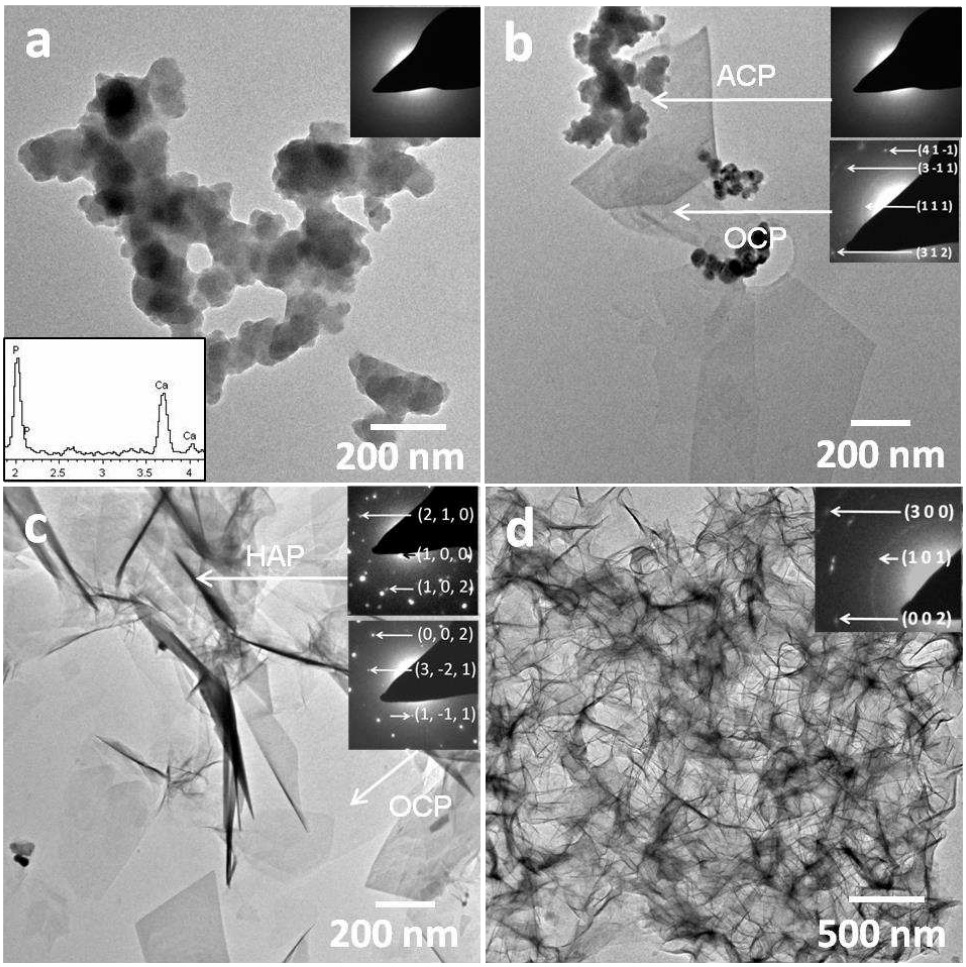


**Figure 4.** TEM images and the corresponding electron diffraction patterns of CaP particles precipitated in buffered bulk solution in the presence of 10 µg $mL^{-1}$ PAsp at 37 °C after a) 1 day, all the particles are ACP. b) 3 days, mixtures of ACP and OCP c) 5 days, mixture of OCP and HAP, no ACP was observed and d) after 1 week, most of the precipitate was HAP.

The combined effects of spatial confinement and soluble additives were then investigated with the goal of gaining insight into the possible role of the non-collagenous proteins in the mineralization of collagen fibrils. Poly(aspartic acid) (PAsp) was selected as it has been considered an effective mimic of the non-collagenous proteins implicated in the mineralization of collagen with CaP. Mineralization of collagen fibrils to give structures comparable to native bone has been achieved by precipitating HAP in the presence of poly(aspartic acid) (PAsp), which acts as an analogue of the non-collagenous proteins.[37, 39, 40]

Control experiments conducted in bulk solution in the presence of 10 μg $ml^{-1}$ PAsp showed that this polymer inhibited crystallization and stabilized ACP so that complete conversion to HAP took 1 week. The precipitates that formed over time were investigated using TEM. After 1 day in the presence of 10 μg $ml^{-1}$ PAsp, most of the particles were ACP (Figure 4a), while after 3 days both amorphous nanoparticles and plate-like crystals could be observed. These thin plate-like crystals were shown to be octacalcium phosphate (OCP) by electron diffraction (Figure 4b). After 5 days all precipitates were crystalline and consisted of a mixture of HAP and OCP (Figure 4c), but by 1 week all crystals observed were HAP, and no OCP remained (Figure 4d).

