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ARTICLE TYPE

# Stabilizing amorphous calcium phosphate phase by citrate adsorption

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**The regulation of citrate on amorphous calcium phosphate (ACP)-mediated crystallization of hydroxyapatite (HAP) is revealed in this work. The surface associated citrate on ACP plays the key role in controlling the nucleation of HAP by inhibiting the reaction of surface nucleation, and the effect of embedded citrate inside ACP or citrate in solution is weak.**

Biomolecules are generally believed to play important roles in controlling biomineralization. The regulation of biomolecule for nucleation is still a mystery in biomineralization. In bone, about one-sixth of bone-apatite crystallites are strongly bound with citrate, a small biomolecule containing three carboxyl groups.[1] The ultrathin bone minerals are thought to be correlated with the strong interaction of citrate with apatite mineral,[1] for which, hydroxyapatite (HAP, $Ca_5(PO_4)_3OH$) is a widely used prototype crystal.[2] It is well documented that citrate will be associated with HAP,[1,3] and in crystallization kinetics, citrate can inhibit both nucleation and crystal growth of HAP.[4] However, in both biomimetic mineralization (*in vitro*)[2c-d, 5] and biomineralization (*in vivo*),[6] a transient precursor phase, amorphous calcium phosphate (ACP, $Ca_3(PO_4)_2$), has been widely observed prior to HAP formation. These findings complicate the classical understanding of biomineralization in the scenario of the molecular interaction between biomolecule and mineral, and arose some important questions, which remain unanswered: At what stage does citrate enrol in HAP crystallization?[1,7] Is citrate present in ACP precursor phase? What is the role of citrate in nucleation kinetics?

The purpose of this work is to reveal the regulation mechanism of citrate on ACP-mediated crystallization under simulated body fluid (SBF),[8] which has the similar ionic species, *p*H and ionic strength to that of physiological solutions (see recipes in Table S1 in ESI†).

As the crystallization of HAP is accompanied by the drop in *p*H,[9] the kinetics of HAP nucleation can be monitored by *p*H meter. All *p*H curves were repeated for at least four times, and the relative standard deviation for induction time is within 6% (see Fig.S1 in ESI†). In *p*H curves, the crystallization processes can be divided into three stages (Fig. 1). In the stage I, after the mixing of a calcium solution and a phosphate solution, the solution *p*H fast dropped to designated *p*H and then, it kept stable. ACP was precipitated in this stage (see below), and it remained stable till crystallization. This stage can be regarded as the induction period for HAP nucleation.

In the stage II, the fast drop of *p*H was observed, suggesting the occurrence of HAP crystallization (cf. eq. 1). In the stage III, the *p*H levelled off (cf. Fig.S1 in ESI†). It was HAP ripening. The induction time, $t_i$, is determined by the intersection of tangents on the *p*H curve for the stage I and II as shown in Fig. 1. The induction time is an indication for the stability of ACP. The shorter the induction time, the less stable the ACP.

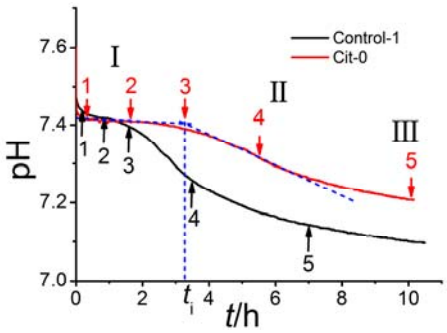


**Fig. 1**Representative *p*H curves of HAP crystallization in the present (Cit-0) and absent (Control-1) of citrate.The *p*H curves can be divided into three stages, named I, II, III, and the induction time, $t_i$, is determined by the intersection of tangents drawn on the first (I) and the second (II) stage of *p*H curve.

