Electronic supplementary information for

Counting Crystal Clusters – A Neutron Reflectometry Study of Calcium Phosphate Pre-nucleation Cluster Adsorption and Composition

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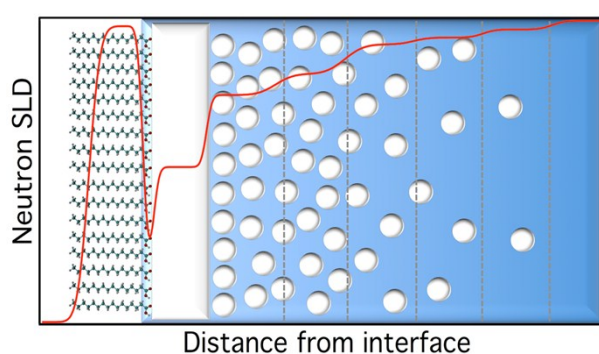

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Experimental Methods

Deuterated hexadecanoic acid (d-HDA) was synthesized at the National Deuteration Facility, ANSTO, Sydney, by hydrothermal H/D exchange reaction using Pt/C in D_2O at 220°C. The d-HDA (98% D) was dissolved in chloroform to make a 0.1 mg mL^{-1} solution. Zein protein (CAS 9010-66-6) was obtained from Sigma Aldrich and used without further purification. Ethanol-water solutions (80:20 by volume) of zein were prepared at a concentration of 1 mg mL^{-1}. Calcium phosphate nucleation experiments were conducted using a simulated body fluid (SBF) which was prepared by dissolving various salts in D_2O, following the method described by Tas and Bhaduri[1]. The concentration of the SBF used for the neutron reflectivity experiments were half of the standard concentration, this was done to ensure only the nucleation process was examined by neutron reflectivity. A Langmuir trough was used to hold 50 mL of SBF solution in D_2O. Then 170 μL of d-HDA solution was

spread onto the surface of the SBF (D_2O) solution. For zein protein calcium phosphate mineralisation experiments 100 μL of zein solution was spread. The surface pressure was set to 33 and 10 N m^{-1} for d-HDA and zein, respectively. (Fig. S1)

Neutron reflectometry (NR) was performed on the PLATYPUS[2] time-of-flight reflectometer at ANSTO, Lucas Heights, Sydney, Australia. The neutron reflectometry measurements taken were of the d-HDA and zein protein layers assembled at the air/SBF interface, using SBF solution prepared with D_2O. The reflectivity profiles were co-refined and fitted using optical matrix formalism in the program MOTOFIT[3] to obtain the thickness, scattering length density and solvent % for each modeled layer. An eight-layer fit was the minimum required to adequately model the features in the data for the d-HDA system and a three-layer fit was required for the zein system. The reflectivity profiles were obtained by measuring at one angle (1.1°) using a wavelength range of 2 Å – 20 Å, providing a useable Q_z-range between 0.0017 Å^{-1} < Q_z < 0.0097 A^{-1}. Measurements were taken approximately 10 minutes after spreading d-HDA/zein over the SBF solutions.

Ellipsometry measurements were done on a Beaglehole Instruments Imaging Ellipsometer[4] at variable angles (40° - 60°) using a quartz halogen light source with a wavelength of 632 nm selected by an interference filter. Data were fitted using the software Thin Film Companion, a refractive index of 1.433 was used for modelling the amorphous calcium phosphate[5] layer, and 1.450 for pure zein protein.[6] Measurements were taken at 20°C every 5 minutes during mineralisation and plotted to show the mineral film growth over time.