An identical precipitation solution was then employed in the crossed-cylinder apparatus, and samples were analyzed after 1 week (Figure 5). In common with results obtained in the absence of PAsp, clusters of HAP crystals precipitated at surface separations of 2-5 μm and above (Figure 5a, as compared with Figure 2). The effect of the additive was clearly observed at smaller separations, however. Here, plate-like OCP particles were observed after 1 week at a SS ≈ 1.5 μm, as compared with a SS ≈ 1 μm after 3 days in the additive-free system (Figure 3a). In the presence of additive in confinement most of the OCP had transformed to HAP after 2 weeks (Figure S2). Interestingly, aggregates of nanospheres of 20-30 nm diameter were also observed at SS ≈ 1 μm (Figure 5d), and electron diffraction showed that they were OCP. At yet greater degrees of confinement only ACP was observed, with clumps of aggregated ACP nanoparticles present at SS ≈ 500 nm and at SS ≈ 200 nm (Figures 5e and 5f).

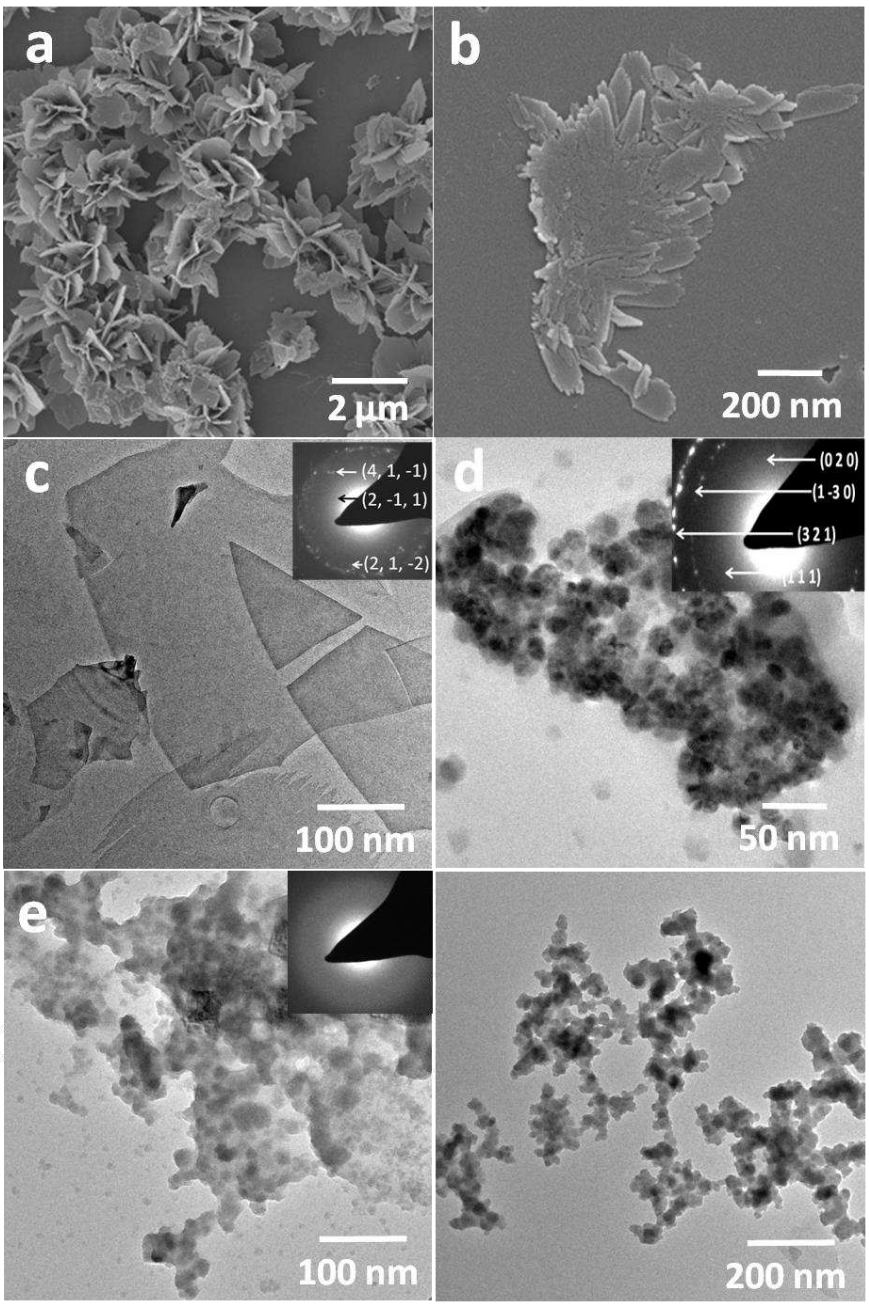


**Figure 5** SEM (a, b) and TEM (c-f) images of CaP precipitates after 1 week in the presence of 10 µg $mL^{-1}$ PAsp in the crossed cylinders apparatus at surface separations of (a) 5 µm, where all the particles were HAP , (b) 2 µm, where flattened HAP particles were observed. (c) 1.5 µm, where all the particles were plate-like OCP, (d) 1 µm, where aggregated nanoparticles of OCP were observed. Insets show the corresponding electron diffraction patterns, (e) 0.5 µm and (f) 0.2 µm, where only amorphous particles were observed. Insets show the corresponding electron diffraction patterns.

## DISCUSSION

The experiments demonstrate that confinement alone can have a considerable effect on the precipitation of CaP by increasing significantly the lifetime of metastable polymorphs (a comparison of the principal results obtained in bulk solution and in different degrees of confinement, in the absence and presence of PAsp is provided in Figure S3). CaP precipitation is complex, with intermediate phases frequently forming prior to the thermodynamically favoured phase.[13, 36, 46-48] HAP is the thermodynamically most stable phase from weakly acidic to basic conditions, but as it can be significantly slower to form than ACP or OCP, kinetic factors can support the formation of these polymorphs as intermediate species.[36] Study of the transformation of OCP to HAP has suggested that this can occur either by a dissolution/ reprecipitation mechanism or by a solid-state transformation in which hydrolysis of an OCP unit cell leads to a layer of HAP two units cells thick.[36] ACP, in turn, is unstable in solution and its lifetime varies according to the presence of additives, the solution composition, pH and temperature.[49] A recent detailed study of the mechanism of CaP precipitation in solutions supersaturated with HAP has shown that this process is based on the aggregation of calcium triphosphate complexes.[48] These complexes are initially present in the solution and subsequently aggregate, with uptake of calcium ions, to form ACP particles. Continued calcium uptake converts ACP into OCP, probably via a dissolution/ reprecipitation mechanism. Finally, OCP takes up calcium ions and hydrolyzes to the structurally very similar HAP.[50]