The phase of minerals at each stage has been examined by *ex situ* characterizations. At designated time intervals (marked by numbers in Fig.1), slurry samples were withdrawn, filtered, and examined. At early induction period (Time **1** in Fig. 1), sphere aggregates have been observed by Transmission Electron Microscopy (TEM) (cf. Fig. 2a, b). The diffusive electron diffractions (ED) (inset in Fig. 2a, b) indicate the mineral to be an amorphous phase. The evolution of mineral phase has been tracked by Fourier Transformed Infrared spectroscopy (FTIR). As the mineralization, FTIR spectra show the splitting of absorption peaks out of broad absorption bands at 1055cm$^{-1}$ (phosphate $\nu_3$ vibrations) and 570 cm$^{-1}$ (phosphate $\nu_4$ bending) (Fig. 2c, d), suggesting the transformation of the amorphous phase to the crystalline phase.[10] After the crystallization, sheet-like crystallites were formed (cf. Fig. S2). X-ray Diffraction patterns confirm the crystalline phase to be poorly crystallized HAP instead of octacalcium phosphate (OCP) as the absence of diffraction peak at about 4.7 degree for the initial crystallized phase (see Fig.S3 in ESI†). The above phase characterizations confirm

the ACP-mediated crystallization pathway, and corroborate that the fast drop in solution *p*H is correlated to the crystallization of HAP. In the present of citrate (2 mM) in solutions, ACP minerals show additional broad bands around 1595 and 1413 $cm^{-1}$ in FTIR spectra (Fig. 2d), which are, respectively, the anti-symmetrical and symmetrical vibrations of the carboxyl group for associated citrate.[3a,4b,11] These results support the citrate is associated with ACP during the precipitation (Note: the solution is under-saturated for calcium citrate, so calcium citrate minerals should not be precipitated in this situation.).

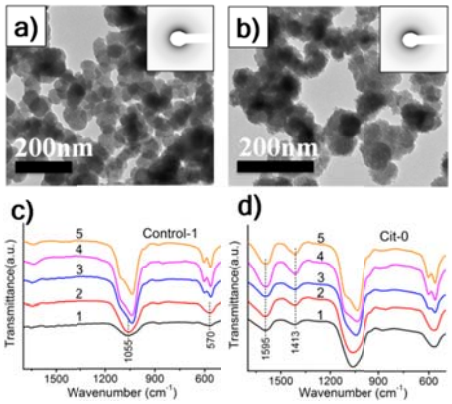


**Fig. 2** The *ex situ* characterizations of minerals during crystallization indicate the crystallization pathway. TEM images of initial formed ACP in the absent (a) and present (b) of citrate. (c, d) FTIR spectra of the minerals at designated time (marked in Fig. 1) indicate ACP-mediated crystallization in the absence (c) and presence (d) of citrate.

The nucleation kinetics (*p*H curves in Fig. 1) indicate that as the introduction of citrate into phosphate solutions (called Cit-0), the induction time is prolonged. This proves that citrate can stabilize ACP. Here, citrate was introduced into solution before the precipitation of ACP. So, there are three possible locations for citrate: in solution, incorporated into ACP particles, or adsorbing on ACP surface. It is of fundamental importance to make clear which one is responsible for the stabilization of ACP. In the following, we performed a series of experiments to explore this.

For citrate in solution, it will chelate with calcium ions.[4b,11] In solution chemistry, it will reduce the free calcium ions in solution, therefore, reduce the supersaturation for both ACP and HAP (Table S2 in ESI†), which may influence the crystallization kinetics.[9d] To check the nucleation kinetics at lower supersaturation, we do another control experiment (called Control-2), in which the activities of calcium and phosphate are identical to that of Cit-0 system (Table S2 in ESI†). It turns out that the induction time does not change much comparing with that of Control-1 (Fig.3). This means the reduction of calcium activity by chelation of citrate is unlikely responsible for the stabilization of ACP.