Fourier transform infrared (FT-IR) measurements were conducted using a PerkinElmer UATR Two. The mineral films were collected from the air–water interface and dried. The obtained powder was collected and spectra were collected from 500–4000 cm^{1} .
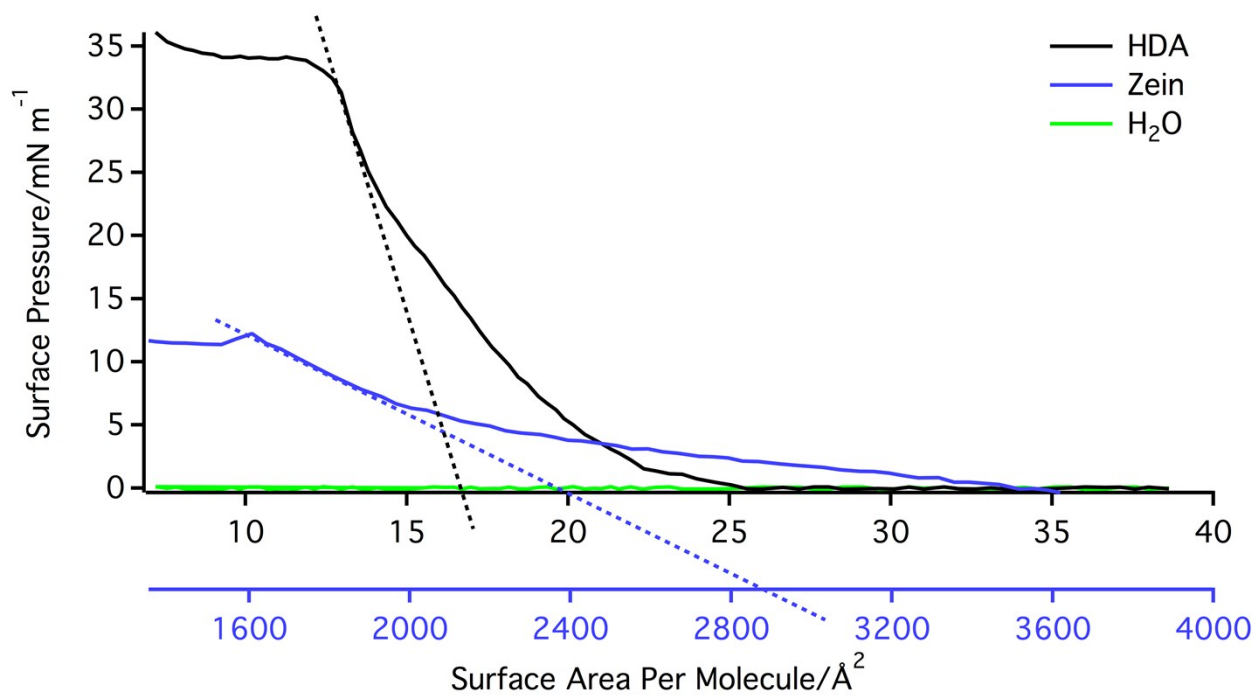
Fig S1. Surface pressure-area measurements of the HDA monolayer and zein protein layer at the air-water interface. Dashed lines show the mean molecular area.
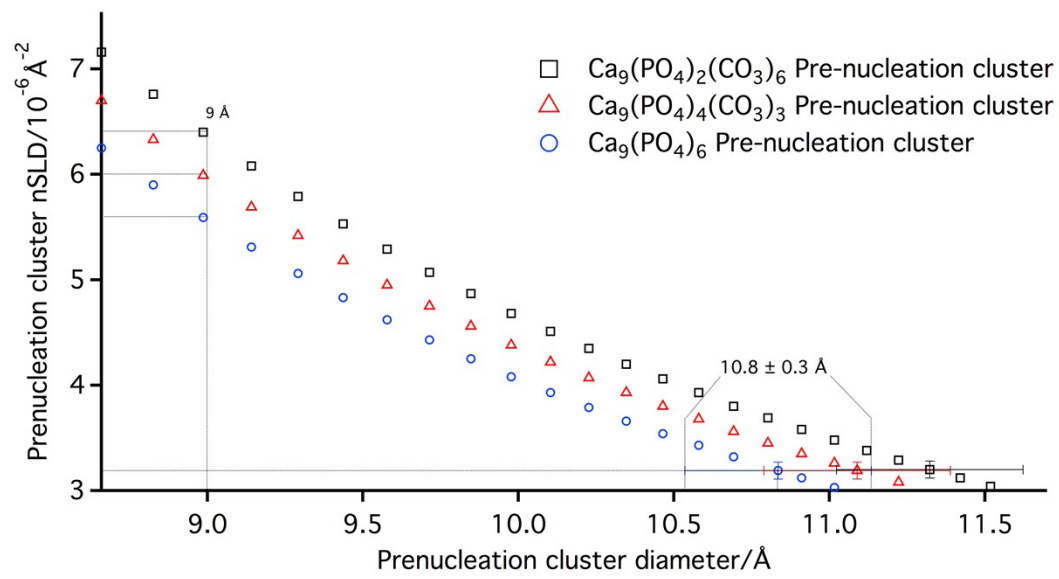