These mechanisms of CaP precipitation/ transformation provide the basis for interpreting the confinement effects observed here. Firstly, we note that true thermodynamic stabilization of the metastable polymorphs would only be expected below surface separations of a few nanometers, so these confinement effects must all be kinetic in origin.[14, 16] Systematic studies

of the precipitation of $CaCO_3$ and $CaSO_4$ have suggested that there are various mechanisms by which confinement can affect the kinetics of the precipitation. The considerable stabilization observed for amorphous calcium carbonate (ACC) within unilamellar vesicles[17, 19] has been attributed to the exclusion of nucleating impurities from these small volumes. An alternative mechanism was proposed to explain the observed stabilization of ACC with respect to calcite in the crossed-cylinder apparatus.[14] Here, dense, micron-size ACC particles are effectively sandwiched between the two glass surfaces. As the crystallization of ACC is strongly dependent on the presence of water,[51, 52] the fact that most of the surface of the ACC lumps is remote from the interface with water would lead to a retardation of the crystallization.

In the case of $CaSO_4$, small calcium sulfate hemihydrate and amorphous calcium sulfate (ACS) particles are also stabilized with respect to the thermodynamically stable phase calcium sulfate dihydrate (gypsum) with increasing degrees of confinement in the crossed-cylinder apparatus.[16] As these particles are not isolated from the solution, the stabilization of these metastable polymorphs cannot be attributed to the absence of water. Instead, we proposed that the aggregation-based transformation of the metastable polymorph hemihydrate to gypsum[53, 54] would be affected by the restricted volume.[16] The diffusive transport of particles is severely hindered when the surface separation approaches the particle diameter such that aggregation would be slower or not occur to the same extent in confinement. As only one nucleation event per particle or aggregate of particles should be required to initiate a phase transformation, it is clear that this will be much less likely to occur if the material is mostly in the form of single particles or smaller aggregates. Confined hemihydrate did indeed show much less, if any, aggregation at the smallest surface separations (0.2 μm). We

could not confirm a similar explanation for the longer lifetime of confined amorphous calcium sulphate due to the very small amounts that were observed.