Citrate may be associated with ACP either by incorporation into ACP or adsorbing on the surface. To exclude the possible incorporation of citrate into ACP, citrate was introduced into solutions after the precipitation of ACP. After mixing for about 10 min (to ensure ACP is formed and remain stable), citrate was introduced into suspensions (called Cit-10). In this protocol, it also prolongs the induction time, close to that of Cit-0 (cf. Fig.3). FTIR spectra indicate that the citrate is also associated with ACP when it is introduced after ACP formation (cf. Fig.S4 in ESI†).

The possible surface association of citrate on ACP was supported by the change of zeta potentials of ACP particles. It shows the zeta potential changed from 3.30 ±0.22 mV (mean±s.d., n=6) (Control-1) to -9.72 ±0.46 mV (n=6) (Cit-0) and to -11.70±0.39 mV (n =6) (Cit-10) as the introduction of citrate into solutions. The reduction of zeta potentials may be resulted from the adsorption of negative charged citrate ions (the major species at *p*H 7.4 [4b,12]) on ACP surface (eqs. 1-2).

$$ACP(s) + HCit^{2-} \rightarrow ACP\text{-}HCit^{2-}(s) \quad (1)$$

$$ACP(s) + Cit^{3-} \rightarrow ACP\text{-}Cit^{3-}(s) \quad (2)$$

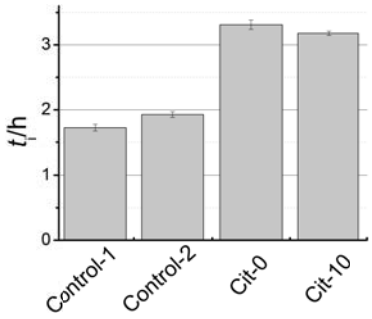


**Fig.3**Induction times for different solutions.

The ACP associated citrate are quantified by HPLC chromatographic analysis (see Experiments in ESI†). More citrate is associated with ACP minerals as the aging (Fig. 4). When citrate was introduced into the system before ACP formation (Cit-0 system), sufficient amount of citrate was found (about 6.2wt%) at the early precursor stage (at 20 min). This proves that citrate is enrolled in HAP formation during the early amorphous phase. When citrate was introduced after ACP formation (Cit-10 system), about 1.5wt% amount of citrate was adsorbed on ACP surface at early stage (at 20 min), far before HAP nucleation (at about 3 hrs).The Ca/P element ratio of initial ACP mineral was increased from 1.36±0.01 (mean±s.d., n=3) (Control-1) to 1.47±0.01 (n=3) (Cit-0), and 1.40±0.01 (n=3) (Cit-10), indicating the partial replacement of phosphate ions by citrate ions for calcium phosphate minerals. The initial amount of citrate that associated with ACP is much less if it is introduced into suspension after ACP formation (Fig.4). This indicates that some citrates may be incorporated into mineral as the precipitation of ACP. In this situation, more phosphate will be replaced by citrate, and Ca/P ratio is less for Cit-0 system (see above). Even though, more citrate is associated with ACP when it is introduced before ACP formation (cf. Fig. 4), there is no big difference in ACP stability (cf. Fig. 3). We consider the surface associated citrate might play the key role in stabilizing ACP.

The amount of associated citrate keeps on increasing as the aging, even after two hours (Fig. 4), indicating that ACP particles might be porous, and the adsorption process is slow. So, the amount of citrate on ACP surface (outer and inner surface if it is porous) can be controlled by adsorption time, which is defined as the time period from the introduction of citrate to the occurrence of HAP nucleation. Delayed adding of citrate into ACP slurries leads to a shorter adsorption time,

and in this manner, the amount of citrate on ACP surface can be controlled. Figure 5a shows the induction time increases as the elongation of the adsorption time, indicating that the more citrate adsorbed, the more stable ACP became. The stabilization effect levels off after about three hours' adsorption (cf. Fig. 5a), suggesting a saturation limit for the adsorption of citrate on ACP.