Fig 2. The relationship between pre-nucleation cluster diameter and the calculated nSLD. The measured nSLD of 3.19×10^{-6}Å^{-2} obtained from neutron reflectivity is used to provide an estimate of the $Ca_9(PO_4)_6$ pre-nucleation cluster diameter, along with carbonated equivalents.
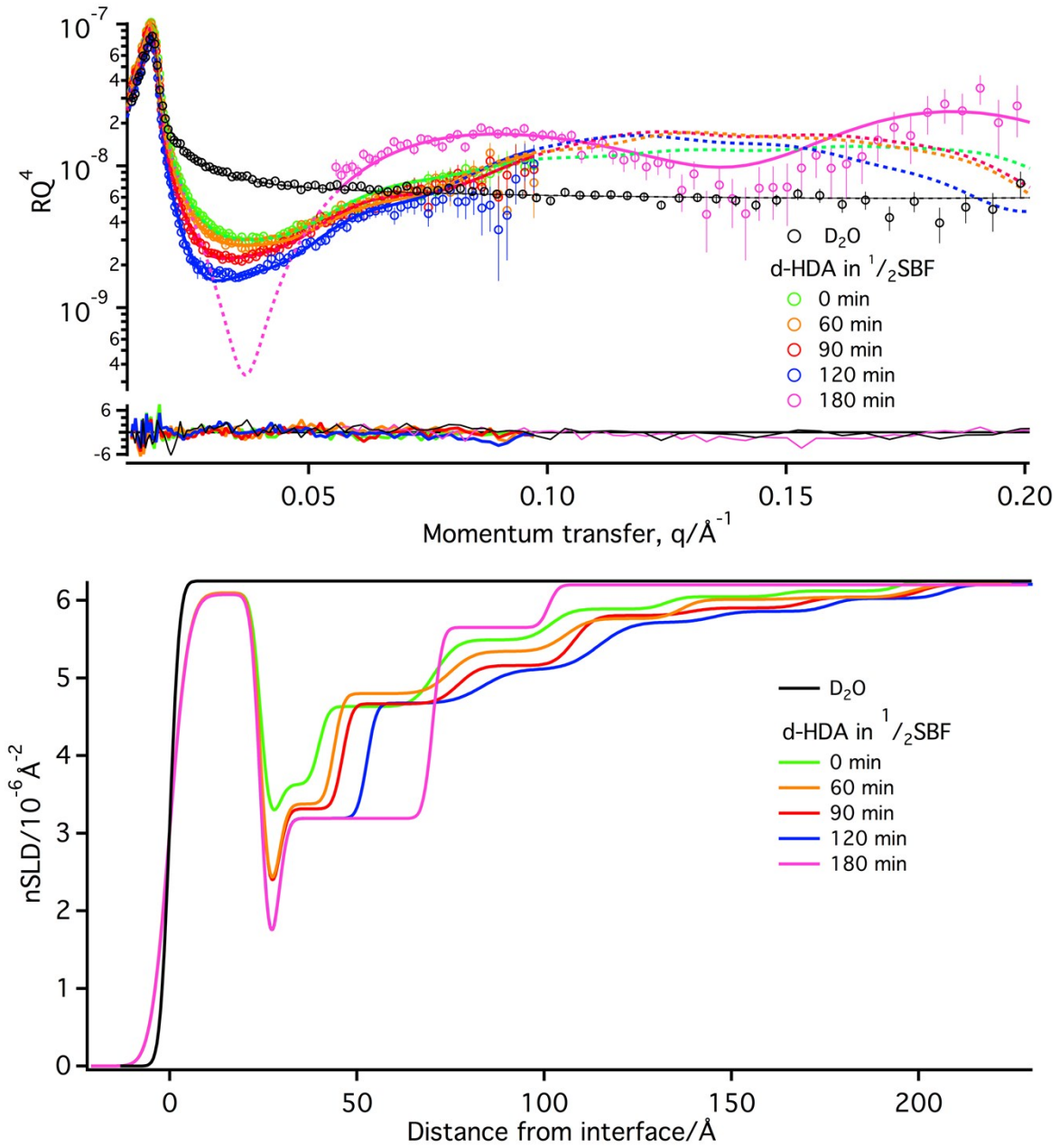
Fig S3. Neutron reflectivity profiles presented in RQ^4 for pure D_2O and d-HDA (top) on a ½ SBF, includes 180 min measured at an angle of 4.8°, residual plot shown within. The nSLD profiles for the D_2O and