In the calcium phosphate system, the morphology of the precipitates and the very limited contact between the crystal faces and the glass surfaces likewise precludes hindered dehydration as a stabilising mechanism (the water content decreases from 15-20 % for ACP via 9 % for OCP to zero for HAP[47]). Nor can hindered aggregation of ACP explain the observations, as aggregation has not been implicated in the transformation to OCP and/or HAP, which often occurs via a dissolution/reprecipitation mechanism. It is therefore intriguing to speculate as to why significant stabilization of the metastable CaP phases is observed in confinement

We can suggest two quite general mechanisms that may account for this effect. Firstly, convective transport due to small but unavoidable temperature gradients would be reduced by the proximity of surfaces. Since it is believed that advection is often the dominant mechanism for transport of material to a growing crystal,[55] reduced rates of transport away from a metastable phase (towards a growing, more stable crystal) would provide a means of extending its lifetime. Secondly, changes in the mean inter-particle separation will occur when particles are confined between two surfaces, as between the glass cylinders in our apparatus. On the assumption that the metastable phase (here ACP) nucleates homogeneously, the number of crystals per unit volume should be independent of the degree of confinement. In bulk, and at large surface separations, the mean distance between crystals is constant, but for surface separation that are less than the mean inter-crystal separation in bulk, the separation between the confined particles must increase. This can be easily seen by considering each particle in bulk to be located at the centre of a cube of side S; the average

interparticle separation is then also S. If the system is now confined by two surfaces so that the surface separation SS is less than S, the solution volume associated with the particle can only remain constant if the cube flattens and expands outwards. The separations between neighbouring particles must increase, so that if SS is reduced to 10% of S, the other two sides must each lengthen by a factor of $\sqrt{10}$. The distance mean separation between adjacent particles hence increases by a factor of about 3. This increase in the inter-particle separation would result in an extended lifetime of the metastable phases because the time taken for diffusive or convective transport of material would increase. It should be noted that this is not a surface effect on ion diffusion, which will only be significantly hindered for surface separations below 20 nm.[56]

Our experiments also demonstrate that the lifetime of the metastable ACP and OCP phases in confinement are yet further extended when PAsp is present. PAsp stabilizes ACP in bulk solution and OCP can be clearly identified as an intermediary during its conversion to HAP. In bulk solution in the absence of additives, ACP is fully converted to HAP after 3 h, but in the presence of 10 µg $mL^{-1}$ PAsp some ACP still persists after 3 d. In the confined system at SS ≈ 0.2 µm, ACP without PAsp is stable for at least 3 d, whereas at the same SS with PAsp the stability is extended to at least 7 d. Similarly, at SS ≈ 0.5 µm, without PAsp, there was partial conversion of ACP to OCP after 3 d. In bulk with PAsp there was also partial conversion after 3 d, while no ACP converted even after 7 days at SS ≈ 0.5 µm in the presence of PAsp. Confinement and PAsp therefore appear to act in synergy to extend the lifetime of the amorphous phase beyond the sum of what each would have achieved on its own.

Considering the relevance of these results to biomineralization processes, and bone formation in particular, much less is known about the mechanism of HAP formation in vivo than in bulk solution, due to the inevitable problems associated with studying real biomineralization processes. After many decades of debate, it has finally been accepted that bone tissues can form via an ACP precursor phase, where this was determined by studying the forming cranial suture of a mouse[57] and the continuously forming bony fin rays of zebrafish.[58, 59] That OCP can act as an intermediate between ACP and HAP during bone formation has also been widely discussed, but remains controversial.[36, 60] In its support, the so-called central dark line observed by TEM in biogenic HAP has been attributed to a central OCP occlusion which may derive from the lattice mismatch between OCP and HAP.[61, 62] OCP has also been observed in the forming cranial suture of a mouse using Raman spectroscopy, where it is notable that these samples were characterised without dehydration.[57] While the biological system is far more complex than the simple model apparatus employed here, our results suggest that it is entirely possible that crystallization of ACP to HAP may occur via an OCP intermediate phase during bone mineralization, where both ACP and OCP can be stabilized by confinement. Indeed, the extent of confinement offered by the collagen fibrils is far greater than the comparatively modest degree seen to provide significant stabilization here.