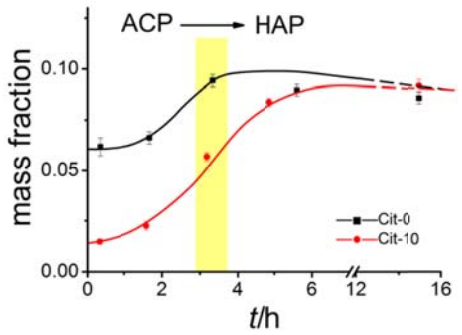


**Fig.4** The change of mass fraction of citrate in mineralsas the evolution (determined by quantitative HPLC chromatographic analysis).

As the amount of surface adsorbed citrate should be depended upon the chemical potential of citrate in solution, the amount of citrate on ACP surface can also be controlled by the initial concentration of citrate in solution (also introduce citrate after ACP formation for about 10 min). Figure 5b shows the effect of initial citrate concentration on the stability of ACP. At lower citrate concentration (< 0.5 mM), there is no obvious effect on stabilizing ACP. As the citrate concentration increases from 0.5mM to 3.0 mM, the induction time increases with citrate concentration. At higher citrate concentration (>3.0mM), the stabilizing effect levels off, suggesting a saturation limit for the adsorption of citrate on ACP. These results also prove the more citrate adsorbed, the more stable ACP became.

Once citrate was introduced into system after the precipitation of ACP, the most possible site for the association of citrate is to be on the surface of ACP (supposing citrate cannot penetrate into ACP solid), replacing the site of phosphate ion (by the strong interaction of citrate with calcium ions on mineral surface[3a]). TEM study revealed that ACP particle surface became rough in the presents of citrate (cf. Fig. 2 a, b, Fig.S5 in ESI†). It should be caused by the surface chelation of $HCit^{2-}$ with surface $Ca^{2+}$. This process accompanied with the releasing of $H^+$ (cf. eq. 3), which would induce local dissolution of ACP. Worth to note, except for surface modification of ACP by citrate, the size of ACP particles in the presents and absence of citrate or under different protocols are similar (cf. dynamic light scattering results in Fig.S6 in ESI†).

$$(ACP)Ca^{2+}(s) + HCit^{2-} \rightarrow (ACP)CaCit^{-} + H^{+} \quad (3)$$

Considering amorphous mineral as the precursor phase, crystal nucleation may take place on precursor surface[13] or inside.[14] Here we found the surface modification of ACP by citrate had obvious effect in retarding the nucleation of HAP (cf. Fig. 3), which indicate that HAP is nucleated from ACP surface. The surface nucleation model has been corroborated by both the nucleation kinetics data,[9d] and TEM observations.[5c] In this work, we confirm that the stability of ACP is sensitive to the amount of citrate associated with ACP surface (cf. Fig. 4, 5). This can be explained by the surface nucleation model. As the association of citrate on ACP, the surface of ACP will be covered by citrate. So, the surface reaction sites may be blocked by citrate. In this manner, the more citrate associate, the more stable ACP became. This is the mechanism for citrate in controlling the nucleation of HAP from ACP precursor.

In conclusion, citrate takes active roles during early amorphous stage, far before crystal nucleation, in HAP mineralization. We confirm that the association of citrate on ACP surface has paramount effect in controlling HAP nucleation. In this regard, special attentions should also be given to the interaction of additives with amorphous precursor phase, in addition to final crystallites. As citrate is widely found in bone mineral, this work can be regarded as a simple model system for biomimetic mineralization. The blocking of ACP phase transformation by surface modification might also be achieved by citrate analogs, such as carboxyl-rich proteins or polymers, in ACP-mediated bio-, and biomimetic-mineralization.[5b,15]