the d-HDA (bottom) induced calcium phosphate mineralisation, obtained from the fitted neutron reflectivity data.
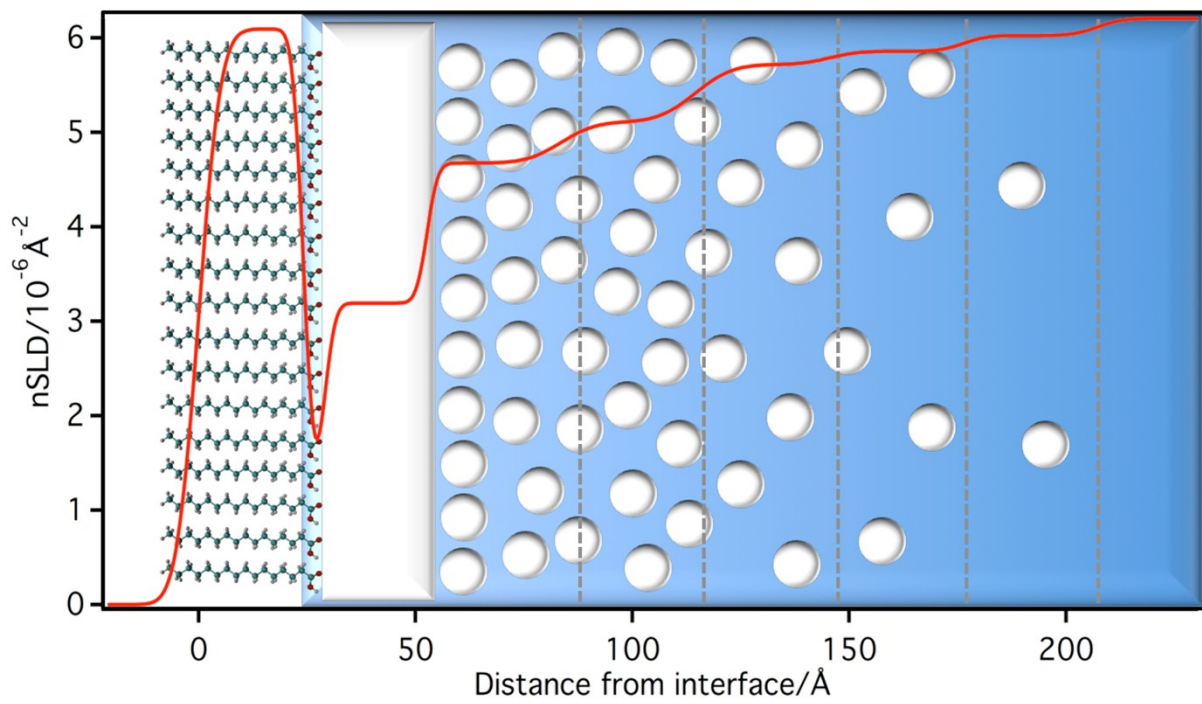
Fig S4. Schematic showing how the nSLD profile displays the structure of the air-liquid (SBF) interface. The first two layers represent the tail and head group of the d-HDA monolayer, the third layer is the dense mineral film and the following five layers represent the pre-nucleation cluster density within the subphase 150 Å below the mineralising layer.