## CONCLUSIONS

We have described a systematic study of the effects of confinement on the precipitation of calcium phosphate, and have demonstrated that both amorphous calcium phosphate (ACP) and octacalcium phosphate (OCP) are stabilized with respect to hydroxyapatite (HAP) at relatively large separations in the crossed-cylinder apparatus. This stabilization cannot be due to impeded access to the solution, as in the case of amorphous calcium carbonate,[14] or to hindered aggregation, as with calcium sulfate hemihydrates.[16] Instead, we speculate that the

reason may be a slower transport of ions due to a reduced rate of convection in confinement, and/or to a greater mean distance between particles or particle aggregates in confinement. Both effects lead to slower rates of dissolution of metastable particles and growth of the more stable phases. Importantly, this mechanism is quite general, and would also be expected to contribute to the stabilization of metastable phases observed in the $CaCO_3$ and $CaSO_4$ systems. However, in the $CaCO_3$ system, in which the ACC particles are so different in size and morphology from the CaP particles observed here, dehydration would be expected to dominate. The stabilization effect of confinement is further enhanced in the presence of poly(aspartic acid), which is widely used as an analogue of acidic non-collagenous proteins. Along with our previous results, which showed that precipitation of CaP within an anisotropic, rod-shaped membrane pore can cause orientation of HAP, these data indicate that the confinement offered by the collagen fibril structure may be important in governing the mechanism and product of collagen fibril mineralization.

## SUPPORTING INFORMATION

Further characterization of CaP particles. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interests.

**REFERENCES**


1. Holbrough, J. L.; Campbell, J. M.; Meldrum, F. C.; Christenson, H. K., Cryst. Growth Des. **2012,** 12, 750-755.

2. Asanithi, P., J Biomed Mater Res Part A **2013**.

3. Christenson, H. K., J. Phys.-Condes. Matter **2001,** 13, R95-R133.

4. Beiner, M.; Rengarajan, G. T.; Pankaj, S.; Enke, D.; Steinhart, M., Nano Lett. **2007,** 7, 1381-1385.

5. Vallet-Regi, M.; Balas, F.; Arcos, D., Angew. Chem.-Int. Edit. **2007,** 46, 7548-7558.

6. Jiang, Q.; Hu, C. H.; Ward, M. D., J. Am. Chem. Soc. **2013,** 135, 2144-2147.

7. Lowenstam, H. A.; Weiner, S., On Biomineralization. Oxford University Press: New York, 1989.

8. Meldrum, F. C.; Colfen, H., Chem. Revs. **2008,** 108, 4332-4432.

9. Yue, W. B.; Kulak, A. N.; Meldrum, F. C., J. Mater. Chem. **2006,** 16, 408-416.

10. Yue, W. B.; Park, R. J.; Kulak, A. N.; Meldrum, F. C., J. Cryst. Growth **2006,** 294, 69-77.

11. Wucher, B.; Yue, W.; Kulak, A. N.; Meldrum, F. C., Chem. Mater. **2007,** 19, 1111-1119.

12. Kim, Y. Y.; Hetherington, N. B. J.; Noel, E. H.; Kroger, R.; Charnock, J. M.; Christenson, H. K.; Meldrum, F. C., Angew. Chem.-Int. Edit. **2011,** 50, 12572-12577.

13. Brecevic, L.; Furedimi.H, Calcif. Tiss. Res. **1972,** 10, 82-&.

14. Stephens, C. J.; Ladden, S. F.; Meldrum, F. C.; Christenson, H. K., Adv. Funct. Mater. **2010,** 20, 2108-2115.

15. Stephens, C. J.; Kim, Y.-Y.; Evans, S. D.; Meldrum, F. C.; Christenson, H. K., J. Am. Chem. Soc. **2011,** 133, 5210-5213.

16. Wang, Y.-W.; Christenson, H. K.; Meldrum, F. C., Adv. Funct. Mater. **2013,** 23, 5615–5623.

17. Tester, C. C.; Brock, R. E.; Wu, C.-H.; Krejci, M. R.; Weigand, S.; Joester, D., CrystEngComm **2011,** 13, 3975-3978.

18. Rodriguez-Ruiz, I.; Veesler, S.; Gomez-Morales, J.; Delgado-Lopez, J. M.; Grauby, O.; Hammadi, Z.; Candoni, N.; Garcia-Ruiz, J. M., Cryst. Growth Des. **2014,** 14, 792-802.

19. Tester, C. C.; Whittaker, M. L.; Joester, D., Chem. Commun. **2014,** 50, 5619-5622.

20. Orgel, J.; Irving, T. C.; Miller, A.; Wess, T. J., Proc. Natl. Acad. Sci. USA **2006,** 103, 9001-9005.

21. Landis, W. J.; Hodgens, K. J.; Arena, J.; Song, M. J.; McEwen, B. F., Microsc. Res. Tech. **1996,** 33, 192-202.

22. Beniash, E., Wiley Interdiscip. Rev.-Nanomed. Nanobiotechnol. **2011,** 3, 47-69.

23. Traub, W.; Arad, T.; Weiner, S., Proc. Natl. Acad. Sci. U. S. A. **1989,** 86, 9822-9826.

24. Weiner, S.; Traub, W., Faseb J. **1992,** 6, 879-885.

25. Gupta, H. S.; Seto, J.; Wagermaier, W.; Zaslansky, P.; P., B.; Fratzl, P., Proc. Natl. Acad. Sci. USA **2006,** 103, 17741-17746.

