Electronic supplementary information

Effect of aggregation state of amorphous calcium phosphate on

hydroxyapatite nucleation kinetics

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S1 The determination of specific surface area of ACPs

TEM image directly showed the state of aggregates, but care should be taken to the overlap region. The overlap in a TEM image does not represent the actual overlap in real 3D space because a TEM image is a projection of a 3D volume. So, the challenge in the analysis of a TEM image is to obtain the real overlap parameter of aggregates. Fortunately, this problem has been solved in previous work,^{S1} which has been widely applied in the study of aggregates.^{S2-S6} It was corroborated that the real overlap parameter (in 3D volume, C_{ov}) can be obtained from the projected one (in TEM image, $C_{ov,proj}$),^{S1}

$$C_{ov} = 1.1C_{ov, proj} - 0.2$$
(S0)

The projected overlap parameter can be directly obtained from TEM images, where all the position and size of particles can be measured by the ImageJ software.^{S7-S8}

In the followings, we will tell how to calculate the specific surface area of aggregates from the overlap parameter and the radius of particles.



Scheme S1. The scheme of partial overlapped ACP spherules.

For partial merged ACP aggregates, it can be simplified as partial overlapped spheres (Scheme S1). To estimation of specific area (surface area per unit volume of a material) of aggregates, one needs to take into account the overlap parameter (C_{ov}) of primary particles, which is given as,

$$C_{ov} = \frac{h}{2r} \tag{S1}$$

Where h is the overlap of two spheres. The total surface area (A_{ACP}) and the total volume (V_{ACP}) of aggregates can be obtained by,

$$A_{ACP} = A_{spheres} - A_{caps}$$
$$= \sum_{i} A_{i} - \sum_{i,j} A_{cap,i,j} = N([A] - N_{c}[A_{cap,i}])$$
(S2)

$$V_{ACP} = V_{spheres} - V_{caps}$$

= $\sum_{i} V_{i} - \sum_{i,j} V_{cap,i,j} = N(V] - N_{c}[V_{cap,i}])$ (S3)

where both the surface area and the volume of caps that were embedded inside aggregates should be discounted from the each sphere of aggregates. [X] is the mean value for X; N is the total number; N_c is the coordination number of each sphere, which means on average, how many particles are overlapped with a central particle. The surface area and volume of a cap with height of h/2 are given as,

$$A_{cap} = 2\pi r \left(\frac{h}{2}\right) = 4\pi r^2 \frac{1}{2} \left(\frac{h}{2r}\right) = [A] \frac{1}{2} C_{ov} = f[A]$$
(S4)
$$V_{cap} = \frac{1}{3} \pi \left(\frac{h}{2}\right)^2 \left(3r - \frac{h}{2}\right) = \frac{4}{3} \pi r^3 \left(\frac{3}{4} \left(\frac{h}{2r}\right)^2 - \frac{1}{4} \left(\frac{h}{2r}\right)^3\right)$$
(S5)
$$= [V] \left(\frac{3}{4} C_{ov}^2 - \frac{1}{4} C_{ov}^3\right) = g[V]$$

By combing eqn (S2)-(S5),

$$A_{ACP} = N[A](1 - fN_c), \quad f = \frac{1}{2}[C_{ov}]$$
(S6)

$$V_{ACP} = N[V](1 - gN_c), \quad g = \frac{3}{4}[C_{ov}]^2 - \frac{1}{4}[C_{ov}]^3$$
(S7)

The specific surface area (S_a) is give as,

$$S_{a} = \frac{A_{ACP}}{V_{ACP}} = \frac{N[A](1 - fN_{c})}{N[V](1 - gN_{c})} = \frac{3(1 - fN_{c})}{[r](1 - gN_{c})}$$
(S8)

where f and g can be determined by C_{ov} (cf. eqn (S6), (S7)). So, for aggregates, given the overlap parameter (C_{ov}), the mean radius of primary particles ([r]), and the coordination number (N_c), the specific surface area can be obtained by eqn (S8).