Fig S5. *In situ* ellipsometry measurements of the zein protein induced CaP film grown from varying concentrations of PO_4^{3-} (A) and CO_3^{2-} (B) within the SBF, dashed lines show growth rate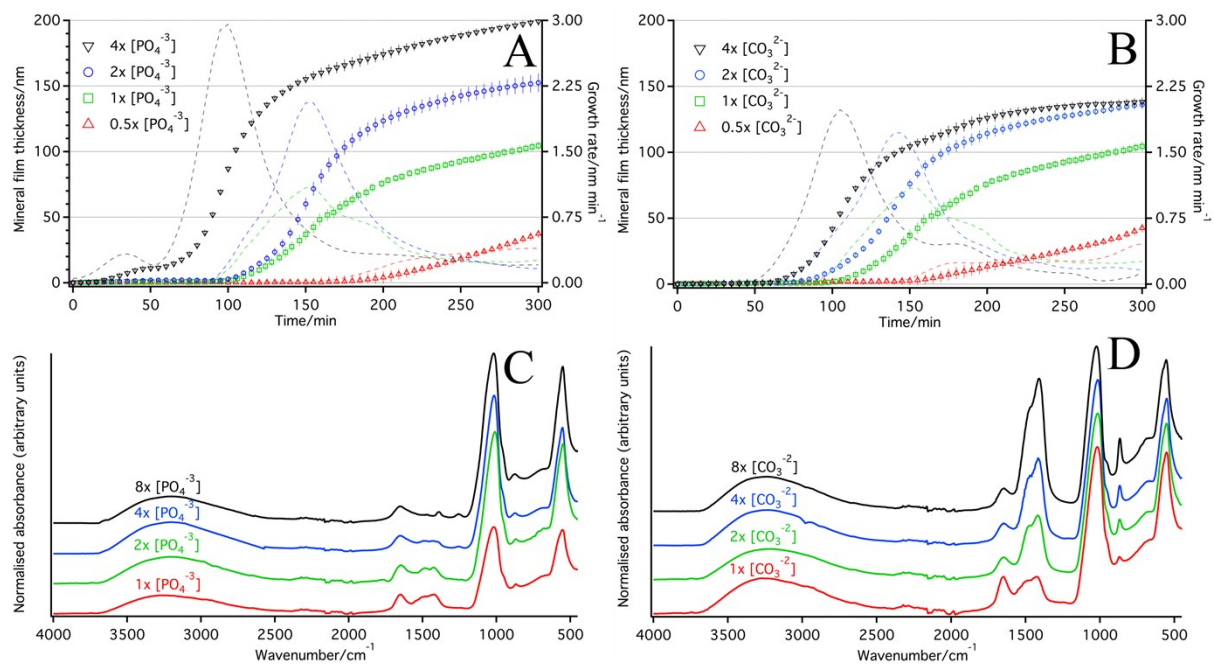s. FT-IR measurements of the zein protein induced CaP film grown from varying concentrations of PO_4^{3-} (C) and CO_3^{2-} (D) within the SBF.
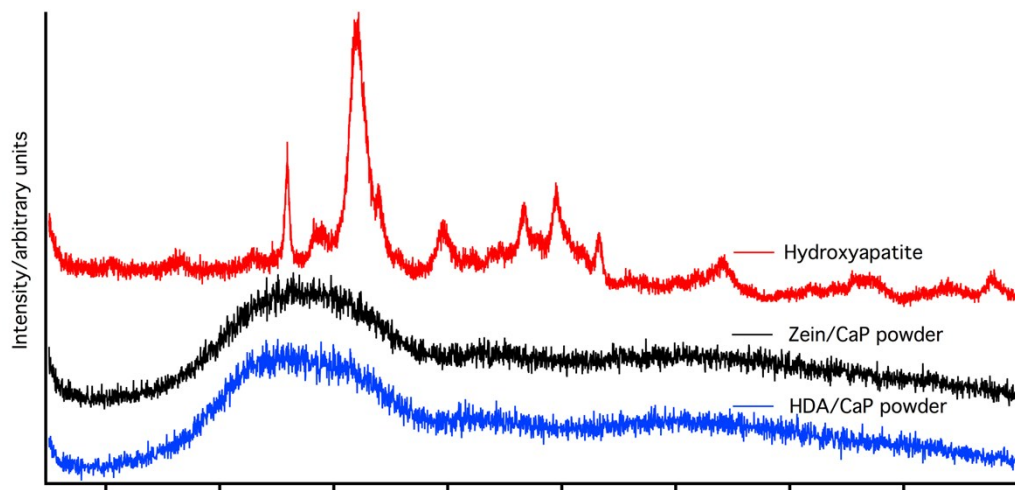