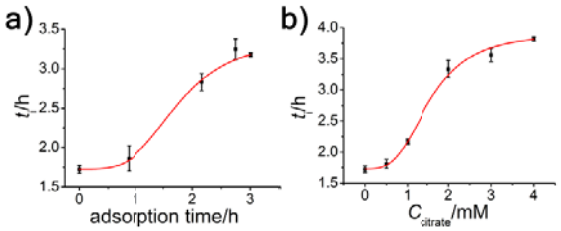


**Fig.5** The influence of adsorption time (a) and initial citrate oncentration (b) on the induction time.

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## Notes and references

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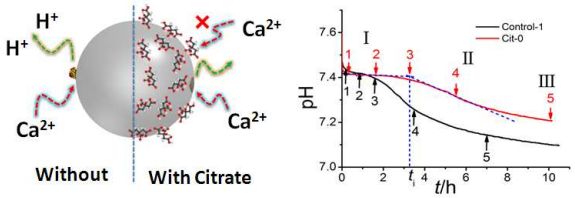
† Electronic Supplementary Information (ESI) available: Experimental details and FTIR, XRD, Zeta potential characterizations, and solution chemistry calculations. See DOI: 10.1039/b000000x/

1. Y.-Y. Hu, A. Rawal and K. Schmidt-Rohr, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 22425.
2. (a) M. Mathew and S. Takagi, *J. Res. Natl. Inst. Stand. Technol.*,2001, **106**, 1035. (b) S. V. Dorozhkin and M. Epple, *Angew. Chem., Int. Ed.*, 2002, **41**, 3130.(c) L. C. Palmer, C. J. Newcomb, S. R. Kaltz, E. D. Spoerke and S. I. Stupp, *Chem. Rev.*, 2008, **108**, 4754. (d) L. Wang and G. H. Nancollas, *Chem. Rev.*, 2008, **108**, 4628.
3. (a) I. Cifuentes, P. González-Díaz and L. Cifuentes-Delatte, *Calcif. Tissue Int.*, 1980, **31**, 147. (b) M. Johnsson, C. Richardson, J. Sallis and G. Nancollas, *Calcif. Tissue Int.*, 1991, **49**, 134. (c) C. Li, L. Zhao, J. Han, R. Wang, C. Xiong and X. Xie, *J. Colloid Interface Sci.*, 2011, **360**, 341.(d)J. M. Delgado-López, M. Iafisco, I. Rodríguez, A. Tampieri, M. Prat and J. Gómez-Morales, *ActaBiomater.*, 2012, **8**, 3491. (e) W. G. Jiang, H. H. Pan, Y. R. Cai, J. H. Tao, P. Liu, X. R. Xu and R. K. Tang, *Langmuir*, 2008, **24**, 12446.