26. Fratzl, P.; Weinkamer, R., Prog. Mater. Sci. **2007,** 52, 1263-1334.

27. Weiner, S.; Wagner, H. D., Ann. Rev. Mater. Sci. **1998,** 28, 271-298.

28. Boskey, A. L.; Gadaleta, S.; Gundberg, C.; Doty, S. B.; Ducy, P.; Karsenty, G., Bone **1998,** 23, 187-196.

29. Boskey, A. L.; Spevak, L.; Paschalis, E.; Doty, S. B.; McKee, M. D., Calcif. Tissue Int. **2002,** 71, 145-154.

30. Ling, Y. F.; Rios, H. F.; Myers, E. R.; Lu, Y. B.; Feng, J. Q.; Boskey, A. L., J. Bone Miner. Res. **2005,** 20, 2169-2177.

31. Boskey, A. L.; Maresca, M.; Doty, S.; Sabsay, B.; Veis, A., Bone and Mineral **1990,** 11, 55-65.

32. Hunter, G. K.; Hauschka, P. V.; Poole, A. R.; Rosenberg, L. C.; Goldberg, H. A., Biochem. J. **1996,** 317, 59-64.

33. Gericke, A.; Qin, C.; Spevak, L.; Fujimoto, Y.; Butler, W. T.; Sorensen, E. S.; Boskey, A. L., Calcif. Tissue Int. **2005,** 77, 45-54.

34. Silverman, L.; Boskey, A. L., Calcif. Tissue Int. **2004,** 75, 494-501.

35. Palmer, L. C.; Newcomb, C. J.; Kaltz, S. R.; Spoerke, E. D.; Stupp, S. I., Chem. Rev. **2008,** 108, 4754-4783.

36. Wang, L. J.; Nancollas, G. H., Chem. Rev. **2008,** 108, 4628-4669.

37. Deshpande, A. S.; Beniash, E., Cryst. Growth Des. **2008,** 8, 3084-3090.

38. Wang, Y.; Azais, T.; Robin, M.; Vallee, A.; Catania, C.; Legriel, P.; Pehau-Arnaudet, G.; Babonneau, F.; Giraud-Guille, M. M.; Nassif, N., Nat. Mater. **2012,** 11, 724-733.

39. Olszta, M. J.; Cheng, X.; Jee, S. S.; Kumar, R.; Kim, Y.-Y.; Kaufman, M. J.; Douglas, E. P.; Gower, L. B., Mater. Sci. Eng. R **2007,** 58, 77-116.

40. Nudelman, F.; Pieterse, K.; George, A.; Bomans, P. H. H.; Friedrich, H.; Brylka, L. J.; Hilbers, P. A. J.; de With, G.; Sommerdijk, N., Nat. Mater. **2010,** 9, 1004-1009.

41. Christenson, H. K., Colloids Surf. A: Physicochem. Eng. Aspects **1997,** 123-124, 355.

42. Cantaert, B.; Beniash, E.; Meldrum, F. C., Chem Eur J. **2013,** 19, 14918–14924.

43. Cantaert, B.; Beniash, E.; Meldrum, F. C., J. Mater. Chem. B. **2013,** 1, 6586-6595.

44. Koutsopoulos, S., J. Biomed. Mater. Res. **2002,** 62, 600-612.

45. Ethirajan, A.; Ziener, U.; Chuvilin, A.; Kaiser, U.; Colfen, H.; Landfester, K., Adv. Funct. Mater. **2008,** 18, 2221-2227.

46. Eanes, E. D.; Termine, J. D.; Nylen, M. U., Calcif. Tissue Res. **1973,** 12, 143-158.

47. Dorozhkin, S. V., Acta Biomater. **2010,** 6, 4457-4475.

48. Habraken, W. J. E. M.; Tao, J. H.; Brylka, L. J.; Friedrich, H.; Bertinetti, L.; Schenk, A. S.; Verch, A.; Dmitrovic, V.; Bomans, P. H. H.; Frederik, P. M.; Laven, J.; van der Schoot, P.; Aichmayer, B.; de With, G.; DeYoreo, J. J.; Sommerdijk, N. A. J. M., Nat. Commun. **2013,** 4, 12.