Effect of image contrast on the size and overlap measurement by TEM observation method

We measured the size and overlap behavior of particles from TEM image by controlling contrast. The results showed that if the contrast is not in extreme case, the measured parameters are almost the same (Fig. S11).

Validate TEM observation method by BET measurement

Due to the amount of formed ACPs were small and BET measurement needs at least 100 mg samples to test, so here we used nano-silica as prototype particles. TEM observation method and BET measurement were used to determinate the surface area of nano-silica, respectively. The results show that the surface area of nano-silica is $53.4 \pm 0.3 \text{ m}^2 \text{ g}^{-1}$ using BET measurement (Fig. S12). For TEM observation method the surface area is $54.8 \pm 4.5 \text{ m}^2 \text{ g}^{-1}$ (Fig. S13). So, the specific surface area of ACP aggregates can be obtained by TEM images, as shown in Fig. S14-S16.

S2 Supplementary figures



Fig. S1 FTIR spectra of minerals formed at different stages (numbers were marked in Figure 1a) (1) amorphous calcium phosphate (ACP) was existed at 10 min; (2) and (3) ACP was transformed into HAP (t = 45 min, 50 min), identified by the splitting of 570 cm⁻¹ absorption peak; (4) crystallized HAP at 100 min. The absence of absorption band at about 917 cm⁻¹ which is P-(OH) stretch and OH inplane bend of HPO₄, indicating there was no OCP existed.



Fig. S2 XRD analysis of minerals formed at different stages (numbers were marked in Figure 1a) (1) amorphous calcium phosphate (ACP) at 10 min; (2) and (3) start of HAP crystallization (t = 45 min, 50 min); (4) crystallized HAP at 100 min.



Fig. S3 Concentrations of calcium in solution during the different stages of mineralization.



Fig. S4 The kinetic growth of ACP particles during different times at induction period at $\ln S = 26.65$. In the absence of collagen: (a) 30 min; (b) 1.5 h; (c) 6 h; in the presence of collagen: (d) 30 min; (e) 1.5 h; (f) 3 h. The collagen concentration was 50 µg mL⁻¹.



Fig. S5 The size of ACP particles obtained from TEM images (N = 100) during different times at induction period (InS = 26.65).



Fig. S6 Particles size measured by DLS (a) and Zeta potentials of formed ACPs (b) without (control) or with 50 μ g mL⁻¹ collagen-I at ln*S* = 26.65, *t* = 10 min.



Fig. S7 Induction time (t_i) of HAP nucleation in the absence (control) and presence of 50 µg mL⁻¹ collagen-I at different supersaturations.



Fig. S8 FTIR spectrum of the mineral during the minealization at $\ln S = 35.44$ in the absence (a) and presence of 50 µg mL⁻¹ collagen-I (b); (c) scheme of splitting function (SF). A₁ is the area enclosed by the spectrum and the straight line and A₂ is the area enclosed by spectrum and straight baseline, the SF is defined as the radio of A₁ to A₂. SF of calcium phosphate is used as the crystallization indicator. (d) kinetic plots of phase transformation from ACP to HAP.



Fig. S9 TEM images of initially formed ACP (t = 30 min) and finally crystallized HAP (t = 24 h) in the presence of collagen-I fibrils.



Fig. S10 TEM images of mineral formed with high collagen-I concentrations at $\ln S = 35.44$, t = 10 min, showed aggregated ACPs. (a,b) in the presence of 80 µg mL⁻¹ collagen-I. (c,d) in the presence of 160 µg mL⁻¹ collagen-I. (e) Induction time (t_i) of HAP nucleation in the presence of collagen-I (80 µg mL⁻¹, 160 µg mL⁻¹). Collagen-I fibrils were marked by black arrows in (a) and (c).



Fig. S11 The size and overlap behavior of particles measured by TEM image with different contrast.



Fig. S12 The BET surface area plot of nano-silica.



Fig. S13 The TEM images of nano-silica and the determination of surface area. (C_{ov} : 0.30 ± 0.21; N_c : 1.32 ± 0.85; r: 20 ± 2 nm; N: 1247). scale bar : 50 nm.



Fig. S14 The mark of primary particles for ACPs at control experiment and the determination of specific surface area.