4. (a) J. W. Wilson, P. G. Werness and L. H. Smith, *J. Urol.*, 1985, **134**, 1255. (b) M. A. Martins, C. Santos, M. M. Almeida and M. E. V. Costa, *J. Colloid Interface Sci.*, 2008, **318**, 210.
5. (a) E.Eanes, I.Gillessen, and A.Posner, *Nature*, 1965, **208**, 365.(b) M. Olszta, X. Cheng, S. Jee, R. Kumar, Y. Kim, M. Kaufman, E. Douglas and L. Gower, *Mater. Sci. Eng., R*, 2007, **58**, 77.(c) H. Pan, X. Liu, R. Tang and H. Xu, *Chem. Commun.*, 2010, **46**, 7415.(d) E. Beniash, R. A. Metzler, R. S. K. Lam and P. Gilbert, *J. Struct. Biol.*, 2009, **166**, 133.
6. (a) S. Weiner, J. Mahamid, Y. Politi, Y. Ma and L. Addadi, *Front. Mater. Sci.*, 2009, **3**, 104. (b) J. Mahamid, A. Sharir, D. Gur, E. Zelzer, L. Addadi and S. Weiner, *J. Struct. Biol.*, 2011, **174**, 527.(c) J. Mahamid,A. Sharir,L. Addadi, and S. Weiner,*Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 12748.(d) J. Mahamid,B. Aichmayer, E. Shimoni, R. Ziblat, C. Li, S. Siegel, O. Paris, P. Fratzl, S. Weiner, and L. Addadi, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 6316.
7. B. Xie and G. H. Nancollas, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 22369.
8. (a) A. Oyane, H.-M. Kim, T. Furuya, T. Kokubo, T. Miyazaki and T. Nakamura, *J. Biomed. Mater. Res., Part A*, 2003, **65A**, 188. (b) T. Kokubo and H. Takadama, *Biomaterials*, 2006, **27**, 2907.
9. (a) J. Christoffersen, M. R. Christoffersen, W. Kibalczyc and F. A. Andersen, *J. Cryst. Growth*, 1989, **94**, 767. (b) P. Ofir, R. Govrin-Lippman, N. Garti and H. Füredi-Milhofer, *Cryst. Growth Des.*, 2004, **4**, 177. (c) X. Yang, B. Xie, L. Wang, Y. Qin, Z. J. Henneman and G. H. Nancollas, *CrystEngComm*, 2011, **13**, 1153. (d) S. Jiang, Y. Chen, H. Pan, Y.-J. Zhang, and R. Tang, *Phys. Chem. Chem. Phys.*, 2013, **15**, 12530.
10. (a) S. Rößler, A. Sewing, M. Stölzel, R. Born, D. Scharnweber, M. Dard and H. Worch, *J. Biomed. Mater. Res., Part A*, 2003, **64A**, 655.(b) H.A.Lowenstam and S.Weiner, Science, 1985, **227**, 51.
11. A. López-Macipe, J. Gómez-Morales and R. Rodríguez-Clemente, *Adv. Mater.*, 1998, **10**, 49.
12. M. J. G. H. M. Lito, M. F. G. F. C. Camões and A. K. Covington, *Anal. Chim. Acta*, 2003, **482**, 137.
13. (a) H. Greer, P. S. Wheatley, S. E. Ashbrook, R. E. Morris and W. Zhou, *J. Am. Chem. Soc.*, 2009, **131**, 17986. (b) E. H. G. Backus, M. L. Grecea, A. W. Kleyn and M. Bonn, *Phys. Rev. Lett.*, 2004, **92**, 236101. (c) O. G. Shpyrko, R. Streitel, V. S. K. Balagurusamy, A. Y. Grigoriev, M. Deutsch, B. M. Ocko, M. Meron, B. Lin and P. S. Pershan, *Science*, 2006, **313**, 77.
14. (a) E. M. Pouget, P. H. H. Bomans, J. A. C. M. Goos, P. M. Frederik, G. de With and N. A. J. M. Sommerdijk, *Science*, 2009, **323**, 1455. (b) T. H. Zhang and X. Y. Liu, *J. Am. Chem. Soc.*, 2007, **129**, 13520.
15. (a) F. Nudelman, K. Pieterse, A. George, P. Bomans, H. Friedrich, L. Brylka, P. Hilbers, G. de With and N. Sommerdijk, *Nat. Mater.*, 2010, **9**, 1004. (b) F. R. Tay and D. H. Pashley, *J. Dent. Res.*, 2009, **88**, 719. (c) A. George and A. Veis, *Chem. Rev.*, 2008, **108**, 4670. (d)J. Wang, Y. Chen, L. Li, J. Sun, X. Gu, X. Xu, H. Pan and R. Tang, *CrystEngComm*, 2013, **15**, 6151.




$3Ca_3(PO_4)_2\,(s) + Ca^{2+} \rightarrow 2Ca_5(PO_4)_3(OH)\,(s) + 2H^+$

$3Ca_3(PO_4)_2\,Cit\,(s) + Ca^{2+} + 2H_2O \rightarrow 2Ca_5(PO_4)_3(OH)\,Cit\,(s) + 2H^+$

Citrate controls nucleation by association with precursor amorphous phase, which inhibits the surface reaction for nucleation.