49. Boskey, A. L.; Posner, A. S., J. Phys. Chem. **1973,** 77, 2313-2317.

50. Taves, D. R., Nature Commun. **1963,** 200, 1312-1313.

51. Ihli, J.; Kulak, A. N.; Meldrum, F. C., Chem. Commun. **2013,** 49, 3134-3136.

52. Ihli, J.; Wong, W.-C.; Noel, E. H.; Kim, Y.-Y.; Kulak, A. N.; Christenson, H. K.; Duer, M. J.; Meldrum, F. C., Nature Commun. **2014,** 5, 3169.

53. Van Driessche, A. E. S.; Benning, L. G.; Rodriguez-Blanco, J. D.; Ossorio, M.; Bots, P.; Garcia-Ruiz, J. M., Science **2012,** 336, 69-72.

54. Wang, Y.-W.; Meldrum, F. C., J. Mater. Chem. **2012,** 22, 22055-22062.

55. Kile, D. E.; Eberl, D. D., Am. Mineral. **2003,** 88, 1514-1521.

56. Lobry, L.; Ostrowsky, N., Phys. Rev. B **1996,** 53, 12050-12056.

57. Crane, N. J.; Popescu, V.; Morris, M. D.; Steenhuis, P.; Ignelzi, M. A., Bone **2006,** 39, 434-442.

58. Mahamid, J.; Aichmayer, B.; Shimoni, E.; Ziblat, R.; Li, C. H.; Siegel, S.; Paris, O.; Fratzl, P.; Weiner, S.; Addadi, L., Proc. Natl. Acad. Sci. U. S. A. **2010,** 107, 6316-6321.

59. Mahamid, J.; Sharir, A.; Addadi, L.; Weiner, S., Proc Natl Acad Sci USA **2008,** 105, 12748–12753.

60. Weiner, S., Bone **2006,** 39, 431-433.

61. Tseng, Y. H.; Mou, C. Y.; Chan, J. C. C., J. Am. Chem. Soc. **2006,** 128, 6909-6918.

62. Nelson, D. G. A.; Wood, G. J.; Barry, J. C.; Featherstone, J. D. B., Ultramicroscopy **1986,** 19, 253-265.

## Supporting Information

## Confinement Increases the Lifetimes of Hydroxyapatite Precursors

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**Figure S1.** CaP particles precipitated after 1 week in the presence of 10 μm $mL^{-1}$ PAsp in the crossed cylinders apparatus at surface separations of (a) 5 μm, where all the particles were HAP , (b) 2 μm, flattened HAP particles were observed. (c) 1.5 μm, all the particles were plate-like OCP, (d) 1 μm, aggregated nanoparticles of OCP were observed. (e) 0.5 μm and (b) 0.2 μm, where amorphous particles were observed in both cases.