Fig S6. X-ray diffraction patterns of tbe zein/CaP and HDA/CaP films as a powder along with a reference hydroxyapatite powder.
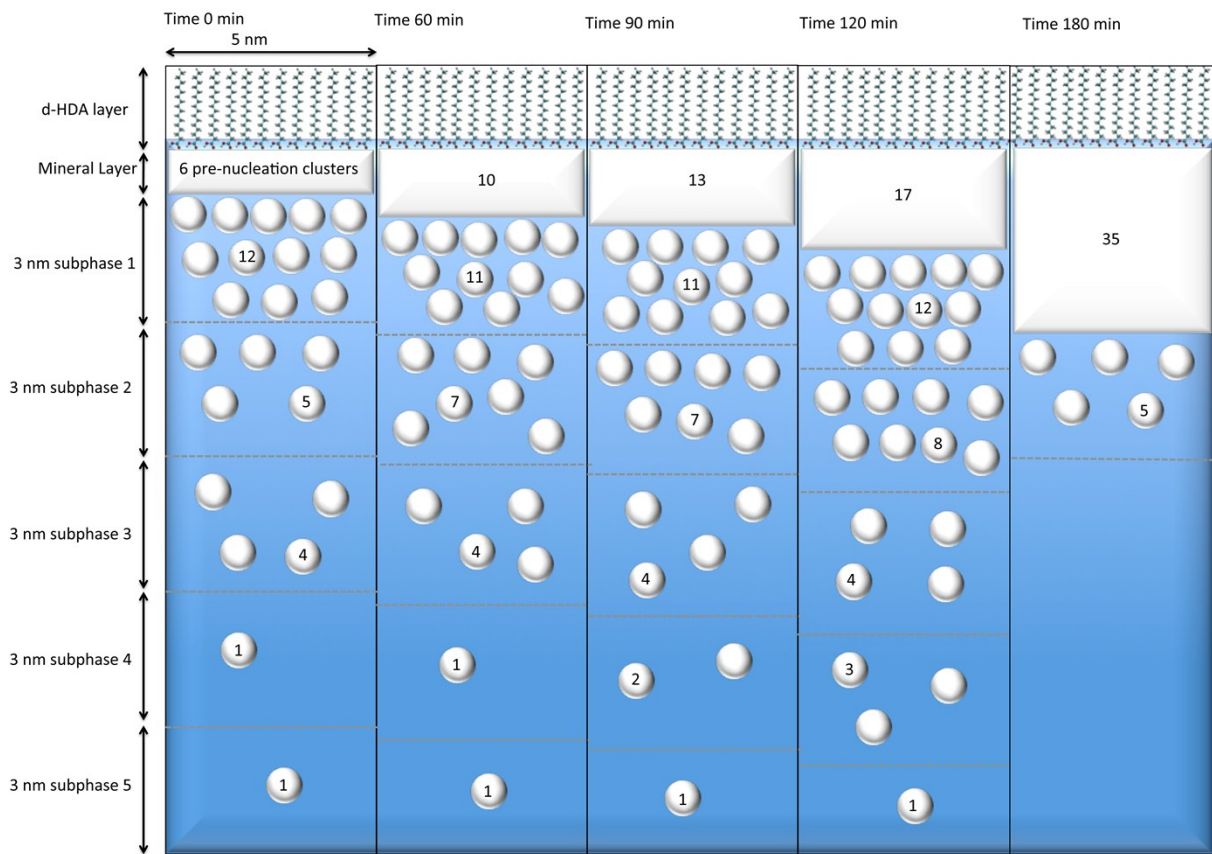
Fig S7. Schematic showing the thickness of the calcium phosphate layer on the d-HDA monolayer along with the pre-nucleation cluster density in the subphase below. Includes the 180 min measurement at an angle of 4.8°