Fig. S15 The mark of primary particles for ACPs for pre-mixing experiment and the determination of specific surface area. Note in this case, ACPs are actually in partial covered by collagen-I fibrils. Assuming about 1/3 of surface area of ACPs are covered by collagen-I fibrils (Fig. S4d-f), the effective S_a should be rescaled as $0.0800^*(2/3) = 0.0533 \text{ nm}^{-1}$.



Fig. S16 The mark of primary particles for ACPs at post-mixing experiment and the determination of specific surface area.

S3 Supplementary tables

Table S1. The composition of SBF solutions with different calcium and phosphate concentrations at pH = 7.4, T = 37 °C. The Ca/P is kept at 1.67. The supersaturation (*S*) of mineral crystal before (*S*) and after the precipitation of ACP (S_{eff}), and the free calcium concentration ({Ca_{eff}}) after ACP precipitation.

Ion. C/(mM)	No.							
	1.	2.	3.	4.	5.	6.		
H^+	12.1	12.2	12.5	13.2	14.2	17.49		
Na ⁺	138.2	138.0	138.0	136.7	135.4	131.0		
K^+	5.0	5.0	5.0	5.0	5.0	5.0		
Mg^{2+}	1.5	1.5	1.5	1.5	1.5	1.5		
Cl-	148.0	148.0	148.0	148.0	148.0	148.0		
SO_4^{2-}	0.5	0.5	0.5	0.5	0.5	0.5		
Ca^{2+}	3.507	3.674	4.175	5.344	7.014	12.508		
PO ₄ ³⁻	2.1	2.2	2.5	3.2	4.2	7.49		
HEPES-	10.0	10.0	10.0	10.0	10.0	10.0		
$\ln(S_{\text{HAP}})$	26.30	26.65	27.60	29.43	31.39	35.44		
$\ln(S_{\rm eff,HAP})$	25.31	25.31	25.31	25.32	25.33	25.37		
$\ln(S_{\rm eff,OCP})$	8.26	8.26	8.25	8.24	8.23	8.19		
{Ca _{eff} }	0.842	0.844	0.850	0.865	0.886	0.962		

Table S2. The chemical analysis of the Ca/P, Ca/Mg ratios and the concentration of incorporated Mg^{2+} of formed mineral at induction period or 24 h at low (InS = 26.65) and high (InS = 35.44) supersaturation. 0 µg mL⁻¹ collagen-I means the absence of collagen-I.

lnS	Collagen-I	Time	Ca/P	incorporated Mg ²⁺	Ca/Mg
	concentration	(h)	$(\text{mean} \pm \text{s.d.})$	$(mM, mean \pm s.d.)$	$(\text{mean} \pm \text{s.d.})$
	(µg mL ⁻¹)				
	0	6.6	1.50 ± 0.04	0.15 ± 0.05	3.32 ± 0.71
26.65	0	24	1.69 ± 0.02	0.04 ± 0.03	34.95 ± 24.96
	50	3.4	1.52 ± 0.06	0.14 ± 0.01	3.29 ± 0.14
	50	24	1.69 ± 0.01	0.06 ± 0.05	33.58 ± 26.48
35.44	0	1.0	1.51 ± 0.01	0.25 ± 0.02	39.52 ± 2.32
	0	24	1.66 ± 0.03	0.05 ± 0.02	226.03 ± 87.81
	50	0.9	1.53 ± 0.02	0.28 ± 0.06	37.86 ± 5.05
	50	24	1.68 ± 0.02	0.04 ± 0.02	257.60 ± 116.55

Table S3. The specific surface area of ACP particles at different protocols

	Control	Pre-mixing	Post-mixing
N	442	727	1580
$C_{\rm ov}$ (mean, s.d.)	0.57(0.31)	0.13(0.23)	0.52(031)
N _c	2.41(0.68)	0.76(0.81)	2.31(0.74)
<i>r</i> (nm)	76(12)	36(7)	61(10)
S_{a} (nm ⁻¹)	0.024(0.004)	0.0533(0.013)*	0.032 (0.004)

S4 Supplementary references

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