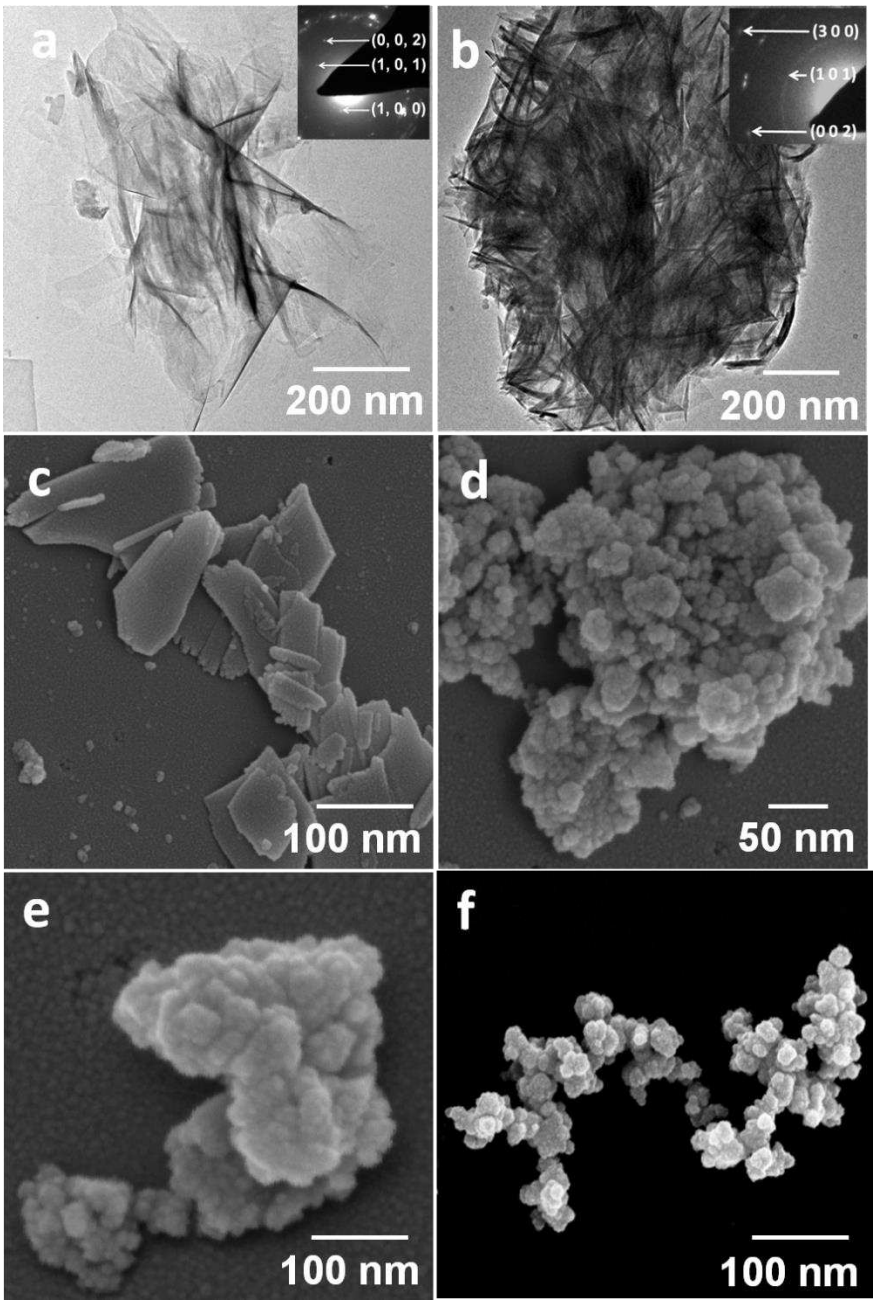

**Figure S2**. CaP particles precipitated in confinement in the presence of 10 µm $mL^{-1}$ PAsp at SS = 0.5 – 1 µm after 2 weeks. No amorphous phase was observed, and the particles were ≈ 70% HAP and ≈ 30% OCP.

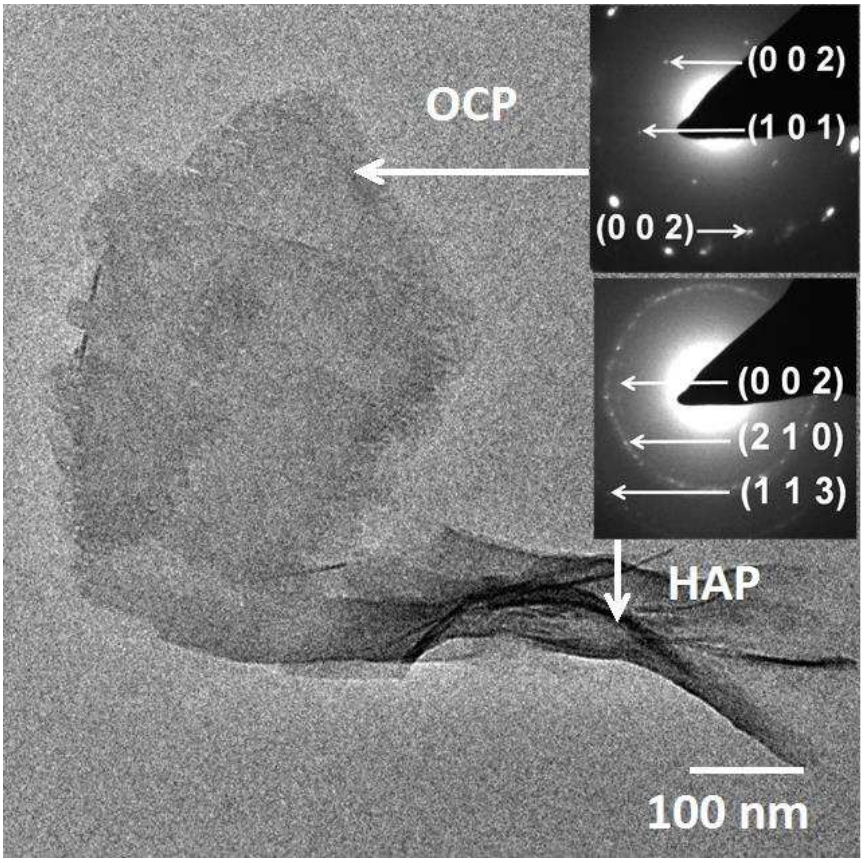

**Figure S3**. Comparison of the results obtained in bulk solution and in different degrees of confinement, in the absence and presence of PAsp.