Fig S8. Neutron reflectivity profiles presented in RQ⁴ for d-HDA in an SBF prepared without Ca² in D2O and null reflecting water (NRW) to provide contrast measurements for co-refined fitting.

Table S1. Concentration of ions in ½SBF solution.

Ion	Na$^+$	K$^+$	Ca^{2+}	Mg^{2+}	Cl$^-$	HPO$_4^{2-}$	CO$_3^{2-}$
mmol L^{-1}	80.0	2.5	1.5	0.5	76.0	0.5	5.0

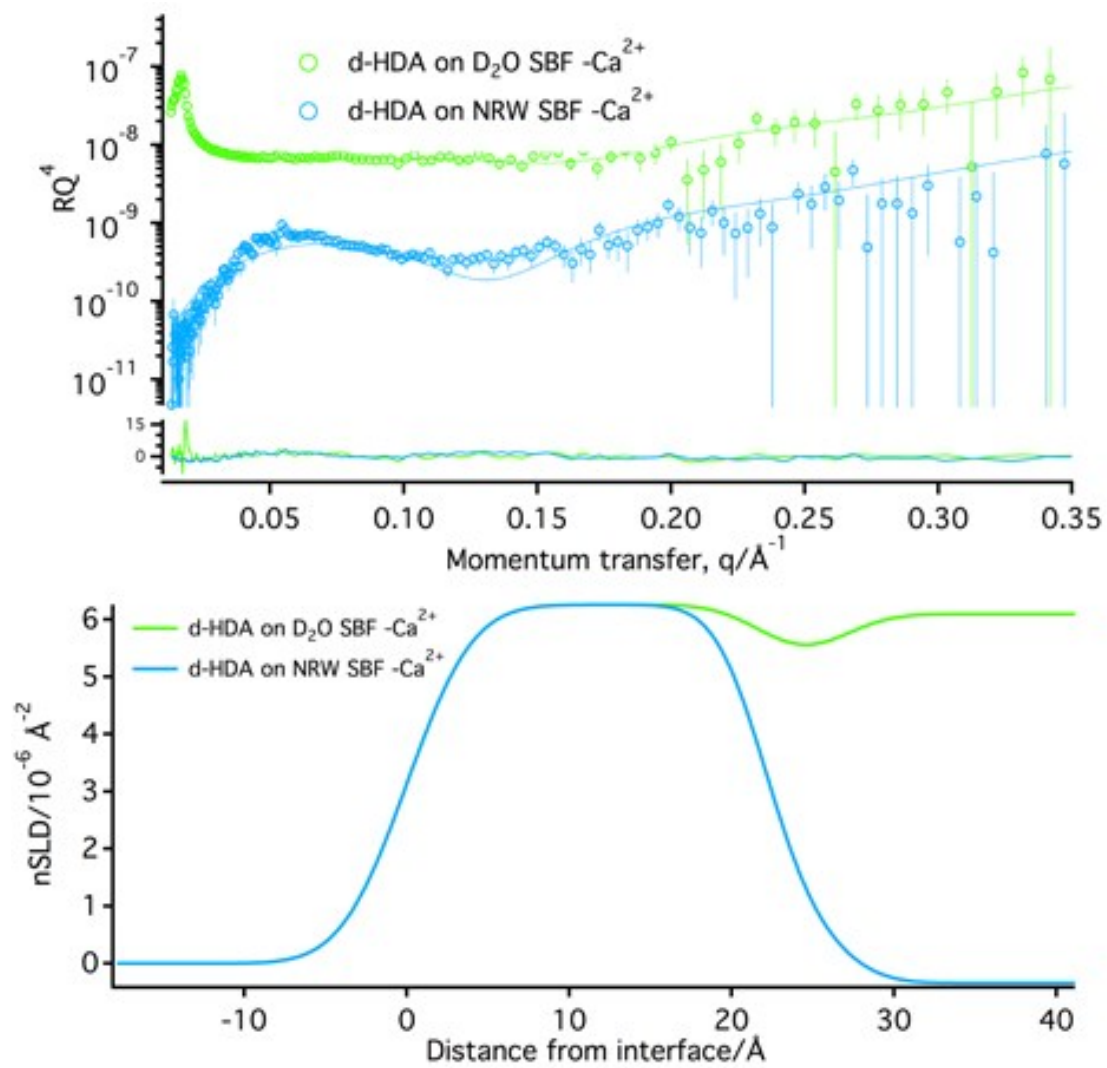Solution pH = 7.4, Ionic strength = 95 mmol L^{-1}.

Table S2. Pre-nucleation cluster composition, nSLD, volume, diameter, molecular mass and scattering length for the un-carbonated and carbonated units. These values were used to calculate pre-nucleation cluster densities within the mineral layer and subphase from the neutron reflectivity data.

Pre-nucleation cluster composition	nSLD (10^{-6} Å^{-2})	Volume (Å^3)	Diameter (Å)	Molecular Mass (g mol^{-1})	Scattering Length (×10^{-3}Å)
Ca$_9$(PO$_4$)$_6$	3.19 ± 0.08	666	10.8 ± 0.3	930.5	2.12
Ca$_9$(PO$_4$)$_4$(CO$_3$)$_3$	3.19 ± 0.08	714	11.1 ± 0.3	920.6	2.28
Ca$_9$(PO$_4$)$_2$(CO$_3$)$_6$	3.19 ± 0.08	766	11.4 ± 0.3	910.7	2.44

Table S3 – Thickness, nSLD, PNC vol.% and PNC cluster densities of the mineral and subphase layers obtained from the fitted results of the d-HDA on ½ SBF neutron reflectivity experiment. Each cell contains the parameter at time periods 0, 60, 90 and 120 min. D_2O vol.% = 100 − PNC vol.%.

	d-HDA tail group	d-HDA head group	Mineral layer	Subphase 1	Subphase 2	Subphase 3	Subphase 4	Subphase 5
τ/Å	22 ± 1	5 ± 1	10 ± 1 (0 min)	31	31	31	31	31
	22 ± 1	5 ± 1	15 ± 1 (60 min)	31	31	31	31	31
	22 ± 1	5 ± 1	18 ± 1 (90 min)	31	31	31	31	31
	22 ± 1	5 ± 1	23 ± 1 (120 min)	31	31	31	31	31
nSLD	6.09 ± 0.15	2.99 ± 0.07	3.63 ± 0.12	4.59 ± 0.07	5.49 ± 0.08	5.84 ± 0.07	6.09 ± 0.06	6.09 ± 0.06
/10^{-6} Å^{-2}	6.09 ± 0.15	1.82 ± 0.07	3.47 ± 0.08	4.75 ± 0.08	5.34 ± 0.08	5.81 ± 0.07	6.03 ± 0.06	6.03 ± 0.04
	6.09 ± 0.15	1.79 ± 0.07	3.31 ± 0.09	4.68 ± 0.08	5.20 ± 0.08	5.75 ± 0.07	5.96 ± 0.06	6.03 ± 0.05
	6.09 ± 0.15	0.93 ± 0.05	3.19 ± 0.08	4.66 ± 0.09	5.12 ± 0.06	5.74 ± 0.06	5.85 ± 0.06	6.04 ± 0.05
PNC vol.%	-	-	83 ± 4	52 ± 3	23 ± 3	12 ± 3	4 ± 2	4 ± 2
	-	-	88 ± 2	46 ± 2	28 ± 2	13 ± 2	5 ± 1	5 ± 1
	-	-	94 ± 3	49 ± 2	32 ± 2	15 ± 2	8 ± 2	5 ± 1
	-	-	100 ± 2	51 ± 4	36 ± 3	15 ± 2	12 ± 2	5 ± 1
PNC mass	-	-	1.93 ± 0.19	3.74 ± 0.22	1.65 ± 0.22	0.86 ± 0.22	0.29 ± 0.14	0.29 ± 0.14
density	-	-	3.06 ± 0.26	3.31 ± 0.14	2.01 ± 0.14	0.93 ± 0.14	0.36 ± 0.07	0.36 ± 0.07
/mg m^{-2}	-	-	3.93 ± 0.34	3.52 ± 0.14	2.30 ± 0.14	1.08 ± 0.14	0.57 ± 0.14	0.36 ± 0.07
	-	-	5.34 ± 0.36	3.67 ± 0.29	2.59 ± 0.22	1.08 ± 0.14	0.86 ± 0.14	0.36 ± 0.07
PNC density	-	-	1.25 ± 0.12	0.78 ± 0.05	0.35 ± 0.05	0.28 ± 0.07	0.06 ± 0.03	0.06 ± 0.03
/PNC nm^{-3}	-	-	1.32 ± 0.11	0.69 ± 0.03	0.42 ± 0.03	0.20 ± 0.03	0.07 ± 0.01	0.07 ± 0.01
	-	-	1.41 ± 0.12	0.74 ± 0.03	0.48 ± 0.03	0.23 ± 0.03	0.12 ± 0.03	0.07 ± 0.01
	-	-	1.50 ± 0.10	0.76 ± 0.06	0.54 ± 0.05	0.23 ± 0.03	0.18 ± 0.03	0.07 ± 0.01

Bulk ½SBF solution contains 0.004 mM of $Ca_9(PO_4)_6$ units, providing a pre-nucleation cluster density of 0.00005 PNC nm^{-3} in solution. The total amount of pre-nucleation clusters in the 50 mL ½SBF is 2.5×10^{18}. After the 120 min experiment a total of 6.1×10^{16} pre-nucleation clusters over the 150 × 45 mm air-liquid surface area was accounted for at the surface. A total of 2.4% of all available pre-nucleation clusters within the bulk solution was consumed during calcium phosphate nucleation at the air-liquid interface. Mass densities were calculated using a PNC molar mass of 930.5 gmol^{-1} and a PNC scattering length of 2.12×10^{-4} Å for the $Ca_9(PO_4)_6$ unit.

Table S4. Thickness, nSLD, zein/PNC/D_2O vol.% and PNC cluster densities of the mineralised layers obtained from the fitted results of the zein on ½SBF neutron reflectivity experiment. Each cell contains the parameter at time periods 0, 60, 90 and 120 min.

	Layer 1	Layer 2	Layer 3
τ/Å	7 ± 1	24 ± 1	45 ± 1
nSLD/10^{-6} Å^{-2}	1.37 ± 0.2	5.11 ± 0.08	5.94 ± 0.02
		4.87± 0.07	5.78 ± 0.02
		4.69± 0.07	5.63 ± 0.02
		4.70± 0.07	5.44 ± 0.02
Zein vol.%	81 ± 2	31 ± 1	6 ± 1
PNC vol.%	-	43 ± 3	13 ± 1
	-	48 ± 1	18 ± 1
	-	52 ± 1	23 ± 1
	-	52 ± 1	29 ± 1
D_2O vol.%	(19 % Air)	26 ± 3	81 ± 1
	(19 % Air)	21 ± 1	76 ± 1
	(19 % Air)	17 ± 1	71 ± 1
	(19 % Air)	17 ± 1	65 ± 1
PNC mass density /mg m^{-2}	-	2.37 ± 0.08	1.35 ± 0.09
	-	2.65 ± 0.08	1.87 ± 0.09
	-	2.87 ± 0.09	2.39 ± 0.09
	-	2.87 ± 0.09	3.01 ± 0.09
PNC density /PNC nm^{-3}	-	0.65 ± 0.02	0.19 ± 0.01
	-	0.72 ± 0.02	0.27 ± 0.01
	-	0.78 ± 0.02	0.27 ± 0.01
	-	0.78 ± 0.02	0.44 ± 0.01

Supplementary references

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